frontiers | Research Topics

Drug-resistant *Mycobacterium tuberculosis*

Edited by
Ben Gold, Helena Ingrid Boshoff
and Digby Warner

Published in
Frontiers in Cellular and Infection Microbiology

ISSN 1664-8714
ISBN 978-2-8325-2615-6
DOI 10.3389/978-2-8325-2615-6

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Drug-resistant *Mycobacterium tuberculosis*

Topic editors

Ben Gold — Weill Cornell Medicine, United States

Helena Ingrid Boshoff — National Institutes of Health (NIH), United States

Digby Warner — University of Cape Town, South Africa

Citation

Gold, B., Boshoff, H. I., Warner, D., eds. (2023). *Drug-resistant Mycobacterium tuberculosis*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-2615-6

Table of contents

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Editorial
PUBLISHED 19 May 2023
DOI 10.3389/fcimb.2023.1215294

OPEN ACCESS

EDITED AND REVIEWED BY
Anthony Baughn,
University of Minnesota Twin Cities,
United States

*CORRESPONDENCE
Helena I. M. Boshoff
hboshoff@niaid.nih.gov
Digby F. Warner
digby.warner@uct.ac.za
Ben Gold
bsg2001@med.cornell.edu

†These authors have contributed equally to this work

RECEIVED 01 May 2023
ACCEPTED 11 May 2023
PUBLISHED 19 May 2023

CITATION
Boshoff HIM, Warner DF and Gold B (2023) Editorial: Drug-resistant *Mycobacterium tuberculosis*. *Front. Cell. Infect. Microbiol.* 13:1215294. doi: 10.3389/fcimb.2023.1215294

Editorial: Drug-resistant *Mycobacterium tuberculosis*

Helena I. M. Boshoff[1*†], Digby F. Warner[2,3*†] and Ben Gold[4*†]

[1]Tuberculosis Research Section, Laboratory of Clinical Immunology and Microbiology, National Institutes of Health, Bethesda, MD, United States, [2]Molecular Mycobacteriology Research Unit, Department of Pathology and Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa, [3]Wellcome Centre for Infectious Diseases Research in Africa, University of Cape Town, Cape Town, South Africa, [4]Department of Microbiology & Immunology, Weill Cornell Medicine, New York, NY, United States

KEYWORDS

***Mycobacterium tuberculosis*, tuberculosis, drug resistance, phenotypic resistance, drug tolerance, drug discovery, antimicrobial resistance (AMR), drug synergy**

Editorial on the Research Topic
Drug-resistant *Mycobacterium tuberculosis*

Introduction

Tuberculosis (TB) is anticipated to regain its position as the leading cause of death globally due to an infectious agent, having transiently ceded that dubious accolade to SARS-CoV-2/COVID-19 (WHO, 2022a). In 2021, 1.6 million deaths were attributed to TB despite the availability of WHO-approved regimens that can cure 85% of patients in 4-6 months (WHO, 2022b). Drug-resistant TB, which is notoriously difficult to treat, accounted for about half a million of the 6.4 million new TB cases in 2021 (WHO, 2022b). Multidrug-resistant TB (MDR-TB), defined as resistance to at least rifampicin and isoniazid, currently requires 6-9 months' treatment. The duration of antibiotic therapy can increase beyond 12 months for extensively drug-resistant TB (XDR-TB), which is MDR-TB plus resistance to a fluoroquinolone, and bedaquiline or linezolid (WHO, 2022b). Treatment success for MDR-TB is less than 60%, while for XDR-TB it is estimated to be much lower at ~39% (Chakaya et al., 2021). In this Research Topic, 91 authors from locations across the globe contributed 17 reviews and 3 primary research articles covering Drug Resistant *Mycobacterium tuberculosis* (Mtb). Consistent with the complexity of the problem, the collection comprises a diversity of approaches towards understanding, characterizing, and combatting anti-TB drug resistance. With the United Nations General Assembly due to hold its second high-level meeting on the fight against TB in September this year, this collection offers a timely perspective on the threat posed to global health by this major source of antimicrobial resistant (AMR) infections.

Genetic drivers of persistence and the evolution of resistance

The number of new chemical entities (17) currently approved for clinical trials alone or in combination with selections from 9 existing anti-TB drugs represents a major improvement on previous years (Edwards and Field, 2022; Fernandes et al., 2022). We know, however, that *Mycobacterium tuberculosis* (Mtb), the bacterial cause of TB, has developed resistance to all drugs in the clinic. This sobering realization emphasizes not only the need to develop new drugs and regimens, but also the importance of characterizing the mechanisms that enable the pathogen to acquire drug-resistance or to survive chemotherapy despite apparent genetic susceptibility. Jones et al. review progress in our understanding of how Mtb evolves drug resistance, often through a stage in which the bacillary population is tolerant of chemotherapy or enriched for persister organisms. The authors appraise the genetic underpinnings of drug tolerance, and their associations with the formation of heterogeneous bacterial populations characterized by differences in their states of DNA replication or repair, transcription, translation, metabolism, and compound efflux. Notably, this heterogeneity manifests between patients, during disease progression within a single patient, and even within and between lesions in the same lung. Liebenberg et al. discuss the factors that affect the development of drug-resistant TB, the transmission of resistant disease, the importance of diagnosis of drug resistance, and the implications for public health management. The authors review many of the mechanisms that are associated with the evolution of drug resistance, including the role of toxin-antitoxin systems in generating persistent cells. They also assess aspects of host-mediated physiology that create environments favoring the development of drug-tolerant populations, including the development of mycobacterial biofilms at certain sites of disease. As discussed by Jones et al., the ability to evolve genetic drug resistance is lineage-dependent, further complicating any models used to predict this process. Once drug resistance has developed, the fitness of the resistant cells can be ameliorated by compensatory mutations that impact the function of the drug target or the metabolic pathways perturbed by the mutated target.

The proportion of differentially culturable cells in TB patients undergoing chemotherapy might offer important insight in the presence *in vivo* of persisters in heterogeneous Mtb populations. In their contribution, Peters et al. report that TB patient sputum harbors a significant fraction of bacilli which do not grow on standard solid agar media. These organisms (variously referred to as “differentially culturable”, “differentially detectable”, or “viable but nonculturable”) can, however, be detected by limiting dilution in liquid media with addition of culture filtrates – which may in some cases harbor growth enhancing components that improve the detection of these cryptic populations. They are also observed in patients on chemotherapy even when traditional solid agar colony counts fall below the limit of detection, and they are found in a sizeable proportion of patients upon completion of chemotherapy. Though not a measure of treatment success, measuring differentially culturable bacteria in patients during chemotherapy may provide an indication of the bacterial load. Gordhan et al. report that the number of differentially culturable Mtb may provide a better measure of bacterial numbers in sputum than solid agar, especially in patients with low bacterial numbers at the onset of disease, such as HIV-coinfected patients. Notably, these authors found that resuscitation promoting factors present in culture filtrates did not promote liquid growth of MDR Mtb strains when compared to rifampicin mono-resistant strains, for which higher numbers of bacilli were detected in the absence of growth promoting factors.

The metabolism of Mtb that drives the evolution of persistent cells and of drug resistant cells

The propensity for mycobacterial populations to contain sub-populations of cells – in some cases even individual/single cells – in different physiological, metabolic and/or replicative states is increasingly recognized as a major contributor to mycobacterial pathogenicity, as well as an impediment to a full understanding of relevant metabolism of the mycobacteria. Put simply, the genomic clonality which typifies many Mtb populations obscures a capacity for phenotypic diversity that historically has been underestimated. Considering the specific case of antibiotic persistence, Shultis et al. pose key questions around the etiology and characteristics of persister mycobacteria (Where do they come from? Are they all created equally)? and the barriers hindering research into these phenomena. In their article, Eoh et al. argue that metabolomics, by providing detailed insights into the molecular compositions of cells, offers key advantages over other techniques through its ability to identify metabolic differences associated with, or causal of, specific phenotypes in genetically identical populations. Their focus on the role of metabolism in drug tolerance and resistance in Mtb is elaborated by Samuels et al., who consider evidence that changes in central carbon metabolism can promote drug tolerance, identifying metabolic pathways as potential drug targets to inhibit the development of drug tolerance and enhance the efficacy of current anti-mycobacterial therapeutics. Taking a broader view which incorporates both clinical and wet lab settings, Mishra and Saito analyze the consequences of heterogeneity in mycobacterial culturability and growth rates on the use and interpretation of mycobacterial culture data. Reinforcing key themes from these papers, Singh et al. consider the specific case of resistance to fluoroquinolones which, given their role as second-line anti-TB agents, threatens the ability to therapeutically limit the progression of MDR-TB to XDR-TB.

The dependence on multidrug combinations as standard therapy for both drug-susceptible and drug-resistant TB highlights the urgent need to expand the existing, approved TB drug arsenal through the identification of new compounds and combinations. In their article, Tomasi and Rubin provide insights into the genetic tools available to improve the identification and

prioritization of new drug targets, including those that might potentiate existing drugs. Poulton and Rock similarly consider the potential for chemical-genetics to improve understanding of intrinsic drug resistance in Mtb and how it might be disarmed in developing potent new antitubercular therapies. The importance of looking beyond enzymes and structural proteins as potential new targets is highlighted by Miotto et al., who argue that based on transcriptional profiles following drug exposure, the major transcriptional regulators such as sigma factors and the WhiB, GntR, XRE, Mar and TetR family regulators should be considered as targets for novel interventions.

The advent of inexpensive sequencing technologies has transformed mycobacterial genetics. However, as Nimmo et al. highlight, while genomics has obvious utility in rapidly identifying mutations conferring resistance to new anti-TB drugs, this requires awareness of the fact that the genotype-phenotype correlations which are available for established antibiotics will not be possible where, by definition, new mutations are individually rare. For Stanley et al., though, the real power of genomics extends beyond standard susceptibility determinations to predicting the risk of developing genetic resistance and treatment failure, making precision anti-TB therapies an achievable aspiration through the integration of genetic determinants of antibiotic response into treatment algorithms.

Approaches to combat persistent and drug-resistant Mtb

Developing antibiotics that tackle drug resistant Mtb requires innovation at many levels. Anti-TB antibiotics have the daunting task of eradicating Mtb that is replicating, phenotypically resistant (often nonreplicating), and/or nonculturable on standard bacteriological agar (differentially culturable/detectable, etc.) (Nathan, 2012; Nathan, 2017). The enormous diversity of host microenvironments and immune chemistries impact Mtb resistance to antibiotics and the development of genetic resistance.

In their review article, Bhagwat et al. explore drug discovery strategies based on evading pre-existing drug resistance and/or development of drug resistance. Some of these strategies include reversing drug resistance with co-treatment of synergizing agents, developing new generations of existing agents that evade resistance, designing drug combinations to thwart resistance mechanisms, polypharmacology, characterizing large numbers of resistant mutants to understand genetic determinants of drug resistance, and implementing early-stage analysis of drug resistance. Roubert et al. provide Pharma's perspective on strategies to fast-track antibiotic discovery and improve success rates by "upcycling" TB drug discovery. The authors provide blueprints to combat TB drug resistance, including strategies to revisit hit molecules discovered during the golden age of drug discovery, improving drugs currently used to treat TB, modifying TB drugs to evade resistance, and focusing efforts on validated anti-infective targets. In their review article, Greenstein and Aldrich lay out the enormity of the logistical task involved in testing pairwise and multidrug combinations in multiple *in vitro* models thought to mimic host environments and in animal models of TB. Towards addressing this challenge, the authors present an overview of strategies to predict successful drug combinations, including the use of specialized experimental methods such as the hollow fiber model for human PK/PD, as well as the analysis of *in vitro*, animal, and clinical data with computational models and machine learning. Likewise, McNeil et al. highlight the mycobacterial respiratory complexes as a target for drug combinations. Using a careful analysis of data from chemical, genetic, and chemical-genetic experiments, the authors identify points of vulnerability in mycobacterial respiration to exploit with combinations of antibiotics, targets whose genetic or chemical inhibition potentiates the activity of existing drugs, targets whose inhibition is synergistic lethal, and/or combinations anticipated to kill both replicating and nonreplicating Mtb. In their article, Rudraraju et al. propose KasA (beta-ketoacyl synthase) as a high value target to kill both drug-sensitive and drug-resistant TB. In support of their proposal, the authors provide a comprehensive summary of an extensive body of work on KasA genetics, enzymology, inhibitors, structure-based inhibitor design, and KasA inhibitors *in vitro* and in animal models of TB. TB disease occurs through a complex interplay of mycobacterial and host factors, necessitating a broad view of the potential therapeutic approaches that might be adopted. In their primary research article, Glenn et al. test the impact of modulating host inflammation on the formation of differentially culturable Mtb in a murine model of TB. The authors find that the drug dimethyl fumarate, used to manage psoriasis and multiple sclerosis, reduces the numbers of differentially culturable Mtb when dosed alone or when dosed with standard of care TB drugs, rifampicin, isoniazid, and pyrazinamide.

In summary, the primary articles and reviews in this topic collectively emphasize the complexity of drug-resistant Mtb. Translating these concepts to the clinic will require further advancing our knowledge of the biology of Mtb and developing new approaches to TB drug discovery.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Funding

HB is supported by the Division of Intramural Research, NIAID, NIH. DW is supported by the Research Council of Norway (R&D Project 261669 "Reversing antimicrobial resistance"), the South African Medical Research Council, and the National Research Foundation of South Africa. BG is supported in part by the National Institutes of Health (R21AI159572) and Bill and Melinda

Gates Foundation (INV-040480 and INV-047552). The Department of Microbiology & Immunology at WCM acknowledges the support of the William Randolph Hearst Foundation.

Acknowledgments

We give our sincere thanks to all authors included in this Drug Resistant TB collection for graciously contributing their expertise and time to write about their favorite topics. We are also grateful the reviewers whose critique and suggestions enhanced the quality of the articles appearing in this collection.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Chakaya, J., Khan, M., Ntoumi, F., Aklillu, E., Fatima, R., Mwaba, P., et al. (2021). Global tuberculosis report 2020 - reflections on the global TB burden, treatment and prevention efforts. *Int. J. Infect. Dis.* 113 Suppl 1 (Suppl 1), S7–S12. doi: 10.1016/j.ijid.2021.02.107

Edwards, B. D., and Field, S. K. (2022). The struggle to end a millennia-long pandemic: novel candidate and repurposed drugs for the treatment of tuberculosis. *Drugs* 82 (18), 1695–1715. doi: 10.1007/s40265-022-01817-w

Fernandes, G. F. S., Thompson, A. M., Castagnolo, D., Denny, W. A., and Dos Santos, J. L. (2022). Tuberculosis drug discovery: challenges and new horizons. *J. Med. Chem.* 65 (11), 7489–7531. doi: 10.1021/acs.jmedchem.2c00227

Nathan, C. (2012). Fresh approaches to anti-infective therapies. *Sci. Transl. Med.* 4 (140), 140sr2. doi: 10.1126/scitranslmed.3003081

Nathan, C. (2017). Kunkel lecture: fundamental immunodeficiency and its correction. *J. Exp. Med.* 214 (8), 2175–2191. doi: 10.1084/jem.20170637

World Health Organization (2022a). *Global tuberculosis report* (Geneva, Switzerland: World Health Organization).

World Health Organization (2022b). *WHO consolidated guidelines on tuberculosis. module 4: treatment-drug-resistant tuberculosis treatment 2022 Update* (Geneva, Switzerland: World Health Organization).

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 17 August 2022
DOI 10.3389/fcimb.2022.933458

OPEN ACCESS

EDITED BY
Digby Warner,
University of Cape Town,
South Africa

REVIEWED BY
Anh K. Lam,
Indiana University, United States
Babak Javid,
University of California, San Francisco,
United States
Galina Mukamolova,
University of Leicester,
United Kingdom
Anna D. Tischler,
University of Minnesota Twin Cities,
United States

*CORRESPONDENCE
Michael Berney
michael.berney@einsteinmed.edu

SPECIALTY SECTION
This article was submitted to
Molecular Bacterial Pathogenesis,
a section of the journal
Frontiers in Cellular and
Infection Microbiology

RECEIVED 30 April 2022
ACCEPTED 25 July 2022
PUBLISHED 17 August 2022

CITATION
Shultis MW, Mulholland CV and
Berney M (2022) Are all antibiotic
persisters created equal?
Front. Cell. Infect. Microbiol. 12:933458.
doi: 10.3389/fcimb.2022.933458

Are all antibiotic persisters created equal?

Michael W. Shultis, Claire V. Mulholland and Michael Berney*

Department of Microbiology and Immunology, Albert Einstein College of Medicine,
New York, NY, United States

Antibiotic persisters are a sub-population of bacteria able to survive in the presence of bactericidal antibiotic despite the lack of heritable drug resistance mechanisms. This phenomenon exists across many bacterial species and is observed for many different antibiotics. Though these bacteria are often described as "multidrug persisters" very few experiments have been carried out to determine the homogeneity of a persister population to different drugs. Further, there is much debate in the field as to the origins of a persister cell. Is it formed spontaneously? Does it form in response to stress? These questions are particularly pressing in the field of *Mycobacterium tuberculosis*, where persisters may play a crucial role in the required length of treatment and the development of multidrug resistant organisms. Here we aim to interpret the known mechanisms of antibiotic persistence and how they may relate to improving treatments for *M. tuberculosis*, exposing the gaps in knowledge that prevent us from answering the question: Are all antibiotic persisters created equal?

KEYWORDS

mycobacteria, tuberculosis, multidrug, persister, persistence, tolerance, resistance

Introduction

In 2020 the WHO reported an estimated 10 million people contracted tuberculosis and 1.5 million died from the disease, making it the second most deadly infectious disease behind COVID-19 worldwide (WHO 2021). The agent responsible for this previously mysterious disease was first identified in 1882 when Robert Koch discovered the bacterium *Mycobacterium tuberculosis* as its cause (Murray et al., 2015). It wasn't until the 1950s that reliable chemotherapy was developed (Murray et al., 2015). This began with streptomycin and para-aminosalycylic acid in 1944, but monotherapy treatments resulted in drug resistance, emphasizing the need for combination therapies. Isoniazid was introduced in 1951, leading to a combined "triple therapy". This therapy required treatment times lasting up to 24 months long (Murray, 2004). Continued emergence of drug resistant populations led to the development of ethambutol in 1961 (Murray et al., 2015). It wasn't until 5 years later that the introduction of rifampin combined with isoniazid was able to shorten treatment times to 9 months. Finally, the introduction of

pyrazinamide further reduced treatment times to 6 months, bringing us to the combination therapies that remain in use today (Murray et al., 2015). The most common regimen includes the combination of isoniazid (INH), ethambutol (EMB), rifampicin (RIF), and pyrazinamide (PZA) for a period of 6 to 9 months (Nahid et al., 2016). Curiously, the two drugs that enabled greatly shortened treatment times, RIF and PZA, share a common property: the ability to kill persistent bacteria (Zhang et al., 2014; Hu et al., 2015).

What is a persister?

Persistent bacteria are a sub-population of bacteria that demonstrate slower killing kinetics in response to a stress, yielding a bimodal kill curve (Figure 1, blue line) (Boldrin et al., 2020). *Persistence* is distinct from antibiotic *resistance* because the state of persistence is non-heritable (Balaban et al., 2019). If persistent bacteria are regrown and exposed to the same stressor, they will again exhibit a heterogeneous response with a bimodal kill curve. Conversely, if a small subpopulation of resistant bacteria is isolated, regrown, and retreated with the same drug, growth would be observed instead of bimodal killing. Persistent bacteria exist across bacterial species, though are commonly referred to by the umbrella term 'persisters'.

The first observation of antibiotic persistence was made in 1944 when Joseph Bigger demonstrated penicillin was incapable of sterilizing a culture of staphylococci (Bigger, 1944). In 1964 physicians with Cornell medical school made observations of "disappearing" *M. tuberculosis* bacilli in mice (McCune et al., 1966; McCune et al., 1966). Treatment of mice with a specific regimen of INH and PZA could push *M. tuberculosis* into an undetectable state by microscopy, culture, or reinfection. When treatment was removed for 90 days, *M. tuberculosis* became detectable in 1/3 mice (McCune et al., 1966). The undetectable state was only achievable when the mice were treated in order first with INH for a period of 2-4 weeks, followed by PZA for 8 weeks (McCune et al., 1966). If the duration of therapy was extended to 26 weeks no bacterial regrowth was observed up to 6 months after treatment cessation, suggesting these bacteria were persistent to sequential INH then PZA treatment after 12 weeks, but were killed with prolonged 26 week treatment (McCune et al., 1966). In these discussed conditions, treatment was generally carried out immediately following inoculation of mice. When mice were left untreated for 15 weeks, the undetectable state was not achievable with 12- or 26-week therapy. Among the bacilli that were detectable in the 26 week treated condition, only INH and PZA dual resistant mutant strains were identified, indicating that after 15 weeks enough pre-existing INH+PZA resistant mutants were generated, preventing the study of persistent bacteria (McCune et al., 1966). Although persistence is a common phenomenon in bacteria, a mechanism ubiquitously required for antibiotic persistence has yet to be identified, specifically in *M. tuberculosis*. The results described in the above Cornell study indicate that revealing the dynamics of persister cell formation are critical to accelerating the sterilization of *M. tuberculosis* infections.

Comprehensive and systematic reviews of persister phenomena have been presented elsewhere e.g (Boldrin et al., 2020). In the present review we focus on offering interpretations of the available data to illustrate the gaps in knowledge that prevent us from drawing conclusions about the heterogeneity of *M. tuberculosis* persisters to multidrug therapies.

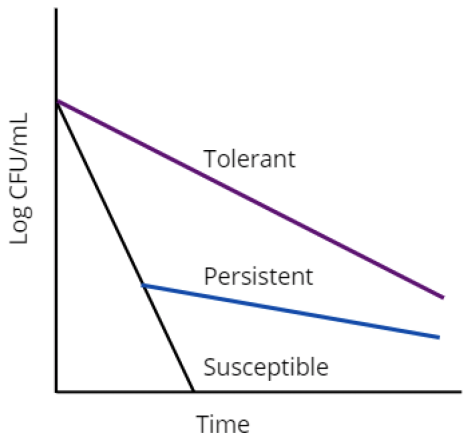


FIGURE 1
Graphical representation of drug susceptible (black), persistent (blue), and tolerant (purple) bacterial populations.

Tolerance versus persistence

The terms persistence and tolerance are often used interchangeably. Some prefer to draw a line between the two, describing them as similar but different phenomena (Brauner et al., 2016). The label of 'persister' appears largely to be definitionally confined. Under the current definition, an antibiotic persister must be a part of a small subpopulation, non-growing in the presence of the drug, genetically identical to the population that was killed by the stressor, vary only slightly with drug concentration at high concentrations of antibiotic, the list goes on (Balaban et al., 2019). By this definition, a bacterium could be labelled tolerant or persistent depending only on the characteristics of surrounding bacteria. If alone, this bacterium is a persister (Figure 1, blue line). If part of a larger population of bacteria exhibiting the same survival advantage, it is tolerant (Figure 1, purple line). There is no evidence to support the mechanistic distinction of persistent and tolerant bacteria, which is ultimately why many use these terms interchangeably. The mechanisms of *M. tuberculosis* tolerance are described in striking similarity to those of persistence. Metabolic slowdown, transcriptional and translational responses to stress, toxin-

antitoxin system utilization, and efflux pumps are used to describe the mechanisms of both persistence and tolerance, [reviewed elsewhere in (Boldrin et al., 2020; Goossens et al., 2020)]. Comparatively it is very easy to draw the line between resistant bacteria and persistent bacteria. Resistance is a heritable adaptation that enables bacteria to grow in the presence of antibiotic (Balaban et al., 2019).

In summary, persistent bacteria are a drug tolerant sub-population that endures bactericidal antibiotic treatment through non-heritable mechanisms. Therefore, is it natural to wonder: If these survival advantages are non-heritable, where do persisters come from?

The origins of persisters

The exact origins of persistent bacteria remain shrouded in mystery. Persisters have been described as belonging to two categories - type 1 and type 2 (Balaban et al., 2004). These types will be referred to here as triggered and stochastic persisters respectively. The distinction between these two categories can generally be understood as a description of when a persister is formed. Does a persister already exist in a population before a stress is introduced? Does a normally growing bacterium respond to stress in its environment by becoming a persister cell? Triggered persisters form in response to a trigger in their environment, whereas stochastic persisters form in the absence of external triggers (Balaban et al., 2004; Balaban et al., 2019). This distinction can be difficult to make when persisters make up 1% or less of a bacterial population (Lewis, 2007) and can only be definitively identified by their survival in response to a stress.

In *Escherichia coli* some studies have reported persistence arising in a stochastic pattern consistent with the decreased availability of nutrients that enhance ATP production in the cell (Shan et al., 2017; Manuse et al., 2021). This increase in persistence to ciprofloxacin and ampicillin is suggested to be due to decreased ATP generation, leading to lower activity of antibiotic targets, resulting in drug tolerance (Shan et al., 2017). This result was interrogated following single cells in solution, revealing 15 out of 16 ampicillin persister cells were not growing prior to ampicillin treatment, supporting their identification as stochastic persisters (Manuse et al., 2021). Other groups have identified *E. coli* persisters that originate from metabolically active cells (Dorr et al., 2010; Goormaghtigh and Van Melderen, 2019). In a single-cell experiment, microfluidics were used to image cells in a culture every 15 minutes, enabling retroactive observation of persister cell growth once they were identified by ofloxacin treatment (Goormaghtigh and Van Melderen, 2019). The authors tracked the cell area of persistent cells and were able to identify a rate of elongation immediately before ofloxacin treatment. Though a small significant decrease in growth rate was identified in persisters compared to the total population, the authors attributed this to a high statistical power as they were unable to find a significant difference when they randomly sampled a smaller subset of the non-persistent population (Goormaghtigh and Van Melderen, 2019). Another group found that ciprofloxacin, also a fluoroquinolone, was able to induce *E. coli* persister cell formation *via* the SOS response triggered by DNA damage (Dorr et al., 2010). In *Pseudomonas aeruginosa*, cells have been observed to upregulate persistence in response to quorum-sensing signals secreted into their media (Moker et al., 2010).

These points together indicate that the mechanisms for persister cell formation can vary between bacterial species and can vary depending on the stress applied to bacteria within the same species. When ampicillin was selected to reveal the *E. coli* persister population in the first experiment endorsing stochastic persistence, bacteria that may have been persistent to another drug were killed, preventing their characterization (Manuse et al., 2021). When ofloxacin or ciprofloxacin were used to reveal the *E. coli* persister population instead, a different group of bacteria capable of triggered persistence may have ended up being analyzed (Dorr et al., 2010; Goormaghtigh and Van Melderen, 2019). Both populations seem to exist, meaning the origins of persister populations can be varied. It is likely that stochastic persistence remains present at a certain level and that triggered persistence can occur with different intensities to different stressors.

Mycobacteria also demonstrate characteristics consistent both with stochastic and triggered persistence. The growth of mycobacteria are inherently heterogenous. The bacilli elongate asymmetrically on one of their two poles (Aldridge et al., 2012). The growing pole deemed the accelerator pole and the nongrowing pole deemed the alternator pole (Aldridge et al., 2012). During each division one daughter cell inherits the accelerator pole while the other inherits the alternator pole (Aldridge et al., 2012). Cell growth continues along the older of the two poles, requiring the alternator pole be converted to an accelerator pole (Aldridge et al., 2012). It was found that daughter cells inheriting the accelerator pole elongated faster than daughter cells inheriting the alternator pole (Aldridge et al., 2012). Further, in *Mycobacterium smegmatis*, it was found that accelerator cells were generally more susceptible to cell wall antibiotics like INH and alternator cells were generally more susceptible to RIF (Aldridge et al., 2012). This result is consistent with the observation that most antimycobacterial drugs have poor activity on slow growing cells, with RIF and PZA being the exceptions (Xie et al., 2005; Pullan et al., 2016). Deletion of *lamA*, the gene responsible for the inhibition of growth at new growth poles, results in a more symmetrical growth from each pole. Of note, these more uniform cells demonstrate a faster killing rate in response to RIF in *M. tuberculosis* (Rego et al., 2017). In *M. smegmatis* these cells were killed faster in response to RIF as well as cell wall targeting drugs (Rego et al., 2017). When considering this slow, asymmetric growth pattern of *M. tuberculosis* it isn't difficult to rationalize that this heterogeneity could lead to stochastic persisters. Indeed, authors Jain et al. demonstrated using a dual reporter mycobacteriophage

that genes linked to persistence in *M. tuberculosis* were upregulated prior to INH treatment, and that the bacteria expressing these genes were enriched in the persistent population (Jain et al., 2016).

In another vein, *M. tuberculosis* excels at responding to stressors in the host environment. Signal transduction systems have been shown to be essential for *M. tuberculosis* to establish latent infection in lung tissue and play a role in the response of *M. tuberculosis* to environmental stressors (Zahrt and Deretic, 2001; Bretl et al., 2011). Mistranslation has been shown to be more prevalent in stressed *M. tuberculosis*, which has led to increased bacterial survival to RIF (Javid et al., 2014). When sequenced, the surviving bacteria contained no mutations in the RIF resistance determining region (RRDR), suggesting these bacteria were demonstrating triggered persistence (Javid et al., 2014). Collectively these observations indicate that persistent *M. tuberculosis* is composed of a mixed population of pre-existing and triggered persistent bacteria. This heterogeneity is likely further exacerbated in the host environment where *M. tuberculosis* encounters a variety of stressors (Warner and Mizrahi, 2006; Adams et al., 2011; Liu et al., 2016).

The nature of a persister

Regardless of its origin, it is important to consider the nature of a persister when attempting to design therapies to sterilize these bacteria. As discussed above, many drugs that impact the bacterial cell wall require actively growing cells to impact their targets (Xie et al., 2005). This fact has led to the belief that all persisters are non-growing cells, but is this truly the case?

Perhaps the most accepted characteristic of a persister is the characteristic of dormancy (Wood et al., 2013). Persisters are generally thought of as metabolically stunted bacteria that are inaccessible by antibiotics because the systems the antibiotics impact are inactive. It has been suggested that vitamin C and cysteine can prevent this metabolic shutdown by stimulating respiration, leading to sterilization of *M. tuberculosis in vitro* (Vilcheze et al., 2013; Vilcheze et al., 2017). In starved or stationary phase *E. coli* and *P. aeruginosa*, where bacteria metabolize more slowly, populations seem to be enriched for persistence (Keren et al., 2004a; Volzing and Brynildsen, 2015). This effect is observed in starved *M. tuberculosis*, where activation of the stringent response mediates persister formation, and deletion of a stringent response enzyme reduces persistence (Dutta et al., 2019). This stringent response enzyme, Rel, initiates metabolic arrest in *M. tuberculosis* (Dahl et al., 2003). The enrichment of persisters in stationary phase has been theorized to be a result of ATP-depletion (Manuse et al., 2021).

Contrarily, some groups presented evidence that persister cells can be metabolically active, and even actively dividing, in *E. coli* and *M. smegmatis* (Orman and Brynildsen, 2013; Wakamoto et al., 2013). In the case of *E. coli* 20/100 persisters were identified to be metabolically active by fluorescence-activated cell sorting (Orman and Brynildsen, 2013). This result comes with scrutiny as the experimental design is accused of carrying over persistent bacteria in the inoculum of the assay (Wood et al., 2013). *M. smegmatis* cells were observed to grow and divide in the presence of INH so long as expression of KatG, the activator of the prodrug INH, was suppressed (Wakamoto et al., 2013). Proponents of dormancy believe this to be an outlier, stating these cells are not persisters but instead normally growing cells that haven't expressed KatG (Wood et al., 2013). This interaction is seen as unique because the cells aren't under any stress from the antibiotic until converted to the active form, making this case inapplicable to persisters as a whole (Wood et al., 2013). Though model *M. smegmatis* is often used in experiments due to its faster growth and reduced biosafety requirements, it is important to be cautious when making direct comparisons to *M. tuberculosis* given the inherent differences of these two bacteria. However, we believe that when considering persistence in *M. tuberculosis* this result is important to consider given it is a direct study of mycobacterial persistence to a clinically relevant antimycobacterial drug. Furthermore, even *E. coli* persisters have been reported as having a reduced, but nonzero growth rate prior to antibiotic treatment (Balaban et al., 2004).

Briefly mentioned above, work in *M. smegmatis* has demonstrated bacterial growth in the presence of RIF, despite cells remaining genotypically sensitive to the drug (Javid et al., 2014). In this study, investigators generated a strain of *M. smegmatis* that resulted in higher rates of protein mistranslation, and these bacteria demonstrated 1000-fold more colonies on RIF containing agar, despite containing no mutations in the RRDR (Javid et al., 2014). The authors then utilized a bacterial strain with a higher fidelity ribosome, resulting in less mistranslation. This strain resulted in a decrease of bacterial survival to RIF compared to wild-type, suggesting that bacterial mistranslation is a unique way mycobacteria can survive antibiotic stress, though it is unknown if a resistance conferring mutation existed beyond the RRDR in the high protein mistranslation strain (Javid et al., 2014).

Another study was carried out in *M. smegmatis* and *M. tuberculosis* that demonstrated "semi-heritable" growth in the presence of RIF (Zhu et al., 2018). The cell wall of *M. smegmatis* was fluorescently labelled and then exposed to increasing concentrations of RIF. Cells that can grow will become less fluorescent as they "dilute" the fluorescent labels in their cell walls. It was found that unique to RIF, a small subset of cells was able to grow in the presence of RIF, up to 36 μg/mL (Zhu et al., 2018). The peak serum concentration for RIF appears to reach 3 to 5 μg/mL in humans (Seth et al., 1993; Lei et al., 2019). When *M. tuberculosis* or *M. smegmatis* exposed to RIF were plated on RIF-containing agar, a significant sub-population of colonies were observed (Zhu et al., 2018). When sequenced, all

30 of the present *M. smegmatis* colonies were found to be wild-type in the RRDR of the *rpoB* gene, the gene controlling the target of RIF. When these colonies were picked and re-plated onto RIF containing agar, there was a 10-fold increase in survival compared to the first exposure to the drug. When the same procedure was performed plating RIF-sensitive clinical isolates from active *M. tuberculosis* infections, a larger surviving sub-population was observed the longer the patient was on RIF therapy (Zhu et al., 2018). When these colonies were picked and regrown in non-selective media for 16 hours, no survival advantage was observed when compared to cells never exposed to RIF, making this effect "semi-heritable" (Zhu et al., 2018). This effect was correlated with increased transcription of *rpoB*.

Another study assessed the impact of asymmetric mycobacterial growth on RIF tolerance, and it was found that RIF tolerance was correlated with large cell size and older inherited growth poles (accelerator poles) (Richardson et al., 2016). This is consistent with the previously mentioned study demonstrating that alternator cells are more susceptible to RIF, since alternator cells will tend to be smaller on average, given the need to convert the nongrowing alternator pole to a growing pole (Aldridge et al., 2012). A study of persistent *M. tuberculosis* revealed persisters to PZA or RIF continue to engage in active transcription despite the apparent growth arrest associated with persistence (Hu et al., 2000). Given that mycobacteria seem to operate on a time based replication schedule, rather than a size based replication schedule (Aldridge et al., 2012), it becomes unclear if a persistent population is truly nongrowing or if this population is in a state of dynamic equilibrium between life and death, similar to the *M. smegmatis* cells observed by Wakamoto et al. (Aldridge et al., 2012; Wakamoto et al., 2013).

In this section we have discussed three instances of mycobacteria growing in the presence of antimycobacterial drugs, RIF and INH, despite remaining genotypically sensitive (Wakamoto et al., 2013; Javid et al., 2014; Zhu et al., 2018). These observations lend themselves to the conclusion that while most persisters are 'dormant', some persistent bacteria can grow in the presence of antimycobacterial drugs when treated with monotherapy.

Phenotypic resistance

Now, with the arguments for non-dormant persister cells established, we introduce with this section the phenomenon of phenotypic resistance and discuss the roles this phenomenon may play alongside persistence and tolerance in complicating the treatment of *M. tuberculosis*.

As already discussed in the above section on tolerance and persistence, the line between tolerance and persistence is very thin and often crossed in discussions of both topics. In contrast, drug resistance has been clearly differentiated based on two factors, heritability and growth. However, discussion of metabolically active bacteria that survive antibiotic treatment begs the question: Are both parameters, heritability and growth, necessary to exclude the persister label or is heritability alone sufficient?

Phenotypic resistance describes a phenomenon where bacteria can grow in the presence of antibiotics, but the mechanism that enables their growth is non-heritable. This phenomenon was discussed above in the context of protein mistranslation (Javid et al., 2014), and is reviewed in greater depth elsewhere (Corona and Martinez, 2013). The bacteria mentioned previously did not contain mutations in the RRDR, indicating that if these bacteria were regrown and exposed to RIF under conditions that did not promote mistranslation, they would remain drug sensitive unless a resistance mutation was present outside the RRDR (Javid et al., 2014). To some the growth of these bacteria excludes the persister label, but why? As discussed previously, we highlight here three instances of mycobacteria that survive treatment with antimycobacterial drugs through a non-heritable mechanism (Wakamoto et al., 2013; Javid et al., 2014; Zhu et al., 2018). Each author used a different descriptor for their population of cells, dynamic persistence (Wakamoto et al., 2013), phenotypic resistance (Javid et al., 2014; Zhu et al., 2018), and tolerance (Zhu et al., 2018). These studies all focus on a sub-population of bacteria undergoing non-heritable (or semi-heritable), heterogenous mechanisms of survival in response to an applied antibiotic stress. If any of these mechanisms arise during an infection, they would likely prolong treatment times and warrant a solution. Though these mechanisms seem to be drug specific, there is evidence, expanded on in the next section, that patient noncompliance and drug pharmacokinetics can impact the effective concentrations of certain drugs in the lesions where *M. tuberculosis* is present (Kimerling et al., 1998; Tostmann et al., 2013; Prideaux et al., 2015). In these cases, bacteria exhibiting drug-specific persistence mechanisms that we would otherwise expect combination therapies to kill, may be of greater clinical concern.

The important take away from these experiments is the identification of the mechanisms that lead to bacterial survival. If these mechanisms, transcriptional downregulation and protein mistranslation, impacted more general pathways, the resulting bacteria may exhibit survival to a wider array of drugs, making them of even greater clinical concern. Therefore, it is important to consider these as persister mechanisms, rather than being concerned whether the resulting phenotype is labelled tolerant, phenotypically resistant, or 'persistent'. These definitional limitations introduce obstacles in communication making it difficult to solve the underlying issue that motivates all this research, how do we improve treatment of *M. tuberculosis?*

Persisters and drug resistance

As discussed above, *M. tuberculosis* is capable of adapting to changes to its environment, including entering states of

metabolic inactivity, rendering most antimycobacterials ineffective until the bacteria reactivate (Connolly et al., 2007). Although, if indeed some bacteria exist in a state of 'dynamic persistence' (Wakamoto et al., 2013) or 'phenotypic resistance' (Javid et al., 2014; Zhu et al., 2018), there is some plausibility that these growing persistent populations may undergo spontaneous mutations that drive them towards resistance and out of persistence. Though there is little evidence demonstrating that resistant populations arise directly from persistent populations (Cohen et al., 2013; Sebastian et al., 2017), any bacteria that survive antibiotic stress can reactivate and grow (Hu et al., 2000; Wood et al., 2013; Boldrin et al., 2020). This cycle of reactivation in the event of improper treatment or poor adherence enables the rise of drug resistant populations (Zhang et al., 2012). This concern is amplified when considering the evidence that mycobacteria can develop phenotypic resistance due to errors in transcription or translation triggered by various stressors (Hu et al., 2000; Wakamoto et al., 2013; Zhu et al., 2018). Though these results were only demonstrated with monotherapy, the spectrum of drug-noncompliant patients is vast. Non-compliant patients range from those that take no drugs to those that miss some of their doses (Munro et al., 2007). In a study of *M. tuberculosis* infected patients in New York City in 1997, 48% were found to be nonadherent. Non-adherent patients took longer to recover and were more likely to develop drug resistant tuberculosis (Pablos-Mendez et al., 1997).

Even if patients remain adherent to therapy, there have been observations of patients with serum drug concentrations below the therapeutic range for these drugs (Kimerling et al., 1998; Tostmann et al., 2013). When drug concentrations fall below the level required to inhibit bacterial growth, drug resistant populations can arise. Furthermore, even when patients maintain therapeutic serum concentrations, authors Prideaux et al. demonstrated that different drugs INH, RIF, PZA, and moxifloxacin (MXF) have "different spatial and temporal patterns of distribution across TB lesion types and compartments" (Prideaux et al., 2015). Though it appears that INH demonstrated good penetration into critical compartments, INH never reached its minimum anaerobic cidal concentration (MAC), as the drug has poor activity on non-replicating bacteria. Inversely, MXF, a drug that has demonstrated promising activity against non-replicating bacteria *in vitro* but failed to shorten treatments in clinical trials (Li et al., 2015) demonstrated sub-cidal concentrations in regions of cavities containing non-replicating bacteria (Prideaux et al., 2015). The two drugs most active against persisters in this study, RIF and PZA, achieved cidal concentrations within relevant compartments of the studied lesions (Prideaux et al., 2015). These results indicate that bacteria exhibiting drug-specific persistence mechanisms may still contribute to the rise of drug resistant bacteria in compartments of relative monotherapy. Of particular concern are persisters to RIF and PZA or the persisters generated to second line therapies used to treat RIF and PZA drug resistant strains, discussed later this section.

The development of drug resistant *M. tuberculosis* can be considered a stepwise process (Allue-Guardia et al., 2021). Before acquiring "high-level" resistance mutations typically associated with clinical strains of *M. tuberculosis*, strains may first accrue "low-level" resistance mutations that enable the bacteria to survive higher concentrations of antibiotics before undergoing cell death (Safi et al., 2013). The importance of low-level resistance to the pathogenesis of *M. tuberculosis*, has been discussed well in a review on the evolution of antibiotic resistance in *M. tuberculosis* (Fonseca et al., 2015). Scientists studying the development of EMB resistance in clinical isolates suggest that these low-level resistant mutants are preferentially selected in patients exposed to sub-therapeutic drug concentrations (Safi et al., 2013). These low-level resistance mutations are typically thought to be associated with efflux pump systems (Machado et al., 2012). As the strains accumulate low-level resistance mutations, they provide a background for high-level resistant mutants to arise from (Martins et al., 2009). Aside from the upregulation of efflux pumps, other mutations that increase antibiotic tolerance have been implicated in the development of high-level drug resistant *M. tuberculosis* (Allue-Guardia et al., 2021). Two such mutations include transcription factor *prpR* and the gene encoding glycerol-3-kinase, *glpK*, which have been demonstrated to promote drug tolerance to clinically relevant antimycobacterial drugs (Hicks et al., 2018; Bellerose et al., 2019). These examples demonstrate the role that persistence could be playing in the development of drug resistant *M. tuberculosis.*

Among persister populations may exist low-level resistant mutations that would otherwise not survive if not for the protection offered by persistence. As time goes on, in some patient's compliance decreases (Jin et al., 2008), in others their metabolism induces sub-therapeutic drug levels (Kimerling et al., 1998), or in some lesions drug concentrations poorly penetrate compartments of lesions (Prideaux et al., 2015) providing the opportunity for either dynamic persistence (Wakamoto et al., 2013; Javid et al., 2014; Zhu et al., 2018) or low-level resistance mutations to exert their survival benefit. Subsequent growth leads to accumulation of low-level resistance or drug tolerance mutations until eventually strains are fully drug resistant. Once fully drug resistant, strains begin to undergo compensatory mutations to reduce the fitness cost of preliminary resistance mutations, resulting in clinically relevant drug resistant strains (Comas et al., 2012). The concept of compensatory mutations is reviewed well elsewhere (Castro et al., 2020). The bacterial mechanisms discussed in previous sections result in greater bacterial survival to monotherapy and therefore increased risk of spontaneous drug resistance, further underscoring the need for multidrug therapy when treating *M. tuberculosis*.

Once drug resistant organisms develop, the most concerning strains are those resistant to RIF and PZA. So long as strains are susceptible to these two drugs, treatment times remain between 6-9 months (Mase and Chorba, 2019). PZA resistant but RIF susceptible strains require 9-month treatment, RIF resistant but PZA susceptible strains require 12–18-month treatment, and strains resistant to both of these drugs require 18-month treatment, the same time required prior to the discovery of these two drugs (Mase and Chorba, 2019). Development of new drugs for *M. tuberculosis* has been slow, with only 3 new drugs, pretomanid, delamanid, and bedaquiline being approved in the last 40 years (Murray et al., 2015). Two of these drugs, bedaquiline and delamanid, have demonstrated activity against dormant bacteria (Koul et al., 2008; Chen et al., 2017). These drugs are typically reserved for multidrug resistant cases, but as evidenced by the treatment times of multidrug resistant bacteria, neither of these have demonstrated the same impact on treatment times as RIF and PZA (Mase and Chorba, 2019).

While we are unaware of studies comparing treatment outcomes of INH monoresistant (INHR) *M. tuberculosis* directly to RIF monoresistant (RIFR) *M. tuberculosis* in the same patient populations, studies of these monoresistant strains have been carried out in separate patient populations. In the following paragraph we discuss the general trend that patient outcomes for RIFR *M. tuberculosis* are worse than those of INHR *M. tuberculosis*. Given the knowledge that RIF has greater activity on persistent *M. tuberculosis* than INH, we suggest that the reason for these worse outcomes can be attributed to the reduced killing of persistent bacteria that RIF otherwise provides.

In 2019 a meta-analysis found the success rate for drug susceptible *M. tuberculosis* infections was 80.1%, multidrug resistant *M. tuberculosis* was 58.4%, and extensively drug resistant *M. tuberculosis* was 27.1% (Chaves Torres et al., 2019). Success was defined as patients that fit the criteria for "cure" or "treatment completion". In one retrospective cohort analysis RIF monoresistance was found to occur less than INH monoresistance with 178 cases compared to 3469 (Prach et al., 2013). In this study it was concluded that compared to drug susceptible strains, patients with RIF resistant *M. tuberculosis* were twice as likely to die (Prach et al., 2013). Another study of 39 patients with RIF resistant *M. tuberculosis* identified only 20 patients that were cured. Of the 39 patients only 30 could be assessed for outcome as the other 9 had either died or been lost to follow up (Meyssonnier et al., 2014). In a study of 165 patients with INH resistant *M. tuberculosis* 140 had treatment success, while 12 had an unsuccessful outcome (Romanowski et al., 2017). The issue of RIF monoresistance was reviewed well by Malenfant and Brewer in 2021 (Malenfant and Brewer, 2021).

Given the heterogeneity of patient populations across these studies, it is difficult to draw conclusions about the outcomes of RIF monoresistant *M. tuberculosis* compared directly to other monoresistant strains. However, from the studies presented here it appears that patients with INHR *M. tuberculosis* experience more positive outcomes than those with RIFR *M. tuberculosis*. If further analyses were carried out that validated this trend that RIF resistance leads to worse outcomes than other resistances, this could illustrate further the importance of persisters and RIF's role in killing them to patient outcomes. Persistent bacteria that would otherwise be killed by RIF may serve as a reservoir for the rise of multidrug resistant strains in compliant patients, as discussed above. In lieu of this line of experimentation, it is evident that rifampicin resistant strains require the longest treatment times (Mase and Chorba, 2019). It has been documented that longer treatment regimens have a lower compliance rate than shorter treatment regimens, and as discussed low adherence can lead to drug resistant populations (Jin et al., 2008).

Multidrug persistence

It is common for persisters to be deemed or implied to be 'multidrug tolerant' (Keren et al., 2004b; Willenborg et al., 2014). In this context, multidrug tolerance means that when bacteria are grown, split into different aliquots, and treated with a range of different drugs, each aliquot demonstrates a persister population. Though persister populations are more and more frequently referred to as heterogenous, descriptions such as these paint a less clear picture. In two such studies a range of drugs were administered to *Streptococcus suis* and *E. coli* (Keren et al., 2004b; Willenborg et al., 2014). Analysis of kill curves performed in these two studies reveal persister populations that vary depending on the drug used in the experiment. As discussed earlier in this review, these populations are likely a mixture of pre-formed stochastic persisters and triggered persisters formed in response to the applied antibiotic stress. However, since this is still up for debate its informative to analyze these results under both paradigms.

Going stepwise, the first analysis comes through the lens of stochastic persistence. Since these bacteria are obtained from the same culture prior to antibiotic treatment, it is safe to assume the level of persistent bacteria should be the same prior to treatment. In the case of the *E. coli* experiment (Keren et al., 2004b), the clearest difference is between ofloxacin, a DNA gyrase inhibitor, and tobramycin, a bacterial ribosome inhibitor. Treatment with ofloxacin revealed a persister population approximately 2-3x in size to the tobramycin treated population (Keren et al., 2004b). Assuming pre-existing persister populations of the same size, this indicates that the true persister population size is closer to that revealed by ofloxacin and that the population observed after tobramycin treatment is a smaller portion of that larger population. This begs the question, is something about these bacteria different? Why did these bacteria persist to this point in the presence of ofloxacin, but not in the presence of tobramycin?

This could be attributed to the efficacy of the drug. For example, RIF has a greater capacity to kill *M. tuberculosis* persister cells, but has very little impact on persister cells of *S. suis* or *Borrelia burgdorferi* (Keren et al., 2004b; Hu et al., 2015; Feng et al., 2015).

To illustrate the concepts discussed here, we generated the schematic seen in Figure 2. This schematic illustrates a susceptible population compared to persistent populations observed to two different drugs, x and y (Figure 2A). In this case, assuming the persistent bacteria are the same or persistent *via* the same mechanism, tobramycin may be demonstrating a higher efficiency in killing these persistent bacteria than ofloxacin (Figure 2B1). The alternative explanation is that these bacteria are different, and that the populations revealed are engaging in two distinct mechanisms that confer persistence to one drug or the other (Figure 2B3), possibly with some overlap (Figure 2B2).

Through another lens, this higher amount of persisters in the ofloxacin group could be a result of triggered persistence, since ofloxacin and the similar drug ciprofloxacin have both been shown to induce persistence in metabolically active cells (Dorr et al., 2010; Goormaghtigh and Van Melderen, 2019). This explanation continues to beg the question whether triggered persisters are the same as the pre-existing persisters. The only way to discern this concept of cross-persistence is to sequentially treat a population with one drug and then the other, assessing what is commonly referred to as 'cross-tolerance'.

This line of experimentation was carried out on *E. coli* with the drugs ciprofloxacin, ampicillin, rifampin, streptomycin, tetracycline, and levofloxacin (Wiuff et al., 2005; Singh et al., 2010). The results revealed that while some drugs conferred cross tolerance, this cross tolerance was not observed for all drugs even among those that did exhibit cross tolerance in certain combinations. For example, streptomycin persisters demonstrated cross tolerance to ampicillin, but not ciprofloxacin. Ciprofloxacin persisters demonstrated cross tolerance to RIF, but not streptomycin. Rifampin persisters did not exhibit cross tolerance to any drugs (Wiuff et al., 2005). However, even when cross-tolerance was not observed, sterilization did not occur, indicating possible sub-populations within persister populations that were cross-tolerant (Wiuff et al., 2005). Another experiment showed that *E. coli* persisters to streptomycin, ampicillin, and levofloxacin seem to all exhibit cross tolerance (Singh et al., 2010). These results suggest that persisters to one drug are not the same as persisters to another drug and may arrive in the persister state *via* unique mechanisms. In fact, RelE homologues, a toxin module found in *E. coli* and *M. tuberculosis* have demonstrated differential

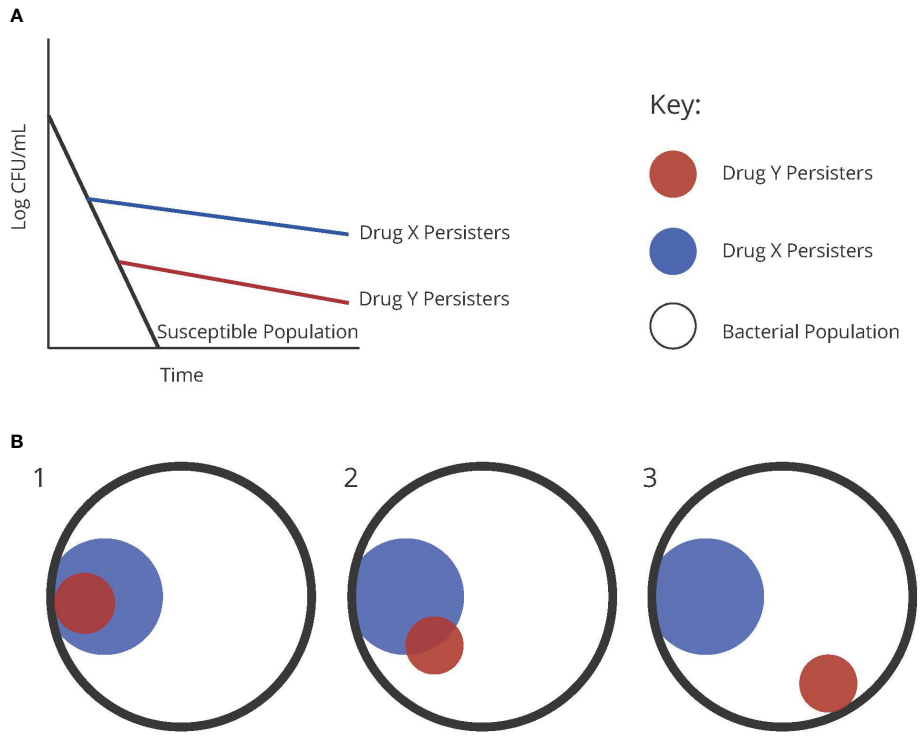


FIGURE 2
(A) Graphical representation of persister populations to two theoretical drugs, x and y, compared to a susceptible population of bacteria. **(B)** Diagrams representing the possible distributions of heterogenous persister populations of two different antibiotics.

impacts on persistence to different antimycobacterial drugs in *M. tuberculosis* (Singh et al., 2010).

Toxin-antitoxin systems are used by many bacterial species. In these systems, bacteria synthesize both a toxin capable of suppressing cellular processes, as well as an anti-toxin capable of binding the toxin and preventing its effects (Boldrin et al., 2020). The anti-toxin tends to degrade much more rapidly than the toxin, requiring active upkeep to maintain antitoxin levels. DNA toxins can impact DNA gyrase, polymerase, or even cleave DNA directly (Bernard and Couturier, 1992; Critchlow et al., 1997). RNA toxins can degrade tRNAs and mRNAs or impair their activity through chemical modifications such as acetylation and phosphorylation (Mets et al., 2017; Culviner and Laub, 2018; Mets et al., 2019). These systems are covered well in a review on toxin-antitoxin systems (Jurėnas et al., 2022).

RelE is an mRNA toxin that cleaves mRNA entering the ribosomal translation site (Pedersen et al., 2003). In the case of RelE homologues in *M. tuberculosis*, RelE2 overexpression increased bacterial persistence to RIF, while its deletion decreased persistence to RIF. RelE3 overexpression increased persistence to INH, while its deletion decreased persistence to INH, but not EMB (Singh et al., 2010). When the authors assessed *M. tuberculosis* for cross-tolerance they found no cross-tolerance between INH, RIF, and PZA (Singh et al., 2010).

Other investigators Grant et al. observed persister populations in *M. smegmatis* and *M. tuberculosis* to drug combinations INH +RIF, Ciprofloxacin (CIP) +RIF, and OFX+INH, suggesting the presence of cross-persistence to these combinations. Interestingly, the persister population generated by 7 days of treatment with a combination of CIP and INH demonstrated cross-tolerance to RIF (Grant et al., 2012). The authors found accelerated killing of these persistent populations when cultures were maintained at a high level of dissolved oxygen (Grant et al., 2012). Though this condition would be difficult to maintain in hypoxic granulomas, this result indicates that an underlying process can be activated by the presence of oxygen in these cross-tolerant populations, either awakening them from the persister state or promoting death of persistent bacteria.

Though most of these studies of persistence have been carried out *in vitro*, experiments performed by Bellerose et al. have shown signs of multidrug-persistence *in vivo* (Bellerose et al., 2019). These authors inoculated mice with wild-type *M. tuberculosis* as well as a *glpK* mutant. As mentioned in the previous section, this *glpK* mutant is unable to phosphorylate glycerol, making the mutant incapable of utilizing glycerol metabolism in the host. The authors demonstrated that this mutant does not exhibit a growth disadvantage in the lungs of mice up to 40 days post infection but when exposed to antimycobacterial PZA, *glpK* mutants in the lungs demonstrated a significant survival advantage compared to the wild-type. Further, when mice were infected with *M. tuberculosis* and treated with INH, EMB, RIF, PZA, the authors identified varying amounts of surviving bacteria. While these surviving bacteria could be interpreted to be persistent, it is difficult to make that claim given bacterial counts were only obtained at a single time point after treatment, so no biphasic killing can be observed (Bellerose et al., 2019). More interestingly, when all four drugs were given in combination, the amount of surviving bacteria most closely resembled the amount surviving after PZA monotherapy (Bellerose et al., 2019). This may be indicative that the mechanism of persistence to PZA that *glpK* mutants undergo *in vivo* demonstrate cross-persistence to other drugs in the standard *M. tuberculosis* drug regimen. One limitation to this interpretation is the fact that drugs were administered as a combination therapy, making the potential mechanisms of persister cell formation much more complex than if drugs were administered in sequence (Bellerose et al., 2019).

It is logical to think that drug-specific INH persisters that are actively growing so long as they downregulate KatG would remain susceptible to other antimycobacterials (Wakamoto et al., 2013) and that those with phenotypic resistance to RIF due to alterations in *rpoB* transcription would remain susceptible to other antimycobacterials (Zhu et al., 2018). It is unclear from any of these studies if the persisters generated will persist when exposed to other antimycobacterials with different mechanisms. Though some investigations begin to show avenues for killing multidrug persistent *M. tuberculosis*, an additional universal mechanism that is more general to all antibiotics may be occurring in the background of these experiments. It would be very illuminating to identify the presence of one of these general mechanisms, as they may lie at the heart of improving treatment of *M. tuberculosis* infections.

Why does it matter?

So far, we discussed that persistent bacteria are relevant for their potential role in *M. tuberculosis* infection severity, treatment times, and development of drug resistant populations. With this importance in mind, we set out to establish the origin, nature, and heterogeneity of persistent *M. tuberculosis*.

The origin of persisters is important to consider in the context of an infected patient. Which persisters are already present in a patient because of the various host environments *M. tuberculosis* finds itself in (Adams et al., 2011; Liu et al., 2016)? Which persisters do we induce when we give treatment? Answering these questions should shape how we configure and administer drug combinations.

The nature of persistent bacteria is important to consider in the evolution of drug resistance. History has already taught us the lesson that *M. tuberculosis* requires a multidrug chemotherapy and never to add a single drug to a failing regimen. Evidence discussed here reveals that dynamic persistence may play a role in this process of single drug resistance.

The heterogeneity of persistent *M. tuberculosis* is important to consider in improving treatment of both drug susceptible and

drug resistant *M. tuberculosis*. Given the lack of study on cross-tolerance, it is unclear whether dynamic persistence exists to the multidrug regimens administered to patients. Patient non-compliance aside, it is also of concern whether drugs remain within the therapeutic range for all compliant patients (Kimerling et al., 1998; Pasipanodya et al., 2012; Tostmann et al., 2013). Drug concentrations outside the therapeutic range may enable dynamic persistence or regrowth despite the administration of combination therapy. The possibilities are endless, but the data are shallow. If persister populations vary in the presence of different drugs as argued here, then what are the next steps?

As illustrated in numerous examples discussed in this review, RIF appears to be key in the killing of persistent bacteria, with pyrazinamide a few steps behind. In the short term, we suggest that it is imperative to find new compounds that replicate RIF's success in killing persistent bacteria in order to shorten treatment times and improve outcomes for RIF resistant *M. tuberculosis*. Salvage of efficacious antibiotics has previously been achieved using adjuvants such as beta-lactamase inhibitors or drug-drug conjugates such as tobramycin-ciprofloxacin (Maiti et al., 1998; Gorityala et al., 2016). One such attempt at a drug-drug conjugate was attempted for RIF by linking it to clofazimine (Saravanan et al., 2021). Though this conjugation did not result in a compound effective against RIF resistant *M. tuberculosis*, it did show activity at lower concentrations than either of the individual compounds. Whether through an adjuvant, RIF analogues that can impact clinical resister mutations, or new drugs that can kill the same persistent bacteria that RIF can, avenues like these could provide a short-term improvement to RIF resistant *M. tuberculosis*.

In the longer term it is imperative to identify general mechanisms of persistence in *M. tuberculosis*, particularly those mechanisms of persistence present in RIF persisters. Any new treatment capable of killing RIF persisters may hold promise to shorten the current treatment of drug susceptible *M. tuberculosis* and provide alternatives in the case of RIF resistance. Before these treatments can be developed, mechanisms of persistence must be further identified and currently known mechanisms of persister enrichment need to be screened for multidrug tolerance to ensure redundancy is avoided.

Conclusions: Are all antibiotic persisters created equal?

Finally, we return to our initial question. Current knowledge on this topic paints a very multifaceted picture of persistence. Convincing data exist to support the existence of some general mechanism of persistence that leads to a basal level of stochastic persisters in a population (Shan et al., 2017; Manuse et al., 2021). Convincing data exist that support the process of triggered persistence, suggesting a more adaptive mechanism to arrive in the persister state (Dorr et al., 2010; Goormaghtigh and Van Melderen, 2019). As argued here, some experiments suggest that persistent bacteria engage in different mechanisms of persistence to survive the presence of different drugs (Singh et al., 2010). Of the data that exist, it appears persistent bacteria to one drug do not necessarily persist in the presence of another (Wiuff et al., 2005; Singh et al., 2010). To our knowledge, neither data exist to suggest stochastic and triggered persisters are composed of the same subpopulation of bacteria, nor does data exist to suggest INH and RIF persisters are composed of the same subpopulation of bacteria. Therefore, based on the current information available, we must assert that no, not all antibiotic persisters are created equal.

Author contributions

MWS, CVM, and MB conceptualized and designed the review. MS wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

This work was supported by Potts Memorial Foundation and NIH grants T32AI007501 (supporting MWS) and R01AI139465 (supporting CVM and MB).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Adams, K. N., Takaki, K., Connolly, L. E., Wiedenhoft, H., Winglee, K., Humbert, O., et al. (2011). Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell* 145 (1), 39–53. doi: 10.1016/j.cell.2011.02.022

Aldridge, B. B., Fernandez-Suarez, M., Heller, D., Ambravaneswaran, V., Irimia, D., Toner, M., et al. (2012). Asymmetry and aging of mycobacterial cells lead to variable growth and antibiotic susceptibility. *Science* 335 (6064), 100–104. doi: 10.1128/mSystems.00396-20

Allue-Guardia, A., Garcia, J. I., and Torrelles, J. B. (2021). Evolution of drug-resistant mycobacterium tuberculosis strains and their adaptation to the human lung environment. *Front. Microbiol.* 12, 612675. doi: 10.3389/fmicb.2021.612675

Balaban, N.Q., Merrin, J., Chait, R., Kowalik, L., and Leibler, S. (2004). Bacterial persistence as a phenotypic switch. *Science* 305 (5690), 1622–1625. doi: 10.1126/science.1099390

Balaban, N.Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D.I., et al. (2019). Definitions and guidelines for research on antibiotic persistence. *Nat. Rev. Microbiol.* 17 (7), 441–448. doi: 10.1038/s41579-019-0196-3

Bellerose, M. M., Baek, S.H., Huang, C.C., Moss, C. E., Koh, E. I., Proulx, M. K., et al. (2019). Common variants in the glycerol kinase gene reduce tuberculosis drug efficacy. *mBio* 10 (4), 1–8. doi: 10.1128/mBio.00663-19

Bernard, P., and Couturier, M. (1992). Cell killing by the f plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. *J. Mol. Biol.* 226 (3), 735–745. doi: 10.1016/0022-2836(92)90629-X

Bigger, J. (1944). Treatment of staphylococcal infections with penicillin by intermittent sterilisation. *Lancet* 244 (6320), 497–500. doi: 10.1016/S0140-6736(00)74210-3

Boldrin, F., Provvedi, R., Cioetto Mazzabo, L., Segafreddo, G., and Manganelli, R. (2020). Tolerance and persistence to drugs: A main challenge in the fight against mycobacterium tuberculosis. *Front. Microbiol.* 11, 1924. doi: 10.3389/fmicb.2020.01924

Brauner, A., Fridman, O., Gefen, O., and Balaban, N. Q. (2016). Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat. Rev. Microbiol.* 14 (5), 320–330. doi: 10.1038/nrmicro.2016.34

Bretl, D. J., Demetriadou, C., and Zahrt, T. C. (2011). Adaptation to environmental stimuli within the host: two-component signal transduction systems of mycobacterium tuberculosis. *Microbiol. Mol. Biol. Rev.* 75 (4), 566–582. doi: 10.1128/MMBR.05004-11

Castro, R. A. D., Borrell, S., and Gagneux, S. (2020). The within-host evolution of antimicrobial resistance in mycobacterium tuberculosis. *FEMS Microbiol. Rev.* 45 (4), 1–24. doi: 10.1093/femsre/fuaa071

Chaves Torres, N. M., Quijano Rodriguez, J. J., Porras Andrade, P.S., Arriaga, M. B., and Netto, E. M. (2019). Factors predictive of the success of tuberculosis treatment: A systematic review with meta-analysis. *PloS One* 14 (12), e0226507. doi: 10.1371/journal.pone.0226507

Chen, X., Hashizume, H., Tomishige, T., Nakamura, I., Matsuba, M., Fujiwara, M., et al. (2017). Delamanid kills dormant mycobacteria in vitro and in a Guinea pig model of tuberculosis. *Antimicrob. Agents Chemother.* 61 (6), 1–11. doi: 10.1128/AAC.02402-16

Cohen, N. R., Lobritz, M. A., and Collins, J. J. (2013). Microbial persistence and the road to drug resistance. *Cell Host Microbe* 13 (6), 632–642. doi: 10.1016/j.chom.2013.05.009

Comas, I., Borrell, S., Roetzer, A., Rose, G., Malla, B., Kato-Maeda, M., et al. (2012). Whole-genome sequencing of RIF-resistant mycobacterium tuberculosis strains identifies compensatory mutations in RNA polymerase genes. *Nat. Genet.* 44 (1), 106–110. doi: 10.1038/ng.1038

Connolly, L. E., Edelstein, P. H., and Ramakrishnan, L. (2007). Why is long-term therapy required to cure tuberculosis? *PloS Med.* 4 (3), e120. doi: 10.1371/journal.pmed.0040120

Corona, F., and Martinez, J. L. (2013). Phenotypic resistance to antibiotics. *Antibiot (Basel)* 2 (2), 237–255. doi: 10.3390/antibiotics2020237

Critchlow, S. E., O'dea, M. H., Howells, A. J., Couturier, M., Gellert, M., Maxwell, A., et al. (1997). The interaction of the f plasmid killer protein, CcdB, with DNA gyrase: induction of DNA cleavage and blocking of transcription. *J. Mol. Biol.* 273 (4), 826–839. doi: 10.1006/jmbi.1997.1357

Culviner, P. H., and Laub, M. T. (2018). Global analysis of the e. coli toxin MazF reveals widespread cleavage of mRNA and the inhibition of rRNA maturation and ribosome biogenesis. *Mol. Cell* 70 (5), 868–880.e10. doi: 10.1016/j.molcel.2018.04.026

Dahl, J. L., Kraus, C. N., Boshoff, H. I., Doan, B., Foley, K., Avarbock, D., et al. (2003). The role of RelMtb-mediated adaptation to stationary phase in long-term persistence of mycobacterium tuberculosis in mice. *Proc. Natl. Acad. Sci. U.S.A.* 100 (17), 10026–10031. doi: 10.1073/pnas.1631248100

Dorr, T., Vulic, M., and Lewis, K. (2010). Ciprofloxacin causes persister formation by inducing the TisB toxin in escherichia coli. *PloS Biol.* 8 (2), e1000317. doi: 10.1371/journal.pbio.1000317

Dutta, N. K., Klinkenberg, L.G., Vazquez, M. J., Segura-Carro, D., Colmenarejo, G., Ramon, F., et al. (2019). Inhibiting the stringent response blocks mycobacterium tuberculosis entry into quiescence and reduces persistence. *Sci. Adv.*, 5(3) eaav2104. doi: 10.1126/sciadv.aav2104

Feng, J., Auwaerter, P. G., and Zhang, Y. (2015). Drug combinations against borrelia burgdorferi persisters in vitro: eradication achieved by using daptomycin, cefoperazone and doxycycline. *PloS One* 10 (3), e0117207. doi: 10.1371/journal.pone.0117207

Fonseca, J. D., Knight, G. M., and McHugh, T. D. (2015). The complex evolution of antibiotic resistance in mycobacterium tuberculosis. *Int. J. Infect. Dis.* 32, 94–100. doi: 10.1016/j.ijid.2015.01.014

Goormaghtigh, F., and Van Melderen, L. (2019). Single-cell imaging and characterization of escherichia coli persister cells to ofloxacin in exponential cultures. *Sci. Adv.* 5 (6), eaav9462. doi: 10.1126/sciadv.aav9462

Goossens, S. N., Sampson, S. L., and Van Rie, A. (2020). Mechanisms of drug-induced tolerance in mycobacterium tuberculosis. *Clin. Microbiol. Rev.* 34 (1), 1–21. doi: 10.1128/CMR.00141-20

Gorityala, B. K., Guchhait, G., Fernando, D. M., Deo, S., Mckenna, S.A., Zhanel, G. G, et al. (2016). Adjuvants based on hybrid antibiotics overcome resistance in pseudomonas aeruginosa and enhance fluoroquinolone efficacy. *Angew Chem. Int. Ed Engl.* 55 (2), 555–559. doi: 10.1002/anie.201508330

Grant, S. S., Kaufmann, B B., Chand, N. S., Haseley, N., and Hung, D. T. (2012). Eradication of bacterial persisters with antibiotic-generated hydroxyl radicals. *Proc. Natl. Acad. Sci. U.S.A.* 109 (30), 12147–12152. doi: 10.1073/pnas.1203735109

Hu, Y., Mangan, J. A., Dhillon, J., Sole, K. M., Mitchison, D. A., Butcher, P.D., et al. (2018). Clinically prevalent mutations in mycobacterium tuberculosis alter propionate metabolism and mediate multidrug tolerance. *Nat. Microbiol.* 3 (9), 1032–1042. doi: 10.1038/s41564-018-0218-3

Hu, Y., Liu, A., Ortega-Muro, F., Alameda-Martin, L., Mitchison, D., and Coates, A. (2000). Detection of mRNA transcripts and active transcription in persistent mycobacterium tuberculosis induced by exposure to rifampin or Pyrazinamide. *J. Bacteriol* 182 (22), 6358–6365. doi: 10.1128/JB.182.22.6358-6365.2000

Hu, Y., Liu, A., Ortega-Muro, F., Alameda-Martin, L., Mitchison, D., Coates, A, et al. (2015). High-dose RIF kills persisters, shortens treatment duration, and reduces relapse rate *in vitro* and *in vivo*. *Front. Microbiol.* 6, 641. doi: 10.3389/fmicb.2015.00641

Jain, P., Weinrick, B. C., Kalivoda, E. J., Yang, H., Munsamy, V., Vilcheze, C, et al. (2016). Dual-reporter mycobacteriophages (Phi2DRMs) reveal preexisting mycobacterium tuberculosis persistent cells in human sputum. *mBio* 7 (5), 1–13. doi: 10.1128/mBio.01023-16

Javid, B., Sorrentino, F., Toosky, M., Zheng, W., Pinkham, J. T., Jain, N., et al. (2014). Mycobacterial mistranslation is necessary and sufficient for RIF phenotypic resistance. *Proc. Natl. Acad. Sci. U.S.A.* 111 (3), 1132–1137. doi: 10.1073/pnas.1317580111

Jin, J., Sklar, G. E., Min Sen Oh, V., and Chuen Li, S. (2008). Factors affecting therapeutic compliance: A review from the patient's perspective. *Ther. Clin. Risk Manag* 4 (1), 269–286. doi: 10.2147/tcrm.s1458

Jurėnas, D., Fraikin, N., Goormaghtigh, F., and Van Melderen, L.. (2022). Biology and evolution of bacterial toxin–antitoxin systems. *Nat. Rev. Microbiol* 20, 335–350. doi: 10.1038/s41579-021-00661-1

Keren, I., Kaldalu, N., Spoering, A., Wang, Y., and Lewis, K. (2004a). Persister cells and tolerance to antimicrobials. *FEMS Microbiol. Lett.* 230 (1), 13–18. doi: 10.1016/S0378-1097(03)00856-5

Keren, I., Shah, D., Spoering, A., Kaldalu, N., and Lewis, K. (2004b). Specialized persister cells and the mechanism of multidrug tolerance in escherichia coli. *J. Bacteriol* 186 (24), 8172–8180. doi: 10.1128/JB.186.24.8172-8180.2004

Kimerling, M. E., Phillips, P., Patterson, P., Hall, M., Robinson, C. A., and Dunlap, N. E. (1998). Low serum antimycobacterial drug levels in non-HIV-infected tuberculosis patients. *Chest* 113 (5), 1178–1183. doi: 10.1378/chest.113.5.1178

Koul, A., Vranckx, L., Dendouga, N., Balemans, W., Van Den Wyngaert, I., Vergauwen, K., et al. (2008). Diarylquinolines are bactericidal for dormant mycobacteria as a result of disturbed ATP homeostasis. *J. Biol. Chem.* 283 (37), 25273–25280. doi: 10.1074/jbc.M803899200

Lei, Q., Wang, H., Zhao, Y., Dang, L., Zhu, C., Lv, X., et al. (2019). Determinants of serum concentration of first-line anti-tuberculosis drugs from China. *Medicine* 98 (41), e17523. doi: 10.1097/MD.0000000000017523

Lewis, K. (2007). Persister cells, dormancy and infectious disease. *Nat. Rev. Microbiol.* 5 (1), 48–56. doi: 10.1038/nrmicro1557

Li, S. Y., Irwin, S. M., Converse, P.J., Mdluli, K. E., Lenaerts, A. J., and Nuermberger, E. L. (2015). Evaluation of moxifloxacin-containing regimens in pathologically distinct murine tuberculosis models. *Antimicrob. Agents Chemother.* 59 (7), 4026–4030. doi: 10.1128/AAC.00105-15

Liu, Y., Tan, S., Huang, L., Abramovitch, R. B., Rohde, K. H., Zimmerman, M. D., et al. (2016). Immune activation of the host cell induces drug tolerance in mycobacterium tuberculosis both in vitro and in vivo. *J. Exp. Med.* 213 (5), 809–825. doi: 10.1084/jem.20151248

Machado, D., Couto, I., Perdigao, J., Rodrigues, L., Portugal, I., Baptista, P., et al. (2012). Contribution of efflux to the emergence of INH and multidrug resistance in mycobacterium tuberculosis. *PloS One* 7 (4), e34538. doi: 10.1371/journal.pone.0034538

Maiti, S. N., Phillips, O. A., Micetich, R. G., and Livermore, D. M. (1998). Beta-lactamase inhibitors: agents to overcome bacterial resistance. *Curr. Med. Chem.* 5 (6), 441–456. doi: 10.2174/092986730566220319110127

Malenfant, J. H., and Brewer, T. F. (2021). RIF mono-resistant tuberculosis–a review of an uncommon but growing challenge for global tuberculosis control. *Open Forum Infect. Dis.* 8 (2), 1–6. doi: 10.1093/ofid/ofab018

Manuse, S., Shan, Y., Canas-Duarte, S. J., Bakshi, S., Sun, W. S., Mori, H., et al. (2021). Bacterial persisters are a stochastically formed subpopulation of low-energy cells. *PloS Biol.* 19 (4), e3001194. doi: 10.1371/journal.pbio.3001194

Martins, A., Iversen, C., Rodrigues, L., Spengler, G., Ramos, J., Kern, W. V., et al. (2009). An AcrAB-mediated multidrug-resistant phenotype is maintained following restoration of wild-type activities by efflux pump genes and their regulators. *Int. J. Antimicrob. Agents* 34 (6), 602–604. doi: 10.1016/j.ijantimicag.2009.06.029

Mase, S. R., and Chorba, T. (2019). Treatment of drug-resistant tuberculosis. *Clin. Chest Med.* 40 (4), 775–795. doi: 10.1016/j.ccm.2019.08.002

McCune, R. M., Feldmann, F. M., Lambert, H. P., and Mcdermott, W. (1966). Microbial persistence. i. the capacity of tubercle bacilli to survive sterilization in mouse tissues. *J. Exp. Med.* 123 (3), 445–468. doi: 10.1084/jem.123.3.445

McCune, R. M., Feldmann, F. M., and McDermott, W. (1966). Microbial persistence. II. characteristics of the sterile state of tubercle bacilli. *J. Exp. Med.* 123 (3), 469–486. doi: 10.1084/jem.123.3.469

Mets, T., Lippus, M., Schryer, D., Liiv, A., Kasari, V., Paier, A., et al. (2017). Toxins MazF and MqsR cleave escherichia coli rRNA precursors at multiple sites. *RNA Biol.* 14 (1), 124–135. doi: 10.1080/15476286.2016.1259784

Mets, T., Kasvandik, S., Saarma, M., Maivali, U., Tenson, T., Kaldalu, N, et al. (2019). Fragmentation of escherichia coli mRNA by MazF and MqsR. *Biochimie* 156, 79–91. doi: 10.1016/j.biochi.2018.10.004

Meyssonnier, V., Bui, T.V., Veziris, N., Jarlier, V., and Robert, J. (2014). RIF mono-resistant tuberculosis in France: a 2005-2010 retrospective cohort analysis. *BMC Infect. Dis.* 14, 18. doi: 10.1186/1471-2334-14-18

Moker, N., Dean, C. R., and Tao, J. (2010). Pseudomonas aeruginosa increases formation of multidrug-tolerant persister cells in response to quorum-sensing signaling molecules. *J. Bacteriol* 192 (7), 1946–1955. doi: 10.1128/JB.01231-09

Munro, S. A., Lewin, S.A., Smith, H. J., Engel, M. E., Fretheim, A., and Volmink, J. (2007). Patient adherence to tuberculosis treatment: a systematic review of qualitative research. *PloS Med.* 4 (7), e238. doi: 10.1371/journal.pmed.0040238

Murray, J. F. (2004). A century of tuberculosis. *Am. J. Respir. Crit. Care Med.* 169 (11), 1181–1186. doi: 10.1164/rccm.200402-140OE

Murray, J. F., Schraufnagel, D. E., and Hopewell, P. C. (2015). Treatment of tuberculosis. a historical perspective. *Ann. Am. Thorac. Soc.* 12 (12), 1749–1759. doi: 10.1513/AnnalsATS.201509-632PS

Nahid, P., Dorman, S. E., Alipanah, N., Barry, P. M., Brozek, J. L., Cattamanchi, A., et al. (2016). Executive summary: Official American thoracic Society/Centers for disease control and Prevention/Infectious diseases society of America clinical practice guidelines: Treatment of drug-susceptible tuberculosis. *Clin. Infect. Dis.* 63 (7), 853–867. doi: 10.1093/cid/ciw566

Orman, M. A., and Brynildsen, M. P. (2013). Dormancy is not necessary or sufficient for bacterial persistence. *Antimicrob. Agents Chemother.* 57 (7), 3230–3239. doi: 10.1128/AAC.00243-13

Pablos-Mendez, A., Knirsch, C. A., Barr, R. G., Lerner, B. H., and Frieden,T. R. (1997). Nonadherence in tuberculosis treatment: predictors and consequences in new York city. *Am. J. Med.* 102 (2), 164–170. doi: 10.1016/S0002-9343(96)00402-0

Pasipanodya, J. G., Srivastava, S., and Gumbo, T. (2012). Meta-analysis of clinical studies supports the pharmacokinetic variability hypothesis for acquired drug resistance and failure of antituberculosis therapy. *Clin. Infect. Dis.* 55 (2), 169–177. doi: 10.1093/cid/cis353

Pedersen, K., Zavialov, A. V., Pavlov, M. Y., Elf, J., Gerdes, K., and Ehrenberg, M. (2003). The bacterial toxin RelE displays codon-specific cleavage of mRNAs in the ribosomal a site. *Cell* 112 (1), 131–140. doi: 10.1016/S0092-8674(02)01248-5

Prach, L. M., Pascopella, L., Barry, P. M., Flood, J., Porco, T. C., Hopewell, P. C., et al. (2013). Rifampin monoresistant tuberculosis and HIV comorbidity in California, 1993-2008: a retrospective cohort study. *AIDS* 27 (16), 2615–2622. doi: 10.1097/01.aids.0000432445.07437.07

Prideaux, B., Via, L. E., Zimmerman, M. D., Eum, S., Sarathy, J., O'brien, P., et al. (2015). The association between sterilizing activity and drug distribution into tuberculosis lesions. *Nat. Med.* 21 (10), 1223–1227. doi: 10.1038/nm.3937

Pullan, S. T., Allnutt, J. C., Devine, R., Hatch, K. A., Jeeves, R. E., Hendon-Dunn, C. L., et al. (2016). The effect of growth rate on PZA activity in mycobacterium tuberculosis - insights for early bactericidal activity? *BMC Infect. Dis.* 16, 205. doi: 10.1186/s12879-016-1533-z

Rego, E. H., Audette, R. E., and Rubin, E. J. (2017). Deletion of a mycobacterial divisome factor collapses single-cell phenotypic heterogeneity. *Nature* 546 (7656), 153–157. doi: 10.1038/nature22361

Richardson, K., Bennion, O. T., Tan, S., Hoang, A. N., Cokol, M., and Aldridge, B. B. (2016). Temporal and intrinsic factors of RIF tolerance in mycobacteria. *Proc. Natl. Acad. Sci.* 113 (29), 8302–8307. doi: 10.1073/pnas.1600372113

Romanowski, K., Chiang, L. Y., Roth, D. Z., Krajden, M., Tang, P., Cook, V. J., et al. (2017). Treatment outcomes for isoniazid-resistant tuberculosis under program conditions in British Columbia, Canada. *BMC Infect. Dis.* 17 (1), 604. doi: 10.1186/s12879-017-2706-0

Safi, H., Lingaraju, S., Amin, A., Kim, S., Jones, M., Holmes, M., et al. (2013). Evolution of high-level EMB-resistant tuberculosis through interacting mutations in decaprenylphosphoryl-beta-D-arabinose biosynthetic and utilization pathway genes. *Nat. Genet.* 45 (10), 1190–1197. doi: 10.1038/ng.2743

Saravanan, P., Dusthackeer, V. N. A., Rajmani, R. S., Mahizhaveni, B., Nirmal, C. R., Rajadas, S. E., et al. (2021). Discovery of a highly potent novel RIF analog by preparing a hybrid of the precursors of the antibiotic drugs RIF and clofazimine. *Sci. Rep.* 11 (1), 1029. doi: 10.1038/s41598-020-80439-2

Sebastian, J., Swaminath, S., Nair, R. R., Jakkala, K., Pradhan, A., and Ajitkumar, P. (2017). De Novo emergence of genetically resistant mutants of mycobacterium tuberculosis from the persistence phase cells formed against antituberculosis drugs *in vitro*. *Antimicrob. Agents Chemother.* 61 (2), 1–25. doi: 10.1128/AAC.01343-16

Seth, V., Beotra, A., Seth, S. D., Semwal, O. P., Kabra, S., Jain, Y., et al. (1993). Serum concentrations of RIF and isoniazid in tuberculosis. *Indian Pediatr.* 30 (9), 1091–1098. doi: 10.4269/ajtmh.2009.81.322

Shan, Y., Brown Gandt, A., Rowe, S. E., Deisinger, J. P., Conlon, B.P., and Lewis, K. (2017). ATP-dependent persister formation in escherichia coli. *mBio* 8 (1), 1–14. doi: 10.1128/mBio.02267-16

Singh, R., Barry, C. E.3rd, and Boshoff, H. I. (2010). The three RelE homologs of mycobacterium tuberculosis have individual, drug-specific effects on bacterial antibiotic tolerance. *J. Bacteriol* 192 (5), 1279–1291. doi: 10.1128/JB.01285-09

Tostmann, A., Mtabho, C. M., Semvua, H. H., Van Den Boogaard, J., Kibiki, G.S., Boeree, M. J., et al. (2013). Pharmacokinetics of first-line tuberculosis drugs in Tanzanian patients. *Antimicrob. Agents Chemother.* 57 (7), 3208–3213. doi: 10.1128/AAC.02599-12

Vilcheze, C., Hartman, T., Weinrick, B., and Jacobs, W. R. Jr. (2013). Mycobacterium tuberculosis is extraordinarily sensitive to killing by a vitamin c-induced fenton reaction. *Nat. Commun.* 4, 1881. doi: 10.1038/ncomms2898

Vilcheze, C., Hartman, T., Weinrick, B., Jain, P., Weisbrod, T. R., Leung, L. W., et al. (2017). Enhanced respiration prevents drug tolerance and drug resistance in mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. U.S.A.* 114 (17), 4495–4500. doi: 10.1073/pnas.1704376114

Volzing, K. G., and Brynildsen, M. P. (2015). Stationary-phase persisters to ofloxacin sustain DNA damage and require repair systems only during recovery. *mBio* 6 (5), e00731–e00715. doi: 10.1128/mBio.00731-15

Wakamoto, Y., Dhar, N., Chait, R., Schneider, K., Signorino-Gelo, F., Leibler, S., et al. (2013). Dynamic persistence of antibiotic-stressed mycobacteria. *Science* 339 (6115), 91–95. doi: 10.1126/science.1229858.

Warner, D. F., and Mizrahi, V. (2006). Tuberculosis chemotherapy: the influence of bacillary stress and damage response pathways on drug efficacy. *Clin. Microbiol. Rev.* 19 (3), 558–570. doi: 10.1128/CMR.00060-05

WHO (2021). *Global Tuberculosis Report 2020*. (Geneva: World Health Organization)

Willenborg, J., Willms, D., Bertram, R., Goethe, R., and Valentin-Weigand, P. (2014). Characterization of multi-drug tolerant persister cells in streptococcus suis. *BMC Microbiol.* 14 (1), 120. doi: 10.1186/1471-2180-14-120

Wiuff, C., Zappala, R. M., Regoes, R. R., Garner, K. N., Baquero, F., and Levin, B. R. (2005). Phenotypic tolerance: antibiotic enrichment of noninherited resistance in bacterial populations. *Antimicrob. Agents Chemother.* 49 (4), 1483–1494. doi: 10.1128/AAC.49.4.1483-1494.2005

Wood, T. K., Knabel, S. J., and Kwan, B. W. (2013). Bacterial persister cell formation and dormancy. *Appl. Environ. Microbiol.* 79 (23), 7116–7121. doi: 10.1128/AEM.02636-13

Xie, Z., Siddiqi, N., and Rubin, E. J. (2005). Differential antibiotic susceptibilities of starved mycobacterium tuberculosis isolates. *Antimicrob. Agents Chemother.* 49 (11), 4778–4780. doi: 10.1128/AAC.49.11.4778-4780.2005

Zahrt, T. C., and Deretic, V. (2001). Mycobacterium tuberculosis signal transduction system required for persistent infections. *Proc. Natl. Acad. Sci. U.S.A.* 98 (22), 12706–12711. doi: 10.1073/pnas.221272198

Zhang, Y., Shi, W., Zhang, W., and Mitchison, D. (2014). Mechanisms of PZA action and resistance. *Microbiol. Spectr.* 2 (4), MGM2–0023-2013. doi: 10.1128/9781555818845.ch24

Zhang, Y., Yew, W. W., and Barer, M. R. (2012). Targeting persisters for tuberculosis control. *Antimicrob Agents Chemother* 56 (5), 2223–2230. doi: 10.1128/AAC.06288-11

Zhu, J.-H., Wang, B.–W., Pan, M., Zeng, Y.–N., Rego, H., Javid, B., et al. (2018). RIF can induce antibiotic tolerance in mycobacteria via paradoxical changes in rpoB transcription. *Nat. Commun.* 9 (1), 4218. doi: 10.1038/s41467-018-06667-3

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 22 August 2022
DOI 10.3389/fcimb.2022.958240

OPEN ACCESS

EDITED BY
Ben Gold,
Weill Cornell Medicine, United States

REVIEWED BY
Babak Javid,
University of California, San Francisco, United States
Robert Sander Jansen,
Radboud University Nijmegen, Netherlands

*CORRESPONDENCE
Hyungjin Eoh
heoh@usc.edu

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 31 May 2022
ACCEPTED 03 August 2022
PUBLISHED 22 August 2022

CITATION
Eoh H, Liu R, Lim J, Lee JJ and Sell P (2022) Central carbon metabolism remodeling as a mechanism to develop drug tolerance and drug resistance in *Mycobacterium tuberculosis*. *Front. Cell. Infect. Microbiol.* 12:958240. doi: 10.3389/fcimb.2022.958240

Central carbon metabolism remodeling as a mechanism to develop drug tolerance and drug resistance in *Mycobacterium tuberculosis*

Hyungjin Eoh*, Rachel Liu, Juhyeon Lim, Jae Jin Lee and Philip Sell

Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles, CA, United States

Suboptimal efficacy of the current antibiotic regimens and frequent emergence of antibiotic-resistant *Mycobacterium tuberculosis* (Mtb), an etiological agent of tuberculosis (TB), render TB the world's deadliest infectious disease before the COVID-19 outbreak. Our outdated TB treatment method is designed to eradicate actively replicating populations of Mtb. Unfortunately, accumulating evidence suggests that a small population of Mtb can survive antimycobacterial pressure of antibiotics by entering a "persister" state (slowly replicating or non-replicating and lacking a stably heritable antibiotic resistance, termed drug tolerance). The formation of drug-tolerant Mtb persisters is associated with TB treatment failure and is thought to be an adaptive strategy for eventual development of permanent genetic mutation-mediated drug resistance. Thus, the molecular mechanisms behind persister formation and drug tolerance acquisition are a source of new antibiotic targets to eradicate both Mtb persisters and drug-resistant Mtb. As Mtb persisters are genetically identical to antibiotic susceptible populations, metabolomics has emerged as a vital biochemical tool to differentiate these populations by determining phenotypic shifts and metabolic reprogramming. Metabolomics, which provides detailed insights into the molecular basis of drug tolerance and resistance in Mtb, has unique advantages over other techniques by its ability to identify specific metabolic differences between the two genetically identical populations. This review summarizes the recent advances in our understanding of the metabolic adaptations used by Mtb persisters to achieve intrinsic drug tolerance and facilitate the emergence of drug resistance. These findings present metabolomics as a powerful tool to identify previously unexplored antibiotic targets and improved combinations of drug regimens against drug-resistant TB infection.

KEYWORDS

drug tolerance, drug resistance, tuberculosis, metabolomics, catalytic shift

Highlights

- Unlike other omics techniques, metabolomics reveals the biochemical state of Mtb as metabolites are the final products of enzymatic, posttranslational, allosteric, and environmental integration.
- High-persistence forms of Mtb have complicated chemotherapeutic options because they are likely to evolve drug-resistant Mtb strains.
- Mtb in a persistent state shifts its metabolic networks to mitigate the production of antibiotic-induced reactive oxygen species and accompanied cellular damage, whereby Mtb persisters evade mycobactericidal activity of antibiotics.
- Multiple metabolic networks within Mtb persisters' central carbon metabolism and their carbon fluxes are simultaneously shifted to mitigate antibiotic effects.
- Metabolic shifts required to form Mtb persisters are also observed in drug-resistant TB clinical isolates, all of which are a source of new antibiotic targets to efficiently control drug-resistant TB infection.

Introduction

The introduction of antibiotics into clinical medicine has drastically changed the societal impact of infectious diseases from fatal bacterial infections into predictably curable maladies (Hutchings et al., 2019). The clinical impact of antibiotic treatment, however, has been seriously compromised by the staggering surge of drug resistance (DR), largely due to uncontrolled abuse of antibiotics (Nathan, 2014; Fair and Tor, 2014; Nathan and Barry, 2015; Schrader et al., 2020). DR emergence is exacerbated by the unforeseen failures of antibiotic research. A key factor contributing to this shortfall is a lack of adequate assays to investigate antibiotic effects and their modes of action. Early methods of antibiotic discovery relied on simple screening assays designed to detect phenotypic viability and metabolic activities. However, most antibiotics kill target pathogens through a series of complicated events that cannot be identified by any single assay. Thus, limitations of these classical methods and underdetermined modes of action frequently result in the development of new antibiotics that are highly vulnerable to the emergence of DR mutants because they often fail to eradicate the target pathogens.

The advent of unbiased systems-level disciplines has helped predict DR emergence in clinically isolated pathogens (Chan and Loscalzo, 2012). Metabolomics is the youngest among these techniques and focuses on metabolites present in a certain biological system, such as bacteria after exposure to a given set of environmental conditions (Rhee, 2013). Metabolites are defined as the substrates or products of enzymes and their cofactors that cover diverse cellular activities (Bennett et al., 2009; Lu et al., 2017). Growing evidence has shown that metabolites play a crucial role in the cellular and physiological processes required for the viability and virulence of infectious agents. Metabolomics has become an integral tool to determine the biochemical state of pathogens, thereby elucidating their virulence factors and pathogenicity. The ability to monitor intrabacterial biochemical states and functional interactions with the host immune system has poised metabolomics to uniquely fill the gaps in antibiotic research by investigating metabolic networks required for DR emergence and discovering conceptually new antibiotics (Warner, 2014).

Although tuberculosis (TB) has afflicted humans for more than 4,000 years, it remains a major public health issue in the top 10 leading causes of human death worldwide (Zaman, 2010). Despite recent reduction in the total TB incidence rate, the situation remains extremely dire due to the high frequency of genetic DR mutants (Connolly et al., 2007; Schrader et al., 2020). The World Health Organization (WHO) estimated that, in 2021, roughly half a million new TB cases were caused by DR TB (WHO, 2021). The DR forms of TB include multidrug-resistant (MDR), pre-extensively drug-resistant (pre-XDR), and extensively drug-resistant (XDR). MDR-TB is defined to be resistant to at least isoniazid (INH) and rifampin (RIF), two of the main first-line TB antibiotics. Pre-XDR is newly included to DR TB forms and defined as MDR plus additional resistance to any fluoroquinolone. XDR-TB is defined as MDR plus additional resistance to any fluoroquinolone and at least one additional group A TB antibiotics such as levofloxacin, moxifloxacin, bedaquiline, and linezolid (Viney et al., 2021). These DR TB pose a serious challenge for effective TB control and hinder successful treatment outcomes.

Unlike mutation-acquired DR, *Mycobacterium tuberculosis* (Mtb) persister cells (persisters) possess intrinsic drug tolerance that is phenotypically reversible and free of genetic mutations (Lewis, 2010; Conlon et al., 2016; Fisher et al., 2017). Several groups have recently identified a growing drug-tolerant subpopulation (Adams et al., 2011; Peyrusson et al., 2020). However, we term Mtb persisters as a slowly replicating or non-replicating subpopulation that transiently resists antibiotic effects. Mtb persisters are known to withstand the mycobactericidal effects of nearly all TB antibiotics, even at lethal concentrations, without a change in antibiotic susceptibility upon regrowth under antibiotic-free conditions (Schrader et al., 2021). Accumulating evidence supports that regrowth populations, stemming from bacterial persisters that survive in the human body for a prolonged period of time, are responsible for causing a wide range of chronic and recurrent

infections and may serve as a source for permanent DR mutants, especially among immune-compromised patients (Levin and Rozen, 2006; Fridman et al., 2014; Levin-Reisman et al., 2017). Thus, Mtb persisters are a critical, yet seriously understudied, component of the TB disease burden.

Here, we review emerging applications of metabolomics to identify Mtb metabolic and biochemical remodeling during persister formation to inform early TB antibiotic discovery and prevent the development of DR mutations. We focus on central carbon metabolism (CCM) remodeling during Mtb persister formation as the major mechanism of their adaptive strategy and altered biochemical states (Eoh, 2014; Hartman et al., 2017; Jansen and Rhee, 2017). Intrinsic drug tolerance aside, acquired DR is often mediated by heritable genetic mutations. These mutations alter antibiotic activity or their access to cognate antibiotic targets, but recent evidence has suggested that these mutations also arise from specific secondary metabolic remodeling, essential to the physiology for DR mutants (Amato et al., 2013; Derewacz et al., 2013; Hartman et al., 2017). Historically, nearly all clinically relevant TB antibiotics emerged from empirical approaches that have proven to be less effective in killing Mtb persisters. The need for new TB antibiotics has prompted concerted efforts to establish more rational antibiotic discovery platforms. We discuss how metabolomics technology in antibiotic target identification will improve precision and efficacy of the antibiotic development pipeline.

Main

The metabolome is a phenotypic fingerprint of biological systems

The genome, transcriptome, and proteome are constructed by polymerization of certain sets of building blocks. Unlike these macromolecules, the metabolome—a collection of metabolites—is not constrained by simple polymerization, which gives rise to vast structural and functional variation and diverse physiological properties (Schwab, 2003; Dettmer et al., 2007; Dunn et al., 2011). Due to this biochemical diversity, there is no single analytic tool to cover the extraction and characterization of all metabolites. Thus, the complete scope of metabolites and their functional interactions remains undefined, which is further complicated by lack of adequate interpretation of analysis. Despite intensive studies, only ~680 metabolites among ~40,000 metabolites present in the Human Metabolome Database (HMDB) have been functionally identified as members of defined pathways (Lu et al., 2017). In studies of Mtb metabolomics, ~300 water-soluble metabolites and ~62 water-insoluble lipids have been identified as the Mtb metabolome (Layre et al., 2011; Eoh and Rhee, 2013; Nandakumar et al., 2014). The extraction rates covering Mtb-specific pathways and the biological relevance of unmapped metabolites remain unknown.

The metabolic phenotype provides a clear readout of Mtb's biochemical state, as metabolites are the final products of enzymatic, posttranslational, allosteric, and environmental integration under a particular condition (Eoh, 2014; Ehrt et al., 2018). Analysis of intra- and extra-bacterial metabolites in Mtb provides phenotypic information that cannot be accurately deduced by other conventional tools such as genotyping, mRNA profiling, or proteomics. The metabolomics profile presents a unique advantage to alternate technologies by providing a top-down, systems-level outcome that integrates cellular biochemistry, physiological state, and adaptive metabolism required to survive continuously changing environments (Jansen and Rhee, 2017).

The metabolic phenotypes of Mtb persisters are adaptive strategies used not only to regulate replication rates *via* permissive carbon/nitrogen source determination, but also to interact with the host immune system as well as with neighboring populations (Parrish et al., 1998; Dhar et al., 2016). Thus, complete understanding of both metabolite abundances/fluxes and their functional interactions within multiple metabolic networks sheds light on how metabolic state is associated with the level of virulence and tolerance to environmental stresses.

The field of metabolomics has evolved to elucidate these complex metabolic profiles and their corresponding phenotypes (Griffin, 2004). Metabolomics is the youngest systems-level discipline to collectively understand metabolic activities and investigate how metabolic shifts lead to phenotypic optimality, how distinct metabolic activities in Mtb produce different pathogenicity, and how metabolic shifts coordinate adaptive strategies allowing Mtb to survive adverse environments (Jansen and Rhee, 2017). Technically, metabolomics results can be integrated with other systems-level data to improve the understanding of the phenotypic state of an organism (Argelaguet et al., 2018; Subramanian et al., 2020). Recently, Kim et al. integrated metabolomics and transcriptomics profiles of sera from nontuberculous mycobacterial (NTM) disease patients and identified the NTM infection-mediated impact on the host gut microbiome, serum metabolome, and immune defense against NTM infection (Kim et al., 2022). Overlaying the data from other omics studies onto the metabolic landscape is extremely useful to build a quantitative and integrative model of a target biological system. The resulting models have a tremendous potential to produce valuable information connecting metabolic phenotypes to the development of conceptually novel therapeutics and antimicrobial agents.

Emerging technology of Mtb metabolomics

The metabolomics platform constitutes a multistep process involving culture preparation, metabolic quenching, extraction, sample concentration, detection, data analysis, and interpretation (Wu et al., 2005; Villas-Boas et al., 2005). As metabolic fluxes change within seconds, special harvesting methods are required to avoid artifacts from handling steps before adequate metabolic quenching. Recently, a filter culture-based method was developed to enable non-disruptive handling, rapid quenching, and collection of Mtb cells (Eoh and Rhee, 2013; Nandakumar et al., 2015; Samuels et al., 2017). This method eliminates additional washing and centrifugation steps that could potentially be associated with metabolic changes or losses. Eoh and Rhee and de Cavalho et al. showed that this filter culture-based method supports almost identical Mtb cell wall architecture, growth kinetics, and phenotypic changes even under hypoxic stress conditions as compared to that of conventional liquid culture systems (de Carvalho et al., 2010; Eoh and Rhee, 2013). Accumulating evidence shows that this method also enables reproducible recovery of core metabolites as well as secondary trace metabolites. The unique advantage that this filter culture-based method has over classical liquid cultures is the ease of handling Mtb cells. For example, Eoh et al. modified this method using a procedure termed ^{13}C pulse chase labeling to study the shifted carbon fluxes between the CCM and carbohydrate core of cell wall glycolipids (Eoh et al., 2017). Mtb-laden filters were incubated on media containing ^{13}C-labeled (U-^{13}C) glucose to fully label all CCM intermediates with ^{13}C. Then, the filters were transferred to media containing unlabeled glucose to reversely wash only CCM intermediates with unlabeled carbon fraction, ultimately producing Mtb bacilli with unlabeled CCM and a fully ^{13}C-labeled carbohydrate core of cell wall glycolipids. This ^{13}C pulse chase labeling allowed tracing of glycolipid carbon fluxes during adaptation to various environmental stresses such as hypoxia. The filter culture-based method also allows for simultaneous collection of intracellular metabolites and extracellular secretome. To this end, Eoh and Rhee replaced the underlying agar media with a plastic inset containing chemically equivalent liquid media in direct contact with the underside of the Mtb-laden filters. Growth atop this liquid medium within the inset was indistinguishable from that achieved on adjacent agar medium and enabled timed start–stop measurements of the secretome or antibiotic uptake by sampling cell-free spent liquid media (Eoh and Rhee, 2013). Using this approach, Eoh and Rhee sought to monitor the amount of secreted succinate from hypoxic Mtb. Using a similar method, Luna et al. and Lim et al. separately sought to measure levels of antibiotic uptake by *Acinetobacter baumannii* and Mtb, respectively, after exposure to various environmental stresses such as nutrient starvation or hypoxia (Luna et al., 2020; Lim et al., 2021).

Mtb persisters are a putative preform of drug-resistant mutants

Metabolomics profiles of Mtb offer a unique perspective to define the mechanistic basis underlying persistence and the development of permanent DR mutations. Persistence in latently infected TB patients contributes to survival of Mtb bacilli and may lead to an active form, followed by transmission to healthy individuals (Bryk et al., 2008; Barry et al., 2009; Ehrt and Schnappinger, 2009; Nathan and Barry, 2015; Gold and Nathan, 2017). The phenotypic transitioning between a persister form and an actively replicating form requires growth rate regulation, yet the specific metabolic activities underlying this cell cycle shift remain largely unknown (Figures 1A, B).

Whether DR evolves from transiently drug-tolerant Mtb persisters and how Mtb drug tolerance is functionally associated with developing DR mutants are the main subjects of debate in the antibiotic discovery community (Gomez and McKinney, 2004; Fridman et al., 2014; Levin-Reisman et al., 2017; Lewis and Shan, 2017). Accumulating evidence using *Escherichia coli* subjected to intermittent exposure to ampicillin showed that cyclic treatment with the antibiotic delayed its growth lag time—a state of drug tolerance—several cycles ahead of achieving increased minimal inhibitory concentration (MIC), a state of permanent DR against the antibiotic. Delayed lag time after treatment with antibiotics was also detected in Mtb (Levin and Rozen, 2006; Windels et al., 2019b; Saito et al., 2021). Recently, Windels et al. calculated the DR mutation frequency using *E. coli* strains harboring a high or low level of persistence to predict the contribution of bacterial persisters to development of DR by mathematical modeling (Windels et al., 2019a; Windels et al., 2019b). The number of DR colonies emerging from viable cells showed the same correlation, even when a correction for the number of surviving cells was applied. This implies that bacterial persisters formed from high-persistence strains have an increased likelihood to develop DR-conferring mutations, while *E. coli* strains from low-persistence are less likely to mutate to gain DR. The positive correlation between persistence levels and DR mutation rates is an important factor to be considered in new antibiotic discovery, as even minor metabolic remodeling to increase persister formation can be evolutionarily associated with the emergence of DR mutants (Castro et al., 2021). Intriguingly, recent studies have shown that Mtb persisters are also clinically relevant to chronic infections in addition to antibiotic treatment (Zhang et al., 2012; Manina et al., 2015; Sarathy et al., 2018; Schrader et al., 2021). High-persistence forms of Mtb are repeatedly identified in chronic infections and have complicated chemotherapeutic options because they are likely to evolve into MDR-, pre-XDR-, and XDR-Mtb strains (Barry et al., 2009; Lewis, 2010; Nathan,

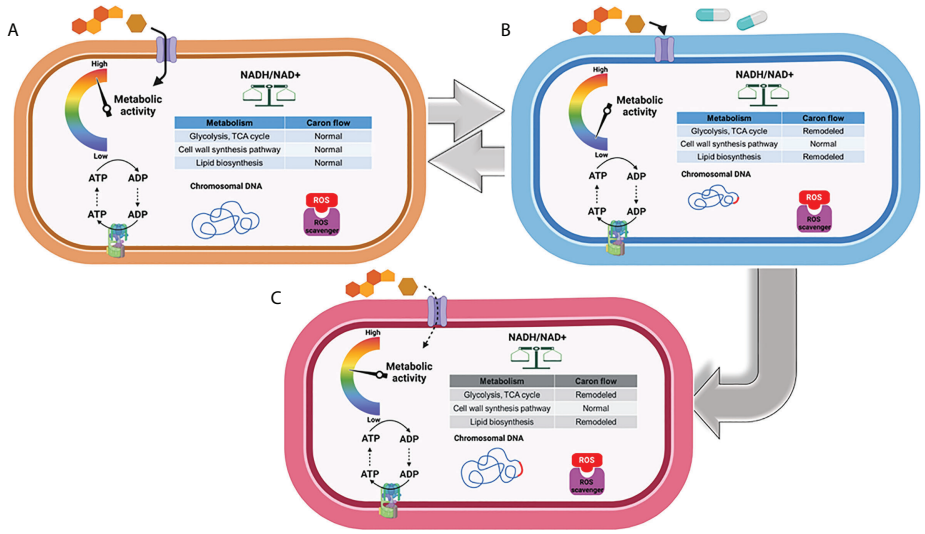


FIGURE 1
Metabolic phenotype associated with replication, transient drug tolerance, and permanent drug resistance in Mtb. **(A)** Under a replicating state, Mtb maintains the carbon fluxes within its CCM (e.g., glycolysis and the TCA cycle) and cell wall biosynthesis to meet the anaplerotic demands for replication. **(B)** Mtb remodels its metabolic networks in response to environmental stress and antibiotic treatment to produce transient drug tolerance or persistence, notably by rerouting its carbon flux from intermediary metabolism towards the biosynthesis of storage compounds and regulatory molecules. **(C)** Accumulation of ROS as a consequence of antibiotic treatment results in DNA mutagenesis and the development of drug-resistant mutations, leading to the emergence of permanent drug resistance. Figures were created by BioRender.com.

2014), albeit a controversial view with a significant amount of evidence that refutes this notion (Figures 1B, C).

As evidenced from other organisms, Mtb persisters that survive antibiotic effects may have an increased likelihood of acquiring genetic mutations that confer DR due to the subsequent high levels of reactive oxygen species (ROS), an active DNA mutagen (Figure 1B) (Davis et al., 2002; Kohanski et al., 2010a; Kohanski et al., 2010b; Long et al., 2016). Thus, the emergence of DR colonies on mycobactericidal antibiotic-containing media over the course of treatment is usually explained by *de novo* DNA mutagenesis, even for Mtb bacilli in a non-replicating state. Spontaneous or antibiotic-induced DNA damage in non-replicating bacilli leads to genetic mutations because, even in the absence of chromosomal replication, these bacilli still display DNA turnover (Cirz et al., 2005; Foster, 2007; Revitt-Mills and Robinson, 2020). Nonetheless, others have proposed that DR mutations in non-growing bacilli are not associated with DNA turnover, but rather stem from a small population of preexisting mutants that grow slowly and give rise to fast-growing mutants that eventually form predominant colonies (Galhardo et al., 2007). *De novo* mutation in non-replicating bacilli can be explained by the gradual accumulation of preexisting DR mutant colonies, shown by the deletion or mutation of several genes that dramatically decreases *E. coli* colony formation on ciprofloxacin-containing media without affecting the number of early colonies or their fitness under growth-restricted conditions (Cirz et al., 2005). Most bacterial pathogens, including Mtb, follow a similar model in which only persisters survive bactericidal antibiotic treatment, forming a viable cell reservoir with an increased likelihood for DR-conferring DNA mutagenesis (Torrey et al., 2016; Windels et al., 2019b; Colangeli et al., 2020).

As previously mentioned, antibiotics kill Mtb by accumulation of ROS, which causes irreversible damage on cellular processes essential for Mtb growth and survival. As a countermeasure, Mtb acquires drug tolerance by entering a persistent state which is triggered by metabolic remodeling to mitigate the ROS-mediated cellular damage and subsequently evade mycobactericidal activity (Figure 2) (Eoh, 2014; Ehrt et al., 2018). Hence, metabolic remodeling for Mtb persister formation not only sustains its viability but also leads to the accumulation of DR mutations.

Metabolic remodeling strategies are required for Mtb persister formation

The hallmark of latent TB infection (in a state of persistence) is its ability to survive various antimicrobial environments inside hosts. Mtb replicates even under a fully functioning immune system: soon after recruitment to the site of infection, immune cells release ROS, nitric oxide (NO), and reactive nitrogen intermediates (RNI), and in response, Mtb slows or ceases its growth rates, which enhances drug tolerance levels within the

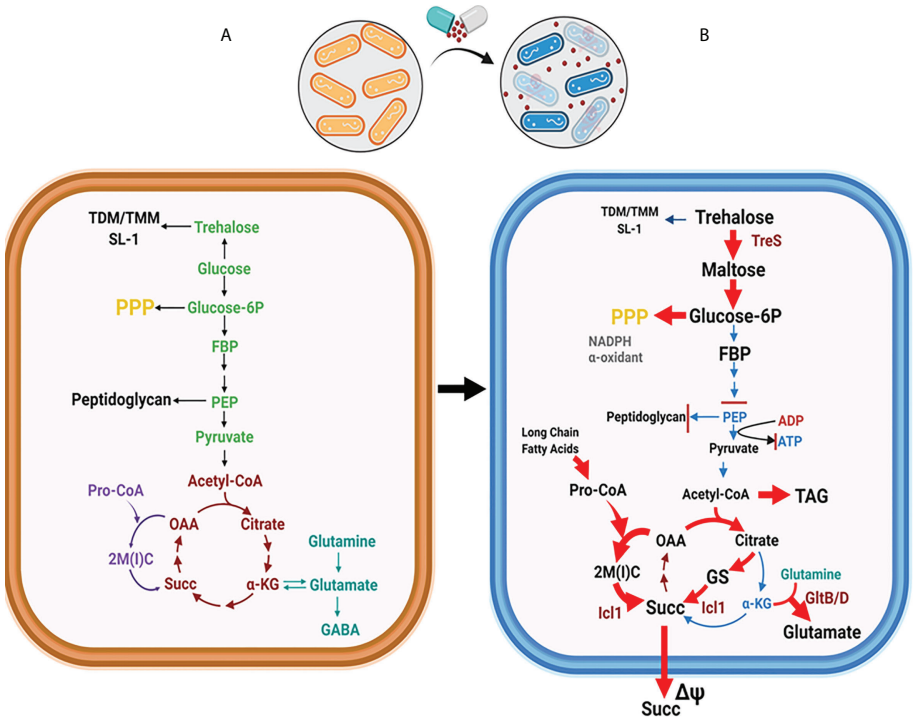


FIGURE 2
Global metabolic shift in Mtb CCM is required for forming persisters. **(A)** Mtb CCM activities are required for its replication. Enzymatic steps dedicated to glycolysis are depicted in green, the TCA cycle in brown, the MCC in purple, and the nitrogen metabolism in teal. **(B)** Mtb shifts CCM carbon fluxes to form persisters. Rerouted pathways are indicated by red arrows. The trehalose-mediated catalytic shift leads to catabolic remodeling of TDM/TMM, which increases the carbon flux towards the biosynthesis of upper glycolytic intermediates and the PPP, while downregulating the flux to the lower glycolysis. Carbon flow through the glyoxylate shunt and MCC is upregulated to increase the biosynthesis of succinate. Toxic accumulation of MCC intermediates is accompanied by increasing glutamate synthase activity (GltB/D), an activity known to neutralize MCC-mediated metabolic intoxication. Abbreviations are as follows: TMM, trehalose monomycoate; TDM, trehalose dimycolate; FBP, fructose 1,6 bisphosphate; PEP, phosphoenolpyruvate, 2M (I)C, 2-methyl (iso)citrate; OAA, oxaloacetate; αKG, alpha-ketoglutarate; Succ, Succinate; Pro-CoA, propionyl-CoA; GABA, γ-aminobutyric acid; GS, glyoxylate shunt; PPP, pentose phosphate pathway. Figures were created by BioRender.com.

population (Nathan and Hibbs, 1991; Chan et al., 1992; Voskuil et al., 2011). During this process, an adequate immune response will recruit various immune cells that convene into a multicellular structure called a granuloma (Davis and Ramakrishnan, 2009; Ramakrishnan, 2012). The interior of the granuloma is full of biochemical stresses including low oxygen tension (hypoxia), limited access to nutrients (starvation), low pH (acidosis), and accumulation of ROS, RNI, and other toxic metabolites. The majority of viable Mtb bacilli reside within the granuloma in a slowly replicating or non-replicating state by entering a persister form through their metabolic and biochemical shift (Flynn and Chan, 2001; Voskuil et al., 2003; Cosma et al., 2003).

The high level of drug tolerance from non-heritable DR in Mtb persisters is the main factor that destabilizes effective chemotherapies (Lewis, 2010; Zhang et al., 2012; Prax and Bertram, 2014; Sarathy et al., 2018; Gollan et al., 2019). This is largely because most conventional TB antibiotics target cellular processes active only during replication, and thus are no longer effective in killing Mtb persisters. The metabolic shift during persister formation has emerged as a central feature of TB pathogenicity and is fertile ground to identify new antibiotic targets (Levin-Reisman et al., 2017; Windels et al., 2019b).

Mtb CCM plays an essential role in enzymatic transformation of carbon substrates through the activities of glycolysis, gluconeogenesis, the pentose phosphate pathway (PPP), trehalose metabolism, the tricarboxylic acid (TCA) cycle, and TCA cycle-branched pathways such as the glyoxylate shunt and the methylcitrate cycle (MCC) (Figure 2) (Baughn and Rhee, 2014; Ehrt et al., 2018). For any organism in an ecosystem, the objective role of their CCM is to meet the stoichiometric demands of sustaining adequate growth rates with optimal efficacy. However, Mtb is microbiologically and metabolically unique in that it is a chronic intracellular pathogen that interacts with humans as its only known host and reservoir (Cosma et al., 2003; Gengenbacher and Kaufmann, 2012). Thus, Mtb bacilli have evolved for survival within the host phagosome as its primary niche. Mtb encounters diverse biochemical stringencies within this adverse environment, many of which play an extrinsic role

that triggers the exit from its normal cell cycle, thereby slowing its growth rate and promoting persister formation. Accompanying this phenotypic shift, Mtb persisters alter CCM activities to sustain their viability and slowly replicating or non-replicating physiology (Eoh, 2014; Ehrt et al., 2018). Relieved of the requirement to double its biomass, Mtb persisters have generally been perceived to have low metabolic activities. Indeed, Mtb initiates persister formation by downregulating key component activities in the electron transport chain (ETC), which decreases oxidative phosphorylation and adenosine triphosphate (ATP) biosynthesis to ~10% to that of their replicating counterparts (Eoh and Rhee, 2013; Lim et al., 2021). However, slowly replicating or non-replicating heterogenous persisters face metabolic challenges to preserve the integrity of the cellular components essential for both survival as persisters and re-entry into the normal cell cycle for transmission to new hosts. Metabolic networks of Mtb in a replicating state have been extensively studied, but knowledge of the specific metabolic activities and altered pathways to exit the normal cell cycle is a major area of unmet scientific and medical need.

The absence of classical catabolic carbon repression (CCR) regulatory circuits in Mtb CCM allows the bacilli to co-catabolize glycolytic and gluconeogenic carbon sources simultaneously during replication, a metabolic capacity that confers a survival advantage in an environment where nutrients are scarce (de Carvalho et al., 2010; Borah et al., 2021). This regulatory concept is also true in Mtb persisters. Accumulating evidence using Mtb persisters shows a simultaneous shift in multiple sets of metabolic networks within their CCM (Figure 2B) (Eoh and Rhee, 2013; Eoh et al., 2017; Dutta et al., 2019; Lee et al., 2019; Lim et al., 2021; Quinonez et al., 2022). The conventional CCM pattern active during replication was remodeled to favor new catalytic patterns aiming to prolong survival under high levels of environmental stresses by mitigating the metabolic and biochemical toxicities from these stresses. Low NAD^+ levels act as an intrinsic factor to trigger a metabolic shift during Mtb persister formation, as NAD^+ is a crucial cofactor involved in a broad range of cellular processes from redox cellular homeostasis to catabolism and energy production (Watanabe et al., 2011; Xie et al., 2020). The absence of lactate dehydrogenase in Mtb metabolic networks causes ETC-mediated oxidative phosphorylation to be strictly essential for virulence and viability (Serafini et al., 2019). The reliance of Mtb virulence on the ETC was supported by the clinical discovery of a new second-line TB antibiotic, bedaquiline (BDQ), with F1F0 ATP synthase of the Mtb ETC as its target (Mahajan, 2013; Kundu et al., 2016). Antibiotics targeting the Mtb ETC are known to be effective against DR TB strains and have potential to shorten TB treatment duration (Rao et al., 2008). For example, the *qcrB* gene of the cytochrome bc1:aa3 complex is the target of Q203, another novel ETC inhibitor (Pethe et al., 2013; Harrison et al., 2019). Intriguingly, Kalia et al. recently reported that the potency of Q203 is determined by CCM shifts that respond to available carbon sources (Kalia et al., 2019). Catalytic activity for glycerol had a positive impact on Q203 antibiotic potency, as the cytochrome bd terminal oxidase became functionally important while consuming glycerol as a carbon source. The potency of BDQ is also affected by CCM shifts and available carbon sources (Wang et al., 2019; Mackenzie et al., 2020). These findings emphasize the potential of Mtb persister metabolic activities for new antibiotic discovery.

Mtb persisters collected after exposure to biochemical growth-limiting stresses that resemble the host environment or from the sputum of active pulmonary TB patients have both long been popular models used to understand the adaptive strategies of Mtb. However, these findings were focused on transcriptional analysis due to suboptimal culture methods and analytical tools. Only recently has research begun to focus on the metabolism of intrinsic drug tolerance. The following are reported examples of metabolic networks shifted during Mtb persister formation.

Metabolic shift from the TCA cycle to TAG biosynthesis

Baek et al. identified the *tgs1* gene using transposon (Tn) mutagenesis while screening for genes whose Tn insertion resulted in Mtb growth or survival advantage under hypoxic stress (Baek et al., 2011). Tgs1 is characterized as a triacylglycerol (TAG) synthase in Mtb and is catalytically involved in condensing glycerol 3-phosphate and acyl-CoA. As Tgs1 is responsible for the dominant TAG biosynthetic activity in Mtb under hypoxia, it is required for optimal virulence within host niches. Targeted deletion of *tgs1* in Mtb (Δtgs1) allows bacilli to continue replication even under hypoxia, indicating the essential role of Tgs1 for the slowly replicating or non-replicating phenotype of Mtb persisters. Metabolomics analysis of Δtgs1 showed that acetyl-CoA-mediated carbon flux towards the biosynthesis of TAG was prevented, with its flux redirected towards the biosynthesis of intermediates in the oxidative arm of the TCA cycle including citrate and α-ketoglutarate (α-KG). This pathway is a major biosynthetic source of NADH, an initial substrate of Mtb ETC. As a result, the carbon flux shift in Δtgs1 significantly increases antibiotic susceptibility against a broad range of clinically relevant antibiotics [e.g., INH, streptomycin (STR), ciprofloxacin (CPF), and ethambutol (EMB)] even under persister-inducing environments and within mouse models. The mechanistic basis underlying the antibiotic susceptibility of Δtgs1 was supported by comparing the drug tolerance and growth pattern of Mtb overexpressing citrate synthase (CitA), an enzyme that condenses oxaloacetate (OAA) and acetyl-CoA in the oxidative arm of the TCA cycle. This study indicates that environmental stress-induced rewiring of acetyl-CoA-mediated carbon flux is an adaptive response of Mtb to environmental stress used to regulate its growth rate and drug tolerance (Figure 3A).

Consistent with the foregoing findings from Δtgs1, accumulation of intrabacterial TAG may act as a metabolic

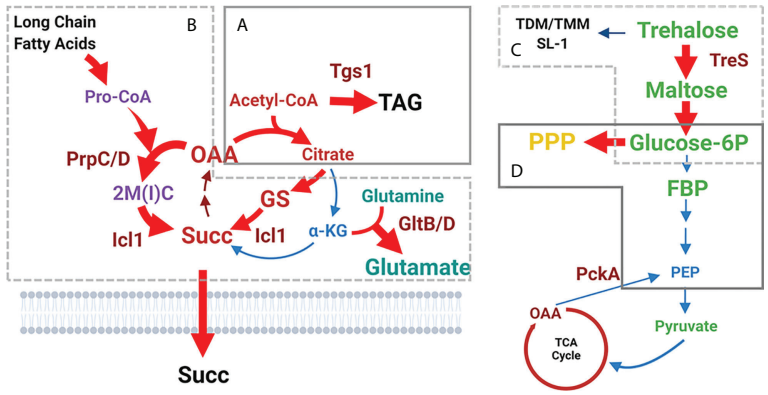


FIGURE 3
Shifted catalytic activities of Mtb persister CCM. **(A)** Acetyl-CoA-mediated carbon flux shift towards the biosynthesis of triacylglycerol (TAG). Consequently, the flux towards the oxidative arm of the TCA cycle is downregulated. **(B)** Reduced carbon flux towards the oxidative arm of the TCA cycle is further facilitated by increasing the carbon flux through the glyoxylate shunt to biosynthesize succinate. Simultaneously, MCC activity is upregulated, resulting in toxic accumulation of MCC intermediates including propionyl-CoA. GltB/D-mediated conversion from glutamine to glutamate is activated as a strategy to neutralize MCC toxicity. **(C)** Trehalose-mediated carbon shift from the cell wall glycolipids towards the biosynthesis of upper glycolytic intermediates such as glucose 6P. **(D)** Glucose 6P-mediated carbon flux shift from downstream glycolysis towards the biosynthesis of PPP intermediates. Abbreviations are the same as depicted in Figure 2. Figures were created by BioRender.com.

biosignature associated with reduced growth rates and increased drug tolerance *in vitro* and in mouse models (Daniel et al., 2011). Mtb expresses the TAG efflux pump, LprG (Rv1410), to adjust intrabacterial TAG levels by pumping TAG out of the cytoplasm (Farrow and Rubin, 2008; Martinot et al., 2016). TAG accumulation within lipid droplets in the cytoplasm of *lprG*-deficient Mtb (ΔlprG) led to reduced growth rates accompanied by drug tolerance. TAG, located at the outer leaflet of the outer membrane, functions as a structural barrier while also providing a source of ATP *via* the β-oxidation pathway of TAG acyl-chains. Thus, intrabacterial TAG levels are metabolically associated with bacterial ATP biosynthesis, another biosignature for bacterial persisters. Like acyl-chains of phthiocerol dimycocerosates (PDIM) (Lee et al., 2013), TAG serves as a catalytic sink to alleviate the accumulation of toxic propionyl-CoA, an initial MCC substrate. Within hypoxic and lipid-rich macrophages, Mtb often uses host fatty acids derived from macrophage TAG acyl-chains after β-oxidation to build up its own TAG stores. Thus, the catalytic shift required for synthesis, accumulation, translocation, and distribution of TAG appears to be critical for cellular homeostasis in Mtb persisters' optimal metabolic state.

Metabolic shift from the oxidative arm of the TCA cycle to the glyoxylate shunt

Recent observations have identified isocitrate lyase (ICL) as a specific mediator to produce the metabolic shifts of Mtb persisters (Eoh and Rhee, 2013; Nandakumar et al., 2014). ICL has been identified as an essential enzyme for Mtb persister formation and high drug tolerance *via* its new role in antioxidant defense rather than its canonical role in fatty acid metabolism. Work by Nandakumar et al. showed that Mtb changes its carbon fluxes through the ICL-mediated glyoxylate shunt as a method to evade antibiotic effects (Nandakumar et al., 2014). In this study, Mtb enhanced its survival rates in response to mycobactericidal concentrations of clinically relevant first-line TB antibiotics such as INH, RIF, and STR, by increasing carbon flux through the glyoxylate shunt and bypassing the oxidative arm of the TCA cycle. Accumulating evidence has shown that this metabolic shift produces glycine and succinate as metabolic end products without overproducing NADH and ROS (Eoh and Rhee, 2013). *icl*-deficient Mtb (Δicl) became hypersensitive to treatment with INH, RIF, and STR, and the normal level of antibiotic sensitivity was restored by supplementation with a chemical antioxidant such as thiourea. These findings collectively suggest that the catalytic shift from the canonical TCA cycle towards biosynthesis of glyoxylate shunt intermediates serves as an adaptive strategy used to evade antibiotic effects. This is accomplished by mitigating the production of respiratory radicals arising from canonical TCA cycle-mediated NADH production and ETC activity (Figure 3B).

Under a physiological context, Giffin et al. explored possible roles of glycine biosynthesis by characterizing a glycine dehydrogenase (Ald)-deficient Mtb (Δald) (Giffin et al., 2016). First, they found that in addition to *icl*, both the mRNA expression of *ald* and the enzymatic activity of its translated product were increased while entering the hypoxia-induced persister state. Intriguingly, Δald showed minor survival disadvantages under *in vitro* hypoxia, but a significant lag phase delay upon resuming growth after reoxygenation. Ald is proposed to maintain optimal NAD^+ recycling during initial regrowth after reoxygenation. Two independent studies

separately identified an essential role of succinate metabolism in the ability of Mtb persisters to survive hypoxic stress (Watanabe et al., 2011; Eoh and Rhee, 2013). Watanabe et al. found a considerable increase in extracellular succinate from Mtb persisters, which is catalytically linked to the reverse TCA cycle activity, identified by stable ^{13}C isotope tracing metabolomics using [U-^{13}C] glucose. This rearrangement of succinate metabolism allows Mtb persisters to maintain an energized membrane potential, despite the lack of a primary ETC terminal electron acceptor, by the reductive branch activity of the TCA cycle followed by accumulation and secretion of succinate. Unexpectedly, knockout of the canonical fumarate reductase in Mtb (Δfrd) was not functionally associated with reduced succinate secretion and impaired membrane potential. Eoh and Rhee used stable ^{13}C isotope tracing metabolomics of Mtb persisters under hypoxia using [U-^{13}C] acetate. This work showed that, upon exposure to hypoxia, Mtb persisters slowed TCA cycle activity and increased succinate production *via* ICL in the glyoxylate shunt. By fermenting fumarate to succinate, Mtb persisters are capable of sustaining both membrane potential and ATP synthesis under low oxygen levels (Figure 3B).

In Mtb persisters, succinate biosynthesized by either the reverse reductive branch of the TCA cycle or the glyoxylate shunt is further catalyzed by succinate dehydrogenase (Sdh1), the enzyme that couples growth processes controlled by the TCA cycle with energy production resulting from the ETC. Hartman et al. characterized the impact of genetically deleting the membrane anchor subunit of Mtb Sdh1 (Δsdh1). Sdh1 oxidizes succinate to biosynthesize fumarate and deliver two electrons to ETC membrane electron carriers such as menaquinone (Hartman et al., 2014). Stable ^{13}C isotope tracing metabolomics of Δsdh1 confirmed that succinate oxidation was downregulated during aerobic growth but uncontrolled during adaptation to hypoxic conditions. As a result, Δsdh1 failed to survive during the stationary phase or during chronic infection in the C3HeB/FeJ mouse model due to several impaired bioenergetic activities arising from a mismatch between glyoxylate shunt-mediated succinate biosynthesis and ETC respiration.

These studies identified enzymes involved in the glyoxylate shunt and succinate metabolism—malate dehydrogenase, succinate dehydrogenase, and ICL—as potential sources of new drug targets to eradicate Mtb persisters.

Metabolic shift from cell wall glycolipids to glycolysis

Eoh et al. showed that the adaptive metabolic shift of Mtb persisters under hypoxic conditions was mediated by an accumulation of upper portion glycolysis intermediates, such as glucose 6-phosphate (Glc 6P) and fructose 1, 6-bisphosphate (FBP) (Marrero et al., 2013; Eoh et al., 2017). This resulted in a subsequent depletion in phosphoenolpyruvate (PEP), the most downstream glycolysis intermediate, and upstream trehalose, a glucose disaccharide (Eoh et al., 2017; Lim et al., 2021). These changes were found to be caused by catabolic remodeling of cell wall glycolipids such as trehalose monomycolate (TMM) and trehalose dimycolate (TDM) *via* trehalose synthase (TreS) activity. A *treS*-deficient Mtb (ΔtreS) was unable to adapt to, or recover from, incubation under hypoxia stress. These results were similarly reproduced by treatment with validamycin A, a validated chemical inhibitor of TreS. These changes were dissociated from TCA cycle remodeling, as confirmed by inclusion of NO_3, a physiologically relevant terminal electron acceptor of ETC capable of supporting near-aerobic levels of canonical TCA cycle activity under hypoxic conditions (Eoh and Rhee, 2013) (Figure 3C).

As alluded, Lee et al. supported the crucial role of the trehalose-mediated catalytic shift as an adaptive strategy of Mtb persisters used to survive many clinically relevant biochemical stresses and antibiotic effects (Lee et al., 2019). This study used an *in vitro* biofilm culture to mimic host-implemented stresses (e.g., hypoxia, nutrient starvation, and toxic metabolites) and to trigger persister formation. Mtb persisters were generated in the biofilm culture in pelleted form at both the wall–media border and air–media interface (Ojha et al., 2005; Kulka et al., 2012; Ojha et al., 2015). Floating and precipitated fractions, which contain replicating and dead bacilli, were removed to selectively collect Mtb culture enriched with persister bacilli. As shown in Mtb persisters under a hypoxic condition, metabolomics analysis of Mtb persisters collected from the biofilm culture showed decreased levels of TMM, TDM, UDP-glucose, and maltose phosphate, which reciprocally matched increases in maltose, Glc 6P, and FBP (Lee et al., 2019). This metabolomics profile differs from that of replicating Mtb, which manages trehalose metabolism for the biosynthesis of the cell wall glycolipids such as TMM and TDM. As glycolysis is a metabolic source of ATP biosynthesis *via* substrate level phosphorylation, the trehalose-mediated catalytic shift serves as an alternate source of ATP for Mtb persisters with a functionally downregulated ETC. Indeed, as seen in ΔtreS or treatment with validamycin A, inhibition of the trehalose-mediated catalytic shift renders Mtb hypersensitive to nearly all TB antibiotics including INH, RIF, and BDQ. Metabolomics profiling of ΔtreS revealed that this hypersensitivity was likely due to ROS damage, and co-treatment with thiol-based antioxidant thiourea afforded a roughly 1.5 $\log_{10}$ increase in colony-forming units (CFUs), approaching nearly complete protection against second-line TB antibiotics such as BDQ. These findings indicate that the trehalose-mediated catalytic shift is an adaptive strategy to primarily sustain ATP levels and additionally serve as a source of antioxidant biosynthesis to protect Mtb persisters from antibiotic-induced antimicrobial damage.

Recently, Ruhl et al. reported that another set of glycolipids in Mtb, termed sulfolipid-1 (SL-1), plays a role as a nociceptive neuron activating molecule (Ruhl et al., 2020). The host cough reflex was significantly decreased during infection with an Mtb

genetic mutant lacking SL-1, suggesting that Mtb persisters take advantage of this reflex to transmit to new healthy individuals. The structure of SL-1 contains a trehalose 2-sulfate core that is esterified with four acyl-chains. As trehalose is present in its carbohydrate core, the SL-1-mediated cough reflex may be reciprocally related with the level of foregoing trehalose-mediated catalytic shift activity (Figure 3C). During latent infection, where Mtb mostly resides in its persister form, the trehalose-mediated catalytic shift activity is enhanced to induce trehalose consumption towards the biosynthesis of glycolysis intermediates. This carbon-flux shift may result in SL-1 downregulation within the cell wall; TB patients with a latent TB infection express weaker SL-1-mediated cough reflex. When exiting from a persister state by resuming its growth, Mtb stops the trehalose-mediated catalytic shift by restoring its flux towards the biosynthesis of cell wall glycolipids including SL-1, which triggers the host cough reflex for immediate transmission.

Metabolic shift from glycolysis towards the biosynthesis of PPP intermediates

Shi et al. proposed an interesting model in which Mtb persisters reroute their carbon and nitrogen fluxes from intermediary metabolism involved in energy production and macromolecule biosynthesis towards the biosynthesis of storage compounds such as TAG or glutamate (Shi et al., 2010). This adaptive metabolism was observed in response to *in vitro* growth arrest or *in vivo* mouse lung infection. Lim et al. recently reported that hypoxia-induced TAG biosynthesis is mediated by a metabolic shift involving downregulation of glycolytic intermediates such as PEP (Lim et al., 2021). Previous metabolomics findings revealed that accumulation of Glc 6P and FBP was kinetically unmatched to that of PEP, indicating disproportionate glycolysis activity (Eoh et al., 2017; Lee et al., 2019; Lim et al., 2021). PEP depletion in Mtb persisters is functionally associated with limited biosynthesis of oxaloacetate (OAA), a substrate of CitA activity, resulting in accumulation of acetyl-CoA and reciprocal induction of acetyl-CoA-mediated carbon flux towards the biosynthesis of TAG. Therefore, enhanced TAG biosynthesis is catalytically associated with downregulation of the lower portion of glycolysis (Figure 3D).

Work by Lee et al. found that reduced carbon flux from Glc 6P to lower glycolysis intermediates is reciprocally related with enhanced carbon flux towards the biosynthesis of PPP intermediates. The PPP is a metabolic pathway parallel to glycolysis conserved in all biological systems and represents the first committed step of glucose metabolism by sharing Glc 6P as a common substrate (Stincone et al., 2015). The PPP intermediates—ribose 5-phosphate (ribose 5P) and sedoheptulose 7-phosphate (sedoheptulose 7P)—function as biosynthetic sources of nucleic acids, histidine, and nicotinamide-adenine dinucleotide phosphate (NADPH) (Lee et al., 2019). The PPP serves a critical role in supporting some cancer cell survival and growth by providing NADPH and generating ribose 5P for nucleic acid biosynthesis (Tozzi et al., 2006; Riganti et al., 2012; Patra and Hay, 2014). NADPH is a cofactor required for the biosynthesis of fatty acids and acts as an electron donor for the biosynthesis of γ-glutamylcysteine, which is involved in ROS scavenging (Blokhina et al., 2003). This study claimed that accumulated Glc 6P can act as the preferred carbon source for biosynthesis of PPP intermediates and not for lower glycolysis intermediates. Intriguingly, an Mtb mutant (ΔdevB) lacking the second enzyme in the PPP, 6-phosphogluconolactonase (DevB), was still able to form persisters at levels similar to those of wild-type Mtb in an *in vitro* biofilm culture (Lee et al., 2019). Together, these findings suggest that the catalytic shift from Glc 6P towards the biosynthesis of PPP intermediates is another metabolic strategy of Mtb persisters used to meet their anabolic demands and combat oxidative stresses (Figure 3D).

A gene termed *pckA* encodes the enzyme PEP carboxykinase (PEPCK), which catalyzes the first committed step in Mtb gluconeogenesis (Liu et al., 2003; Marrero et al., 2010). Transcriptional silencing of *pckA* led to Mtb clearance in mice during the chronic phase of infection. In conjunction with the evidence showing the importance of PEPCK's canonical role in fatty acid metabolism and PEP biosynthesis, mouse infection models implicated an essential role in Mtb gluconeogenesis for their persister physiology. However, the impaired survival of the *pckA* knockout strain (ΔpckA) could not be recapitulated when culturing ΔpckA on fatty acid media or in resting or immune-activated mouse bone marrow-derived macrophages (BMDMs) (Marrero et al., 2010). Similar to Δicl, growing evidence has identified PEPCK as one determinant required for the Mtb metabolic shift, but has failed to clarify its metabolic role because of the inaccuracy in its catalytic annotations and *in vitro* biochemical studies. Separate studies demonstrated that Mtb PEPCK could catalyze the reverse reaction in an anaplerotic, not gluconeogenic, direction when Mtb was exposed to a reducing environment as seen in hypoxic conditions (Machova et al., 2014). Computational modeling of catalytic shifts has similarly suggested that Mtb PEPCK may execute in an anaplerotic direction during intracellular growth within THP-1 macrophages. Unlike these findings, Lim et al. conducted stable ^{13}C isotope tracing metabolomics using ΔpckA under a hypoxic condition in the presence of [U-^{13}C] acetate and found that there were no significant differences in the abundance and ^{13}C enrichment rates for the majority of Mtb CCM intermediates such as the reductive arm of the TCA cycle (e.g., OAA as a substrate of PEPCK and malate) and PEP (a product of PEPCK) as compared to those of wild-type Mtb under the same condition (Lim et al., 2021). This metabolomics profile implies that PEP depletion in Mtb persisters is attributed to downregulation of gluconeogenic carbon flux as a result of defective PEPCK activity in both gluconeogenic and anaplerotic directions.

Induced metabolic fluxes through the MCC

Environmental stress induces catalytic shifts that are associated with altered TCA cycle activity by enhancing the carbon flux through the reductive arm of the TCA cycle while concomitantly downregulating flux through the oxidative arm. This discontinued TCA cycle activity is catalytically attributed to enhanced activity through the MCC, a pathway that branches from the reductive arm of the TCA cycle. Eoh and Rhee conducted stable ^{13}C isotope tracing metabolomics using Δicl in the presence of [U-^{13}C] acetate or [U-^{13}C] propionate as a single carbon source and confirmed that ICL also functions as a methylisocitrate lyase (MCL), the last enzyme in the MCC, which catabolizes propionate after β-oxidation of odd-chain fatty acids (Eoh and Rhee, 2014). Δicl was shown to be incapable of establishing infection in a mouse model of pulmonary TB (McKinney et al., 2000; Munoz-Elias and McKinney, 2005). Loss of MCL activity resulted in accumulation of propionyl-CoA and MCC intermediates, providing a mechanistic basis underlying the Δicl bactericidal phenotype in propionate media or a chronic infection mouse model (Gould et al., 2006). Recent metabolomics studies of Mtb persisters showed that the MCC and the reductive arm of the TCA cycle are two top pathways greatly altered during adaptation to antibiotic treatment (Eoh and Rhee, 2013; Jansen and Rhee, 2017). Quinonez et al. supported this finding by showing that Mtb cultured in media containing short-chain fatty acids (e.g., either propionate or acetate) form a significantly greater portion of antibiotic-induced Mtb persister bacilli than those cultured in media containing glycerol or glucose due to the buildup of MCC and reductive TCA cycle intermediates (Quinonez et al., 2022). Collectively, MCC remodeling in the fatty acid-induced persister state of Mtb is yet another adaptive strategy to achieve improved levels of drug tolerance (Figure 3B).

Despite the phenotypic advantage functionally associated with high level of drug tolerance, MCC remodeling is often accompanied by accumulation of toxic intermediates such as acetyl-CoA, propionyl-CoA, or MCC intermediates (Savvi et al., 2008; Eoh and Rhee, 2014; Puckett et al., 2017). Biological systems always employ adaptive strategies, including catalytic activities, required for importing new host nutrients and degrading these carbon sources into small compounds that can be further catabolized. As part of their adaptive strategies, altered metabolic networks of Mtb persisters also include the ability to neutralize adverse metabolic effects from new carbon catabolism. To elucidate this activity, Lee et al. compared metabolic networks between virulent Mtb and the attenuated Bacillus Calmette-Guérin (BCG) vaccine strain to propose that BCG lacks the same adaptive strategies like glutamate synthase (GltB/D)-mediated activity to convert glutamine to glutamate as a metabolic effort to neutralize propionate and MCC-induced metabolic intoxication (Lee et al., 2018). Thus, the functional crosstalk between active MCC and GltB/D-mediated metabolic neutralization is essential for a high level of drug tolerance of Mtb persisters. Inactivation of this crosstalk will selectively kill Mtb persisters by maximizing the effect from propionate and MCC-meditated metabolic intoxication, providing a source of new antibiotic targets (Figure 3B).

Collectively, conventional TB treatment is not effective in killing Mtb persisters and preventing the development of DR Mtb mutants, as the current regimen targets only the metabolic activities required for replication. Slowly replicating or non-replicating Mtb persisters alter their metabolic networks by remodeling the carbon fluxes previously mentioned. It is critical to map the holistic metabolic fluxes altered while forming Mtb persisters and pinpoint the catalytic enzymes or pathways required to produce these carbon fluxes as a source of new drug targets.

Metabolic shifts shared by *Mtb* persisters and drug-resistant mutants

Even in developed countries including the USA, treating DR TB patients takes ~24 months and ~$393,000 per case, which is substantially more time-consuming and expensive than ~6 months and ~$49,000 for a drug-sensitive TB patient (Schrader et al., 2020). Thus, the major focus of our TB treatment campaign and research is on preventing the emergence of DR through the elucidation of metabolic activities necessary to sustain the metabolism and viability of DR Mtb strains. One possible mechanism suggests that exposure of drug-sensitive Mtb strains to bactericidal concentrations of TB antibiotics can select for Mtb persisters, during which permanent DR Mtb mutants evolve (Bifani et al., 2008; Brauner et al., 2016; Goossens et al., 2020).

Intriguingly, the metabolic shifts required to form Mtb persisters are also often detected in DR TB clinical isolates. Recently, Lim et al. showed that supplementing Mtb persisters with PEP under a hypoxic condition significantly reduced antibiotic-induced persister formation and suggested that metabolic remodeling from PEP depletion plays an important role in shifting Mtb persister metabolism. Intriguingly, this report also found that PEP supplementation significantly reduced the DR mutation rate against RIF, confirming a functional linkage between metabolic remodeling of Mtb persisters and the emergence of DR Mtb mutants (Lim et al., 2021). Metabolomics profiles of DR TB clinical isolates supported this notion, as PEP abundance and PEP/pyruvate ratio were significantly lower in MDR- and XDR-TB clinical isolates than in drug-sensitive clinical isolates (Lim et al., 2021). This indicates that the metabolic shift arising from accumulation of upper glycolysis intermediates, followed by low levels of PEP, is advantageous not only for Mtb persisters but also for the emergence of genetic mutation-mediated DR Mtb mutants.

Two comprehensive genetic studies screened for Mtb genes that are functionally altered to avoid antibiotic effects using DR TB clinical isolates (Bellerose et al., 2019; Safi et al., 2019). Both identified transient frameshift mutations in the hypervariable homopolymeric region of glycerol-3-kinase (GlpK), an enzyme required for optimal glycerol catabolism and associated with tolerance against INH, RIF, pyrazinamide, and moxifloxacin. Loss of GlpK function was shown to be a specific metabolic marker of DR TB clinical isolates. These studies hypothesized that the *glpK* frameshift mutations resulted in drug tolerance through metabolic changes that reduced growth and promoted expression of stress response regulators, such as DosR and SigH, thus diminishing antibiotic effectiveness. This stress response also induced genes related to TAG biosynthesis. In addition, DR TB clinical isolates exhibit a catabolic defect in glycerol consumption due to a lack of functional GlpK that may alter global glycolysis activity, presumably facilitating the depletion of PEP (Lim et al., 2021). Investigations on the effects of PEP supplementation on antimicrobial synergy with second-line TB antibiotics against DR TB clinical isolates are warranted.

Disproportionate activities within glycolysis of DR TB clinical isolates were also identified in a study reported by Lee et al. by collecting and characterizing three drug-sensitive and six DR TB clinical isolates (Lee et al., 2019). The growth of DR TB clinical isolates was impaired in media containing carbohydrates as a single carbon source, while drug-sensitive TB clinical isolates grew at a rate similar to that of the H_{37}Rv lab strain. Butyric acid (a C_4 short-chain fatty acid) supported the growth of both drug-sensitive and DR TB clinical isolates at a similar level. *In vitro* growth patterns of all TB clinical isolates in media containing butyric acid were enhanced by supplementation with trehalose. Intriguingly, the improved growth of DR TB clinical isolates was suppressed by treatment with validamycin A or a trehalose analog, 6-azido-6-α,α'-trehalose (6-treAz), while the enhanced growth of drug-sensitive TB clinical isolates was relatively unaffected even after treatment with validamycin A. The specific sensitivity of DR TB clinical isolates to treatment with a TreS-specific inhibitor indicated that DR TB clinical isolates, unlike drug-sensitive clinical isolates, rely on TreS activity in managing their CCM activities. These findings were further confirmed by metabolomics and lipidomics profiling. Chemical inhibition of the trehalose-mediated catalytic shift afforded more rapid and vigorous depletion in CFUs of DR TB clinical isolates against BDQ by ameliorating its weak early bactericidal activity (Koul et al., 2014; Lee et al., 2019). These results indicate that the trehalose-mediated catalytic shift can serve as an adjunctive target option in combination with second-line TB antibiotics for treatment of DR TB infections.

Although drug tolerance is a reversible DR state, growing evidence indicates that the presence of specific genetic mutations is also associated with the propensity of Mtb to enter an drug-tolerant persister state (Goossens et al., 2020). Hicks et al. used a genome-wide study of TB clinical isolates collected in China to identify previously unappreciated bacterial intrinsic factors that accelerate the development of DR mutations (Hicks et al., 2018; Hicks et al., 2019). This study showed that many DR TB clinical isolates harbor genetic mutations at the gene encoding the transcriptional factor *prpR*. The mutations at *prpR* conferred conditional drug tolerance against some TB antibiotics such as RIF, INH, and ofloxacin, by altering the main propionate metabolism pathways including the MCC and methylmalonyl-CoA pathway. Indeed, *prpR* is a transcriptional factor that is divergently transcribed from the *prpDC* operon in Mtb, which encodes the first two enzymes in the MCC. Intriguingly, *prpR*-mediated drug tolerance is carbon source-dependent *in vitro*. Consistent with previous findings, this conditional drug tolerance depends on metabolic intoxication arising from accumulation of propionyl-CoA and MCC intermediates (Eoh and Rhee, 2014; Lee et al., 2018). Thus, as discussed by Lee et al., the GltB/D-mediated metabolic shifts that presumably function to neutralize foregoing MCC intermediates and propionyl-CoA mediated toxicity should be studied as a new antibiotic target to kill Mtb persisters and DR TB infection by maximizing the level of metabolic intoxication.

High persisting (*hip*) mutants of genes in fatty acid metabolism and CCM have been isolated after treatment with bactericidal concentrations of RIF and STR (Torrey et al., 2016). The *hip* mutants with reduced fatty acid degradation activities, such as those catalyzed by FadE30, are likely to manifest a reduction in fatty acid metabolism. This is consistent with the metabolic shift of carbon fluxes away from the TCA cycle in favor of the biosynthesis of long-chain fatty acids. The upregulation of *icl* and *tgs1* in a condition lacking wild-type *hip* supports the foregoing metabolic examples seen in drug-tolerant Mtb persisters.

Conclusion

Metabolic remodeling strategies serve as an intrinsic virulence factor of Mtb through multiple roles beyond nutrient uptake and consumption required for increasing its biomass. These roles include cellular intrinsic processes such as carbon flux homeostasis, membrane bioenergetics, antioxidant defense, and cell cycle remodeling, as well as extrinsic factors such as regulation of immunoreactivity, drug tolerance, and DR. Thus, new antibiotic discovery should be implemented by elucidating such roles, as metabolic shifts are the biochemical foundation of all physiological processes (Miethke et al., 2021). It is of particular importance to elucidate phenotype-specific metabolic shifts and vulnerability to condition-specific modulation that explain the diverse roles of metabolic remodeling in the pathogenicity of Mtb persisters. The advent of new technologies has made it possible to revise old-fashioned identification of new antibiotic targets based on structure–activity relationship (SAR) or high-throughput screening.

Metabolomics-based antibiotic discovery combined with complete knowledge about the Mtb persister metabolic shifts promises to not only expand our specific knowledge about TB pathogenicity, but also enable intelligence technology-based computational simulations to address more complex questions that go beyond reconstruction of drug to target interactions. In the new antibiotic discovery platform, knowledge about metabolic remodeling specific to host-induced persistence and drug tolerance remains considerably important to identify a more effective TB drug regimen and shorten its duration.

Author contributions

HE, RL, and JL participated in conceptualization and writing of the original draft. JJL and PS substantially contributed to the discussion of the content, review, and editing. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by NIH (R21AI139386 and R56AI143870), American Lung Association Innovation Award, and Donald E. & Delia B. Baxter Foundation to HE.

Acknowledgments

We would like to thank all Eoh lab members for reading and commenting on the manuscript. We are grateful to C. Stallings for kindly sharing her unpublished manuscript. We apologize that many papers could not be discussed owing to the lack of space.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Adams, K. N., Takaki, K., Connolly, L. E., Wiedenhoft, H., Winglee, K., Humbert, O., et al. (2011). Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell* 145, 39–53. doi: 10.1016/j.cell.2011.02.022

Amato, S. M., Orman, M. A., and Brynildsen, M. P. (2013). Metabolic control of persister formation in *Escherichia coli*. *Mol. Cell* 50, 475–487. doi: 10.1016/j.molcel.2013.04.002

Argelaguet, R., Velten, B., Arnol, D., Dietrich, S., Zenz, T., Marioni, J. C., et al. (2018). Multi-omics factor analysis-a framework for unsupervised integration of multi-omics data sets. *Mol. Syst. Biol.* 14, e8124. doi: 10.15252/msb.20178124

Baek, S. H., Li, A. H., and Sassetti, C. M. (2011). Metabolic regulation of mycobacterial growth and antibiotic sensitivity. *PloS Biol.* 9, e1001065. doi: 10.1371/journal.pbio.1001065

Barry, C. E.,3RD, Boshoff, H. I., Dartois, ,. V., Dick, T., Ehrt, S., Flynn, J., et al. (2009). The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat. Rev. Microbiol.* 7, 845–855. doi: 10.1038/nrmicro2236

Baughn, A. D., and Rhee, K. Y. (2014). Metabolomics of central carbon metabolism in *Mycobacterium tuberculosis*. *Microbiol. Spectr.* 2 (3). doi: 10.1128/microbiolspec.MGM2-0026-2013

Bellerose, M. M., Baek, S. H., Huang, C. C., Moss, C. E., Koh, E. I., Proulx, M. K., et al. (2019). Common variants in the glycerol kinase gene reduce tuberculosis drug efficacy. *MBio* 10 (4), e00663–19. doi: 10.1128/mBio.00663-19

Bennett, B. D., Kimball, E. H., Gao, M., Osterhout, R., Van Dien, S. J., and Rabinowitz, J. D. (2009). Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat. Chem. Biol.* 5, 593–599. doi: 10.1038/nchembio.186

Bifani, P., Mathema, B., Kurepina, N., Shashkina, E., Bertout, J., Blanchis, A. S., et al. (2008). The evolution of drug resistance in *Mycobacterium tuberculosis*: from a mono-rifampin-resistant cluster into increasingly multidrug-resistant variants in an HIV-seropositive population. *J. Infect. Dis.* 198, 90–94. doi: 10.1086/588822

Blokhina, O., Virolainen, E., and Fagerstedt, K. V. (2003). Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann. Bot.* 91, 179–194. doi: 10.1093/aob/mcf118

Borah, K., Mendum, T. A., Hawkins, N. D., Ward, J. L., Beale, M. H., Larrouy-Maumus, G., et al. (2021). Metabolic fluxes for nutritional flexibility of *Mycobacterium tuberculosis*. *Mol. Syst. Biol.* 17, e10280. doi: 10.15252/msb.202110280

Brauner, A., Fridman, O., Gefen, O., and Balaban, N. Q. (2016). Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat. Rev. Microbiol.* 14, 320–330. doi: 10.1038/nrmicro.2016.34

Bryk, R., Gold, B., Venugopal, A., Singh, J., Samy, R., Pupek, K., et al. (2008). Selective killing of nonreplicating mycobacteria. *Cell Host Microbe* 3, 137–145. doi: 10.1016/j.chom.2008.02.003

Castro, R. A. D., Borrell, S., and Gagneux, S. (2021). The within-host evolution of antimicrobial resistance in *Mycobacterium tuberculosis*. *FEMS Microbiol. Rev.* 45 (4), fuaa071. doi: 10.1093/femsre/fuaa071

Chan, S. Y., and Loscalzo, J. (2012). The emerging paradigm of network medicine in the study of human disease. *Circ. Res.* 111, 359–374. doi: 10.1161/CIRCRESAHA.111.258541

Chan, J., Xing, Y., Magliozzo, R. S., and Bloom, B. R. (1992). Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. *J. Exp. Med.* 175, 1111–1122. doi: 10.1084/jem.175.4.1111

Cirz, R. T., Chin, J. K., Andes, D. R., De Crecy-Lagard, V., Craig, W. A., and Romesberg, F. E. (2005). Inhibition of mutation and combating the evolution of antibiotic resistance. *PloS Biol.* 3, e176. doi: 10.1371/journal.pbio.0030176

Colangeli, R., Gupta, A., Vinhas, S. A., Chippada Venkata, U. D., Kim, S., Grady, C., et al. (2020). *Mycobacterium tuberculosis* progresses through two phases of latent infection in humans. *Nat. Commun.* 11 (1), 4870. doi: 10.1038/s41467-020-18699-9

Conlon, B. P., Rowe, S. E., Gandt, A. B., Nuxoll, A. S., Donegan, N. P., Zalis, E. A., et al. (2016). Persister formation in *Staphylococcus aureus* is associated with ATP depletion. *Nat. Microbiol.* 1, 16051. doi: 10.1038/nmicrobiol.2016.51

Connolly, L. E., Edelstein, P. H., and Ramakrishnan, L. (2007). Why is long-term therapy required to cure tuberculosis? *PloS Med.* 4 (3), e120. doi: 10.1371/journal.pmed.0040120

Cosma, C. L., Sherman, D. R., and Ramakrishnan, L. (2003). The secret lives of the pathogenic mycobacteria. *Annu. Rev. Microbiol.* 57, 641–676. doi: 10.1146/annurev.micro.57.030502.091033

Daniel, J., Maamar, H., Deb, C., Sirakova, T. D., and Kolattukudy, P. E. (2011). *Mycobacterium tuberculosis* uses host triacylglycerol to accumulate lipid droplets and acquires a dormancy-like phenotype in lipid-loaded macrophages. *PloS Pathog.* 7, e1002093. doi: 10.1371/journal.ppat.1002093

Davis, E. O., Dullaghan, E. M., and Rand, L. (2002). Definition of the mycobacterial SOS box and use to identify LexA-regulated genes in *Mycobacterium tuberculosis*. *J. Bacteriol.* 184, 3287–3295. doi: 10.1128/JB.184.12.3287-3295.2002

Davis, J. M., and Ramakrishnan, L. (2009). The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell* 136, 37–49. doi: 10.1016/j.cell.2008.11.014

de Carvalho, L. P., Fischer, S. M., Marrero, J., Nathan, C., Ehrt, S., and Rhee, K. Y. (2010). Metabolomics of *Mycobacterium tuberculosis* reveals compartmentalized co-catabolism of carbon substrates. *Chem. Biol.* 17, 1122–1131. doi: 10.1016/j.chembiol.2010.08.009

Derewacz, D. K., Goodwin, C. R., Mcnees, C. R., Mclean, J. A., and Bachmann, B. O. (2013). Antimicrobial drug resistance affects broad changes in metabolomic phenotype in addition to secondary metabolism. *Proc. Natl. Acad. Sci. U.S.A.* 110, 2336–2341. doi: 10.1073/pnas.1218524110

Dettmer, K., Aronov, P. A., and Hammock, B. D. (2007). Mass spectrometry-based metabolomics. *Mass Spectrom Rev.* 26, 51–78. doi: 10.1002/mas.20108

Dhar, N., Mckinney, J., and Manina, G. (2016). Phenotypic heterogeneity in *Mycobacterium tuberculosis*. *Microbiol. Spectr.* 4 (6). doi: 10.1128/microbiolspec.TBTB2-0021-2016

Dunn, W. B., Broadhurst, D. I., Atherton, H. J., Goodacre, R., and Griffin, J. L. (2011). Systems level studies of mammalian metabolomes: the roles of mass spectrometry and nuclear magnetic resonance spectroscopy. *Chem. Soc. Rev.* 40, 387–426. doi: 10.1039/B906712B

Dutta, N. K., Klinkenberg, L. G., Vazquez, M. J., Segura-Carro, D., Colmenarejo, G., Ramon, F., et al. (2019). Inhibiting the stringent response blocks *Mycobacterium tuberculosis* entry into quiescence and reduces persistence. *Sci. Adv.* 5, eaav2104. doi: 10.1126/sciadv.aav2104

Ehrt, S., and Schnappinger, D. (2009). Mycobacterial survival strategies in the phagosome: defence against host stresses. *Cell Microbiol.* 11, 1170–1178. doi: 10.1111/j.1462-5822.2009.01335.x

Ehrt, S., Schnappinger, D., and Rhee, K. Y. (2018). Metabolic principles of persistence and pathogenicity in *Mycobacterium tuberculosis*. *Nat. Rev. Microbiol.* 16, 496–507. doi: 10.1038/s41579-018-0013-4

Eoh, H. (2014). Metabolomics: a window into the adaptive physiology of *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)* 94, 538–543. doi: 10.1016/j.tube.2014.08.002

Eoh, H., and Rhee, K. Y. (2013). Multifunctional essentiality of succinate metabolism in adaptation to hypoxia in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6554–6559. doi: 10.1073/pnas.1219375110

Eoh, H., and Rhee, K. Y. (2014). Methylcitrate cycle defines the bactericidal essentiality of isocitrate lyase for survival of *Mycobacterium tuberculosis* on fatty acids. *Proc. Natl. Acad. Sci. U.S.A.* 111, 4976–4981. doi: 10.1073/pnas.1400390111

Eoh, H., Wang, Z., Layre, E., Rath, P., Morris, R., Branch Moody, D., et al. (2017). Metabolic anticipation in *Mycobacterium tuberculosis*. *Nat. Microbiol.* 2, 17084. doi: 10.1038/nmicrobiol.2017.84

Fair, R. J., and Tor, Y. (2014). Antibiotics and bacterial resistance in the 21st century. *Perspect. Med. Chem.* 6, 25–64. doi: 10.4137/PMC.S14459

Farrow, M. F., and Rubin, E. J. (2008). Function of a mycobacterial major facilitator superfamily pump requires a membrane-associated lipoprotein. *J. Bacteriol.* 190, 1783–1791. doi: 10.1128/JB.01046-07

Fisher, R. A., Gollan, B., and Helaine, S. (2017). Persistent bacterial infections and persister cells. *Nat. Rev. Microbiol.* 15, 453–464. doi: 10.1038/nrmicro.2017.42

Flynn, J. L., and Chan, J. (2001). Tuberculosis: latency and reactivation. *Infect. Immun.* 69, 4195–4201. doi: 10.1128/IAI.69.7.4195-4201.2001

Foster, P. L. (2007). Stress-induced mutagenesis in bacteria. *Crit. Rev. Biochem. Mol. Biol.* 42, 373–397. doi: 10.1080/10409230701648494

Fridman, O., Goldberg, A., Ronin, I., Shoresh, N., and Balaban, N. Q. (2014). Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature* 513, 418–421. doi: 10.1038/nature13469

Galhardo, R. S., Hastings, P. J., and Rosenberg, S. M. (2007). Mutation as a stress response and the regulation of evolvability. *Crit. Rev. Biochem. Mol. Biol.* 42, 399–435. doi: 10.1080/10409230701648502

Gengenbacher, M., and Kaufmann, S. H. (2012). *Mycobacterium tuberculosis*: success through dormancy. *FEMS Microbiol. Rev.* 36, 514–532. doi: 10.1111/j.1574-6976.2012.00331.x

Giffin, M. M., Shi, L., Gennaro, M. L., and Sohaskey, C. D. (2016). Role of alanine dehydrogenase of *Mycobacterium tuberculosis* during recovery from hypoxic nonreplicating persistence. *PloS One* 11, e0155522. doi: 10.1371/journal.pone.0155522

Gold, B., and Nathan, C. (2017). Targeting phenotypically tolerant mycobacterium tuberculosis. *Microbiol. Spectr.* 5(1), 10. doi: 10.1128/9781555819569.ch15

Gollan, B., Grabe, G., Michaux, C., and Helaine, S. (2019). Bacterial persisters and infection: Past, present, and progressing. *Annu. Rev. Microbiol.* 73, 359–385. doi: 10.1146/annurev-micro-020518-115650

Gomez, J. E., and McKinney, J. D. (2004). *M. tuberculosis* persistence, latency, and drug tolerance. *Tuberculosis (Edinb)* 84, 29–44. doi: 10.1016/j.tube.2003.08.003

Goossens, S. N., Sampson, S. L., and Van Rie, A. (2020). Mechanisms of drug-induced tolerance in *Mycobacterium tuberculosis*. *Clin. Microbiol. Rev.* 34(1), e00141–20. doi: 10.1128/CMR.00141-20

Gould, T. A., De Langemheen, V., H., M.-E., Mckinney, J. D., and Sacchettini, J. C. (2006). Dual role of isocitrate lyase 1 in the glyoxylate and methylcitrate cycles in *Mycobacterium tuberculosis*. *Mol. Microbiol.* 61, 940–947. doi: 10.1111/j.1365-2958.2006.05297.x

Griffin, J. L. (2004). Metabolic profiles to define the genome: can we hear the phenotypes? *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 359, 857–871. doi: 10.1098/rstb.2003.1411

Harrison, G. A., Bridwell, M., Singh, M., K., W., Kinsella, R. L., Aneke, J. S., et al. (2019). Identification of 4-Amino-Thieno[2,3-d]Pyrimidines as QcrB inhibitors in *Mycobacterium tuberculosis*. *mSphere* 4 (5), e00606–19. doi: 10.1128/mSphere.00606-19

Hartman, T. E., Wang, Z., Jansen, R. S., Gardete, S., and Rhee, K. Y. (2017). Metabolic perspectives on persistence. *Microbiol. Spectr.* 5 (1), 10. doi: 10.1128/9781555819569.ch31

Hartman, T., Weinrick, B., Vilcheze, C., Berney, M., Tufariello, J., Cook, G. M., et al. (2014). Succinate dehydrogenase is the regulator of respiration in *Mycobacterium tuberculosis*. *PloS Pathog.* 10, e1004510. doi: 10.1371/journal.ppat.1004510

Hicks, N. D., Carey, A. F., Yang, J., Zhao, Y., and Fortune, S. M. (2019). Bacterial genome-wide association identifies novel factors that contribute to ethionamide and prothionamide susceptibility in *Mycobacterium tuberculosis*. *MBio* 10 (2), e00616–19. doi: 10.1128/mBio.00616-19

Hicks, N. D., Yang, J., Zhang, X., Zhao, B., Grad, Y. H., Liu, L., et al. (2018). Clinically prevalent mutations in *Mycobacterium tuberculosis* alter propionate metabolism and mediate multidrug tolerance. *Nat. Microbiol.* 3, 1032–1042. doi: 10.1038/s41564-018-0218-3

Hutchings, M. I., Truman, A. W., and Wilkinson, B. (2019). Antibiotics: past, present and future. *Curr. Opin. Microbiol.* 51, 72–80. doi: 10.1016/j.mib.2019.10.008

Jansen, R. S., and Rhee, K. Y. (2017). Emerging approaches to tuberculosis drug development: At home in the metabolome. *Trends Pharmacol. Sci.* 38, 393–405. doi: 10.1016/j.tips.2017.01.005

Kalia, N. P., Shi Lee, B., Ab Rahman, N. B., Moraski, G. C., Miller, M. J., and Pethe, K. (2019). Carbon metabolism modulates the efficacy of drugs targeting the cytochrome bc1:aa3 in *Mycobacterium tuberculosis*. *Sci. Rep.* 9 (1):8608. doi: 10.1038/s41598-019-44887-9

Kim, Y. J., Lee, J. Y., Lee, J. J., Jeon, S. M., Silwal, P., Kim, I. S., et al. (2022). Arginine-mediated gut microbiome remodeling promotes host pulmonary immune defense against nontuberculous mycobacterial infection. *Gut Microbes* 14, 2073132. doi: 10.1080/19490976.2022.2073132

Kohanski, M. A., Depristo, M. A., and Collins, J. J. (2010a). Sublethal antibiotic treatment leads to multidrug resistance *via* radical-induced mutagenesis. *Mol. Cell* 37, 311–320. doi: 10.1016/j.molcel.2010.01.003

Kohanski, M. A., Dwyer, D. J., and Collins, J. J. (2010b). How antibiotics kill bacteria: from targets to networks. *Nat. Rev. Microbiol.* 8, 423–435. doi: 10.1038/nrmicro2333

Koul, A., Vranckx, L., Dhar, N., Gohlmann, H. W., Ozdemir, E., Neefs, J. M., et al. (2014). Delayed bactericidal response of *Mycobacterium tuberculosis* to bedaquiline involves remodelling of bacterial metabolism. *Nat. Commun.* 5, 3369. doi: 10.1038/ncomms4369

Kulka, K., Hatfull, G., and Ojha, A. K. (2012). Growth of *Mycobacterium tuberculosis* biofilms. *J. Vis. Exp.* 60, 3820. doi: 10.3791/3820

Kundu, S., Biukovic, G., Gruber, G., and Dick, T. (2016). Bedaquiline targets the epsilon subunit of mycobacterial f-ATP synthase. *Antimicrob. Agents Chemother.* 60, 6977–6979. doi: 10.1128/AAC.01291-16

Layre, E., Sweet, L., Hong, S., Madigan, C. A., Desjardins, D., Young, D. C., et al. (2011). A comparative lipidomics platform for chemotaxonomic analysis of *Mycobacterium tuberculosis*. *Chem. Biol.* 18, 1537–1549. doi: 10.1016/j.chembiol.2011.10.013

Lee, J. J., Lee, S. K., Song, N., Nathan, T. O., Swarts, B. M., Eum, S. Y., et al. (2019). Transient drug-tolerance and permanent drug-resistance rely on the trehalose-catalytic shift in *Mycobacterium tuberculosis*. *Nat. Commun.* 10, 2928. doi: 10.1038/s41467-019-10975-7

Lee, J. J., Lim, J., Gao, S., Lawson, C. P., Odell, M., Raheem, S., et al. (2018). Glutamate mediated metabolic neutralization mitigates propionate toxicity in intracellular *Mycobacterium tuberculosis*. *Sci. Rep.* 8 (1), 8506. doi: 10.1038/s41598-018-26950-z

Lee, W., Vanderven, B. C., Fahey, R. J., and Russell, D. G. (2013). Intracellular *Mycobacterium tuberculosis* exploits host-derived fatty acids to limit metabolic stress. *J. Biol. Chem.* 288, 6788–6800. doi: 10.1074/jbc.M112.445056

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., and Balaban, N. Q. (2017). Antibiotic tolerance facilitates the evolution of resistance. *Science* 355, 826–830. doi: 10.1126/science.aaj2191

Levin, B. R., and Rozen, D. E. (2006). Non-inherited antibiotic resistance. *Nat. Rev. Microbiol.* 4, 556–562. doi: 10.1038/nrmicro1445

Lewis, K. (2010). Persister cells. *Annu. Rev. Microbiol.* 64, 357–372. doi: 10.1146/annurev.micro.112408.134306

Lewis, K., and Shan, Y. (2017). Why tolerance invites resistance. *Science* 355, 796. doi: 10.1126/science.aam7926

Lim, J., Lee, J. J., Lee, S. K., Kim, S., Eum, S. Y., and Eoh, H. (2021). Phosphoenolpyruvate depletion mediates both growth arrest and drug tolerance of *Mycobacterium tuberculosis* in hypoxia. *Proc. Natl. Acad. Sci. U.S.A.* 118. doi: 10.1073/pnas.2105800118

Liu, K., Yu, J., and Russell, D. G. (2003). *pckA*-deficient *Mycobacterium bovis* BCG shows attenuated virulence in mice and in macrophages. *Microbiology* 149, 1829–1835. doi: 10.1099/mic.0.26234-0

Long, H., Miller, S. F., Strauss, C., Zhao, C., Cheng, L., Ye, Z., et al. (2016). Antibiotic treatment enhances the genome-wide mutation rate of target cells. *Proc. Natl. Acad. Sci. U.S.A.* 113, E2498–E2505. doi: 10.1073/pnas.1601208113

Luna, B., Trebosc, V., Lee, B., Bakowski, M., Ulhaq, A., Yan, J., et al. (2020). A nutrient-limited screen unmasks rifabutin hyperactivity for extensively drug-resistant acinetobacter baumannii. *Nat. Microbiol.* 5, 1134–1143. doi: 10.1038/s41564-020-0737-6

Lu, W., Su, X., Klein, M. S., Lewis, I. A., Fiehn, O., and Rabinowitz, J. D. (2017). Metabolite measurement: Pitfalls to avoid and practices to follow. *Annu. Rev. Biochem.* 86, 277–304. doi: 10.1146/annurev-biochem-061516-044952

Machova, I., Snasel, J., Zimmermann, M., Laubitz, D., Plocinski, P., Oehlmann, W., et al. (2014). *Mycobacterium tuberculosis* phosphoenolpyruvate carboxykinase is regulated by redox mechanisms and interaction with thioredoxin. *J. Biol. Chem.* 289, 13066–13078. doi: 10.1074/jbc.M113.536748

Mackenzie, J. S., Lamprecht, D. A., Asmal, R., Adamson, J. H., Borah, K., Beste, D. J. V., et al. (2020). Bedaquiline reprograms central metabolism to reveal glycolytic vulnerability in *Mycobacterium tuberculosis*. *Nat. Commun.* 11(1), 6092. doi: 10.1038/s41467-020-19959-4

Mahajan, R. (2013). Bedaquiline: First FDA-approved tuberculosis drug in 40 years. *Int. J. Appl. Basic Med. Res.* 3, 1–2. doi: 10.4103/2229-516X.112228

Manina, G., Dhar, N., and Mckinney, J. D. (2015). Stress and host immunity amplify *Mycobacterium tuberculosis* phenotypic heterogeneity and induce nongrowing metabolically active forms. *Cell Host Microbe* 17, 32–46. doi: 10.1016/j.chom.2014.11.016

Marrero, J., Rhee, K. Y., Schnappinger, D., Pethe, K., and Ehrt, S. (2010). Gluconeogenic carbon flow of tricarboxylic acid cycle intermediates is critical for *Mycobacterium tuberculosis* to establish and maintain infection. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9819–9824. doi: 10.1073/pnas.1000715107

Marrero, J., Trujillo, C., Rhee, K. Y., and Ehrt, S. (2013). Glucose phosphorylation is required for *Mycobacterium tuberculosis* persistence in mice. *PloS Pathog.* 9, e1003116. doi: 10.1371/journal.ppat.1003116

Martinot, A. J., Farrow, M., Bai, L., Layre, E., Cheng, T. Y., Tsai, J. H., et al. (2016). Mycobacterial metabolic syndrome: LprG and Rv1410 regulate triacylglyceride levels, growth rate and virulence in *Mycobacterium tuberculosis*. *PloS Pathog.* 12, e1005351. doi: 10.1371/journal.ppat.1005351

McKinney, J. D., Zu Bentrup, H., K., M.-E., Miczak, A., B., C., Swenson, D., et al. (2000). Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* 406, 735–738. doi: 10.1038/35021074

Miethke, M., Pieroni, M., Weber, T., Bronstrup, M., Hammann, P., Halby, L., et al. (2021). Towards the sustainable discovery and development of new antibiotics. *Nat. Rev. Chem.* 5, 726–749. doi: 10.1038/s41570-021-00313-1

Munoz-Elias, E. J., and McKinney, J. D. (2005). *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for *in vivo* growth and virulence. *Nat. Med.* 11, 638–644. doi: 10.1038/nm1252

Nandakumar, M., Nathan, C., and Rhee, K. Y. (2014). Isocitrate lyase mediates broad antibiotic tolerance in *Mycobacterium tuberculosis*. *Nat. Commun.* 5, 4306. doi: 10.1038/ncomms5306

Nandakumar, M., Prosser, G. A., De Carvalho, L. P., and Rhee, K. (2015). Metabolomics of *Mycobacterium tuberculosis*. *Methods Mol. Biol.* 1285, 105–115. doi: 10.1007/978-1-4939-2450-9_6

Nathan, C. (2014). Drug-resistant tuberculosis: a new shot on goal. *Nat. Med.* 20, 121–123. doi: 10.1038/nm.3470

Nathan, C., and Barry, C. E.3rd (2015). TB drug development: immunology at the table. *Immunol. Rev.* 264, 308–318. doi: 10.1111/imr.12275

Nathan, C. F., and Hibbs, J. B. Jr. (1991). Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr. Opin. Immunol.* 3, 65–70. doi: 10.1016/0952-7915(91)90079-G

Ojha, A., Anand, M., Bhatt, A., Kremer, L., Jacobs, W. R., and Hatfull, G. F. (2005). GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria. *Cell* 123, 861–873. doi: 10.1016/j.cell.2005.09.012

Ojha, A. K., Jacobs, W. R.Jr., and Hatfull, G. F. (2015). Genetic dissection of mycobacterial biofilms. *Methods Mol. Biol.* 1285, 215–226. doi: 10.1007/978-1-4939-2450-9_12

Parrish, N. M., Dick, J. D., and Bishai, W. R. (1998). Mechanisms of latency in *Mycobacterium tuberculosis*. *Trends Microbiol.* 6, 107–112. doi: 10.1016/S0966-842X(98)01216-5

Patra, K. C., and Hay, N. (2014). The pentose phosphate pathway and cancer. *Trends Biochem. Sci.* 39, 347–354. doi: 10.1016/j.tibs.2014.06.005

Pethe, K., Bifani, P., Jang, J., Kang, S., Park, S., Ahn, S., et al. (2013). Discovery of Q203, a potent clinical candidate for the treatment of tuberculosis. *Nat. Med.* 19, 1157–1160. doi: 10.1038/nm.3262

Peyrusson, F., Varet, H., Nguyen, T. K., Legendre, R., Sismeiro, O., Coppee, J. Y., et al. (2020). Intracellular *Staphylococcus aureus* persisters upon antibiotic exposure. *Nat. Commun.* 11 (1), 2200. doi: 10.1038/s41467-020-15966-7

Prax, M., and Bertram, R. (2014). Metabolic aspects of bacterial persisters. *Front. Cell Infect. Microbiol.* 4, 148. doi: 10.3389/fcimb.2014.00148

Puckett, S., Trujillo, C., Wang, Z., Eoh, H., Ioerger, T. R., Krieger, I., et al. (2017). Glyoxylate detoxification is an essential function of malate synthase required for carbon assimilation in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 114, e2225–e2232. doi: 10.1073/pnas.1617655114

Quinonez, C. G., Lee, J. J., Lim, J., Odell, M., Lawson, C. P., Anyogu, A., et al. (2022). The role of fatty acid metabolism in drug tolerance of *Mycobacterium tuberculosis*. *MBio*. 13(1), e0355921. doi: 10.1128/mbio.03559-21

Ramakrishnan, L. (2012). Revisiting the role of the granuloma in tuberculosis. *Nat. Rev. Immunol.* 12, 352–366. doi: 10.1038/nri3211

Rao, S. P., Alonso, S., Rand, L., Dick, T., and Pethe, K. (2008). The protonmotive force is required for maintaining ATP homeostasis and viability of hypoxic, nonreplicating *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11945–11950. doi: 10.1073/pnas.0711697105

Revitt-Mills, S. A., and Robinson, A. (2020). Antibiotic-induced mutagenesis: Under the microscope. *Front. Microbiol.* 11, 585175. doi: 10.3389/fmicb.2020.585175

Rhee, K. (2013). Minding the gaps: metabolomics mends functional genomics. *EMBO Rep.* 14, 949–950. doi: 10.1038/embor.2013.155

Riganti, C., Gazzano, E., Polimeni, M., Aldieri, E., and Ghigo, D. (2012). The pentose phosphate pathway: an antioxidant defense and a crossroad in tumor cell fate. *Free Radic. Biol. Med.* 53, 421–436. doi: 10.1016/j.freeradbiomed.2012.05.006

Ruhl, C. R., Pasko, B. L., Khan, H. S., Kindt, L. M., Stamm, C. E., Franco, L. H., et al. (2020). *Mycobacterium tuberculosis* sulfolipid-1 activates nociceptive neurons and induces cough. *Cell* 181, 293–305.e11. doi: 10.1016/j.cell.2020.02.026

Safi, H., Gopal, P., Lingaraju, S., Ma, S., Levine, C., Dartois, V., et al. (2019). Phase variation in *Mycobacterium tuberculosis glpK* produces transiently heritable drug tolerance. *Proc. Natl. Acad. Sci. U.S.A.* 116, 19665–19674. doi: 10.1073/pnas.1907631116

Saito, K., Mishra, S., Warrier, T., Cicchetti, N., Mi, J., Weber, E., et al. (2021). Oxidative damage and delayed replication allow viable *Mycobacterium tuberculosis* to go undetected. *Sci. Transl. Med.* 13, eabg2612. doi: 10.1126/scitranslmed.abg2612

Samuels, D. J., Wang, Z., Rhee, K. Y., and Brinsmade, S. R. (2017). A tandem liquid chromatography-mass spectrometry-based approach for metabolite analysis of *Staphylococcus aureus*. *J. Vis. Exp.* 121, 55558. doi: 10.3791/55558

Sarathy, J. P., Via, L. E., Weiner, D., Blanc, L., Boshoff, H., Eugenin, E. A., et al. (2018). Extreme drug tolerance of *Mycobacterium tuberculosis* in caseum. *Antimicrob. Agents Chemother.* 62 (2), e02266–17. doi: 10.1128/AAC.02266-17

Savvi, S., Warner, D. F., Kana, B. D., Mckinney, J. D., Mizrahi, V., and Dawes, S. S. (2008). Functional characterization of a vitamin B12-dependent methylmalonyl pathway in *Mycobacterium tuberculosis*: implications for propionate metabolism during growth on fatty acids. *J. Bacteriol.* 190, 3886–3895. doi: 10.1128/JB.01767-07

Schrader, S. M., Botella, H., Jansen, R., Ehrt, S., Rhee, K., Nathan, C., et al. (2021). Multiform antimicrobial resistance from a metabolic mutation. *Sci. Adv.* 7 (35), eabh2037. doi: 10.1126/sciadv.abh2037

Schrader, S. M., Vaubourgeix, J., and Nathan, C. (2020). Biology of antimicrobial resistance and approaches to combat it. *Sci. Transl. Med.* 12 (549), eaaz6992. doi: 10.1126/scitranslmed.aaz6992

Schwab, W. (2003). Metabolome diversity: Too few genes, too many metabolites? *Phytochemistry* 62, 837–849. doi: 10.1016/S0031-9422(02)00723-9

Serafini, A., Tan, L., Horswell, S., Howell, S., Greenwood, D. J., Hunt, D. M., et al. (2019). *Mycobacterium tuberculosis* requires glyoxylate shunt and reverse methylcitrate cycle for lactate and pyruvate metabolism. *Mol. Microbiol.* 112, 1284–1307. doi: 10.1111/mmi.14362

Shi, L., Sohaskey, C. D., Pheiffer, C., Datta, P., Parks, M., Mcfadden, J., et al. (2010). Carbon flux rerouting during *Mycobacterium tuberculosis* growth arrest. *Mol. Microbiol.* 78, 1199–1215. doi: 10.1111/j.1365-2958.2010.07399.x

Stincone, A., Prigione, A., Cramer, T., Wamelink, M. M., Campbell, K., Cheung, E., et al. (2015). The return of metabolism: Biochemistry and physiology of the pentose phosphate pathway. *Biol. Rev. Camb. Philos. Soc.* 90, 927–963. doi: 10.1111/brv.12140

Subramanian, I., Verma, S., Kumar, S., Jere, A., and Anamika, K. (2020). Multi-omics data integration, interpretation, and its application. *Bioinform. Biol. Insights* 14, 1177932219899051. doi: 10.1177/1177932219899051

Torrey, H. L., Keren, I., Via, L. E., Lee, J. S., and Lewis, K. (2016). High persister mutants in *Mycobacterium tuberculosis*. *PloS One* 11, E0155127. doi: 10.1371/journal.pone.0155127

Tozzi, M. G., Camici, M., Mascia, L., Sgarrella, F., and Ipata, P. L. (2006). Pentose phosphates in nucleoside interconversion and catabolism. *FEBS J.* 273, 1089–1101. doi: 10.1111/j.1742-4658.2006.05155.x

Villas-Boas, S. G., Mas, S., Akesson, M., Smedsgaard, J., and Nielsen, J. (2005). Mass spectrometry in metabolome analysis. *Mass Spectrom Rev.* 24, 613–646. doi: 10.1002/mas.20032

Viney, K., Linh, N. N., Gegia, M., Zignol, M., Glaziou, P., Ismail, N., et al. (2021). New definitions of pre-extensively and extensively drug-resistant tuberculosis: Update from the world health organization. *Eur. Respir. J.* 57 (4), 2100361. doi: 10.1183/13993003.00361-2021

Voskuil, M. I., Bartek, I. L., Visconti, K., and Schoolnik, G. K. (2011). The response of *Mycobacterium tuberculosis* to reactive oxygen and nitrogen species. *Front. Microbiol.* 2, 105. doi: 10.3389/fmicb.2011.00105

Voskuil, M. I., Schnappinger, D., Visconti, K. C., Harrell, M. I., Dolganov, G. M., Sherman, D. R., et al. (2003). Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *J. Exp. Med.* 198, 705–713. doi: 10.1084/jem.20030205

Wang, Z., Soni, V., Marriner, G., Kaneko, T., Boshoff, H. I. M., Barry, C. E., et al. (2019). Mode-Of-Action profiling reveals glutamine synthetase as a collateral metabolic vulnerability of *M. tuberculosis* to bedaquiline. *Proc. Natl. Acad. Sci. U.S.A.* 116 (39), 19646–19651. doi: 10.1073/pnas.1907946116

Warner, D. F. (2014). *Mycobacterium tuberculosis* metabolism. *Cold Spring Harb. Perspect. Med.* 5 (4), a021121. doi: 10.1101/cshperspect.a021121

Watanabe, S., Zimmermann, M., Goodwin, M. B., Sauer, U., Barry, C. E.3rd, and Boshoff, H. I. (2011). Fumarate reductase activity maintains an energized membrane in anaerobic *Mycobacterium tuberculosis*. *PloS Pathog.* 7, e1002287. doi: 10.1371/journal.ppat.1002287

WHO (2021). *Global tuberculosis report 2021*. (Switzerland: World Health Organization Geneva). Available at: https://www.who.int/publications/i/item/9789240037021.

Windels, E. M., Michiels, J. E., Fauvart, M., Wenseleers, T., Van Den Bergh, B., and Michiels, J. (2019a). Bacterial persistence promotes the evolution of antibiotic resistance by increasing survival and mutation rates. *Isme J.* 13, 1239–1251. doi: 10.1038/s41396-019-0344-9

Windels, E. M., Michiels, J. E., Van Den Bergh, B., Fauvart, M., and Michiels, J. (2019b). Antibiotics: Combatting tolerance to stop resistance. *Mbio* 10 (5), e02095–19. doi: 10.1128/mBio.02095-19

Wu, L., Mashego, M. R., Van Dam, J. C., Proell, A. M., Vinke, J. L., Ras, C., et al. (2005). Quantitative analysis of the microbial metabolome by isotope dilution mass spectrometry using uniformly ^{13}c-labeled cell extracts as internal standards. *Anal. Biochem.* 336, 164–171. doi: 10.1016/j.ab.2004.09.001

Xie, N., Zhang, L., Gao, W., Huang, C., Huber, P. E., Zhou, X., et al. (2020). Nad (+) metabolism: Pathophysiologic mechanisms and therapeutic potential. *Signal Transduct Target Ther.* 5, 227. doi: 10.1038/s41392-020-00311-7

Zaman, K. (2010). Tuberculosis: A global health problem. *J. Health Popul. Nutr.* 28, 111–113. doi: 10.3329/jhpn.v28i2.4879

Zhang, Y., Yew, W. W., and Barer, M. R. (2012). Targeting persisters for tuberculosis control. *Antimicrob. Agents Chemother.* 56, 2223–2230. doi: 10.1128/AAC.06288-11

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Mini Review
PUBLISHED 22 August 2022
DOI 10.3389/fcimb.2022.958555

OPEN ACCESS

EDITED BY
Helena Ingrid Boshoff,
National Institutes of Health (NIH),
United States

REVIEWED BY
Khushboo Borah,
University of Surrey, United Kingdom
Nicole Suzanne Sampson,
Stony Brook University, United States

*CORRESPONDENCE
Christina L. Stallings
stallings@wustl.edu

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 31 May 2022
ACCEPTED 29 July 2022
PUBLISHED 22 August 2022

CITATION
Samuels AN, Wang ER, Harrison GA, Valenta JC and Stallings CL (2022) Understanding the contribution of metabolism to *Mycobacterium tuberculosis* drug tolerance. *Front. Cell. Infect. Microbiol.* 12:958555. doi: 10.3389/fcimb.2022.958555

Understanding the contribution of metabolism to *Mycobacterium tuberculosis* drug tolerance

Amanda N. Samuels†, Erin R. Wang†, Gregory A. Harrison†, Joy C. Valenta and Christina L. Stallings*

Department of Molecular Microbiology, Washington University School of Medicine, Saint Louis, MO, United States

Treatment of *Mycobacterium tuberculosis* (*Mtb*) infections is particularly arduous. One challenge to effectively treating tuberculosis is that drug efficacy *in vivo* often fails to match drug efficacy *in vitro*. This is due to multiple reasons, including inadequate drug concentrations reaching *Mtb* at the site of infection and physiological changes of *Mtb* in response to host derived stresses that render the bacteria more tolerant to antibiotics. To more effectively and efficiently treat tuberculosis, it is necessary to better understand the physiologic state of *Mtb* that promotes drug tolerance in the host. Towards this end, multiple studies have converged on bacterial central carbon metabolism as a critical contributor to *Mtb* drug tolerance. In this review, we present the evidence that changes in central carbon metabolism can promote drug tolerance, depending on the environment surrounding *Mtb*. We posit that these metabolic pathways could be potential drug targets to stymie the development of drug tolerance and enhance the efficacy of current antimicrobial therapy.

KEYWORDS

tuberculosis, antibiotics, tolerance, metabolism, granuloma, cholesterol, hypoxia

Mtb infections are recalcitrant to antibiotic therapy

Tuberculosis, an infection caused by the pathogen *Mycobacterium tuberculosis (Mtb)* is one of the leading causes of death world-wide by an infectious agent (WHO, 2021). Standard of care treatment for drug sensitive *Mtb* infections requires at least 6 months of antibiotic therapy with 4 or more antibiotics (WHO, 2017). Infection with *Mtb* mutants that are resistant to the frontline antibiotics isoniazid and rifampicin constituted approximately half a million tuberculosis cases in 2019 (WHO, 2021) and contributes

to treatment failure (Chen et al., 2020). The treatment regimen for patients harboring drug resistant *Mtb* is even longer and more expensive than drug sensitive cases and has an increased risk of adverse side effects (Nahid et al., 2019; WHO, 2021; Ghazy et al., 2022). Overall, the emergence and prevalence of *Mtb* drug resistance threatens treatment efficacy globally.

In addition, treatment failure and relapse can occur even in the absence of drug resistance. Dating as far back as the 1950's, it is documented that *Mtb* can be recovered from some patients after antibiotic treatment, with a fraction of these isolates remaining drug sensitive *in vitro* (Hobby, 1955; Wallace and Sutherland, 1955). In a 2014 study, 8% of patients that were treated with the standard of care isoniazid, rifampin, pyrazinamide, and ethambutol for 8 weeks followed by 18 weeks of isoniazid and rifampicin had an unfavorable outcome (Gillespie et al., 2014). The most common unfavorable outcome was relapse, which was differentiated from patients re-infected with another strain by using 24-locus mycobacterial-interspersed-repetitive-unit analysis to confirm that the strains isolated during relapse were the same as the primary infection (Gillespie et al., 2014). In this study, only 25% of the patients receiving the standard of care that relapsed after conversion to culture-negative status were suspected to have acquired drug resistance (Gillespie et al., 2014). Shortening the antibiotic regimen results in even further increased rates of treatment failure and relapse (Gillespie et al., 2014; Jindani et al., 2014). Another study collected serial *Mtb* isolates from tuberculosis patients that had relapsed infection after antibiotic treatment, where relapse was defined by paired isolates exhibiting 0-6 single nucleotide polymorphisms by whole genome sequencing (Bryant et al., 2013). In this study, all the relapsed *Mtb* isolates were drug sensitive *in vitro* (Bryant et al., 2013). Collectively, this data support that a reservoir of drug sensitive *Mtb* can persist in the host despite antibiotic therapy, contributing to treatment failure in some patients.

Factors that contribute to *Mtb* surviving antibiotic treatment *in vivo*

Multiple factors have been identified that enable *Mtb* to persist in the host during antibiotic treatment without acquiring a drug resistance mutation. One factor is the pathology that develops within the lung during *Mtb* infection. During infection, the interaction between *Mtb* and the host immune response can result in the development of a granuloma, which is made up of host immune cells, *Mtb*, and tissue debris (Ehlers and Schaible, 2013). Antibiotic penetration into the granuloma can be limited based on the chemical properties of the antibiotic, which creates a challenge for efficient delivery of the antibiotic to the various sites where *Mtb* resides (Kjellsson et al., 2012; Prideaux et al., 2015; Sarathy et al., 2018). In addition, *Mtb* can reside within various compartments inside innate immune cells, which can impact antibiotic efficacy. For example, pyrazinamide preferentially accumulates and is maximally active against *Mtb* in acidified compartments within the macrophage (Santucci et al., 2021; Santucci et al., 2022).

In addition to the host response impacting antibiotic accessibility to *Mtb*, the pathogen itself changes its physiology in response to the host environment, resulting in phenotypic drug tolerance. Importantly, drug tolerance is different from drug resistance in that a drug tolerant population can survive in the presence of an antibiotic but cannot grow until the antibiotic pressure is removed, whereas a drug resistant population can both survive and replicate in the presence of an antibiotic. In unstressed axenic culture conditions, *Mtb* populations display a basal level of heterogeneity such that a subpopulation of bacteria is transiently tolerant to antibiotics (Aldridge et al., 2012; Manina et al., 2015; Rego et al., 2017). Because of this drug tolerant subpopulation, treatment with a bactericidal antibiotic, such as isoniazid or rifampicin, leads to a significant decrease in viable bacteria, but fails to sterilize the culture (Jain et al., 2016; Sukheja et al., 2017; Vilcheze et al., 2017). Some of the drug susceptibility heterogeneity results from *Mtb*'s asymmetric cell division (Aldridge et al., 2012; Rego et al., 2017). Deletion of the gene *lamA/mmpS3* leads to a loss of asymmetric cell elongation and cell size heterogeneity in *Mycobacterium smegmatis*, and an *Mtb lamA/mmpS3* mutant is more susceptible to killing by rifampicin and vancomycin, suggesting that asymmetric cell elongation and cell size heterogeneity contributes to the emergence of drug tolerant subpopulations (Rego et al., 2017). In addition, there are stochastic differences in gene expression within mycobacterial cultures that can affect antibiotic susceptibility. For example, mycobacteria exhibit stochastic variation in the expression of *katG*, which is required to activate the pro-drug isoniazid, leading to a small population of bacteria with transiently low *katG* expression that can survive exposure to isoniazid (Wakamoto et al., 2013).

The proportion of drug tolerant *Mtb* is higher *in vivo* when compared to the small population that exists at basal levels in unstressed axenic cultures. *Mtb* directly isolated from patient sputum samples exhibited a nearly 10-fold reduction in killing by streptomycin, isoniazid, ethambutol, or rifampicin in comparison to when those same isolates were passaged through normal culture conditions (Turapov et al., 2016). *Mtb* in caseum isolated from infected rabbit granulomas also exhibited a >100-fold increase in the minimum bactericidal concentration for rifampicin and isoniazid compared to *Mtb* growing *in vitro* (Sarathy et al., 2018). Therefore, the *Mtb* population at the site of infection is enriched for drug tolerant cells, indicating that the host environment causes the *Mtb* population to shift towards a more drug tolerant state. Understanding the mechanistic basis for this enhanced drug tolerance is essential for developing therapies that target the *Mtb* population that is recalcitrant to treatment.

Stresses encountered in the host promote drug tolerance

During infection of macrophages, *Mtb* may be exposed to low pH, nitrosative stress, oxidative stress, osmotic changes, carbohydrate limitation, and cell envelope damage (Schnappinger et al., 2003; Tan et al., 2013; Larrouy-Maumus et al., 2016; Pisu et al., 2020). The environment within granulomas also poses additional stresses on *Mtb*, where granulomas can be hypoxic (Via et al., 2008), contain host factors that sequester iron (Kurthkoti et al., 2017), and harbor host enzymes that produce reactive oxygen species (Marakalala et al., 2016). Despite this harsh host environment, *Mtb* can survive due to its robust stress response capabilities. *Mtb* responds transcriptionally and metabolically to survive exposure to hypoxia (Wayne and Hayes, 1996), nitric oxide (Voskuil et al., 2003), reactive oxygen species (Voskuil et al., 2011), carbon limitation (Loebel et al., 1933; Betts et al., 2002; Gengenbacher et al., 2010), iron limitation (Kurthkoti et al., 2017), and low pH (Baker et al., 2014). Importantly, when exposed to stress *in vitro*, such as hypoxia, low pH, changes in osmolarity, or nutrient limitation, the proportion of drug tolerant *Mtb* increases, leading to higher minimal inhibitory concentrations or minimum bactericidal concentrations for several antibiotics (Wayne and Hayes, 1996; Deb et al., 2009; Gengenbacher et al., 2010; Larrouy-Maumus et al., 2016; Sarathy et al., 2018; Baker and Abramovitch, 2018; Xie et al., 2005). These data support that exposure to host derived stresses contributes to the increased *Mtb* antibiotic tolerance observed during infection.

Mtb stress responses are complex and involve multiple transcriptional, proteomic, and metabolic changes aimed at promoting pathogen survival. The resulting increase in drug tolerance that emerges in these conditions is indisputably multifactorial. Recent reviews have focused on the role of transcriptional adaptation (Kundu and Basu, 2021), the stringent response (Sharma et al., 2021), bacterial respiration (Hasenoehrl et al., 2021), and drug efflux in *Mtb* drug tolerance (Remm et al., 2021). In this review, we will focus on the role of fluctuations in central carbon metabolism in promoting drug tolerance of *Mtb* and discuss how continued dissection of the link between central carbon metabolism and drug tolerance will provide novel therapeutic approaches to target drug tolerant *Mtb*.

Carbon metabolism in *Mtb*

Mtb grown *in vitro* can metabolize multiple carbon sources, even simultaneously (de Carvalho et al., 2010). Some of the most common carbon sources used to culture *Mtb* include glucose, glycerol, and oleic acid (Larsen et al., 2007). Glucose and other sugars are metabolized primarily through glycolysis and the pentose phosphate pathway to generate ATP and reducing equivalents (Figure 1). Glycerol is also used to generate ATP and reducing equivalents through glycolysis, or it can be anabolized *via* gluconeogenesis to synthesize sugars. To assimilate into these pathways, glycerol must first be converted to glycerol-3-phosphate by GlpK and then oxidized to dihydroxyacetone phosphate (DHAP)(Figure 1). Oleic acid and other even-chain fatty acids are catabolized to acetyl-CoA, which enters the tricarboxylic acid (TCA) cycle (Figure 1). The TCA cycle is critical for the generation of the reducing equivalents NADH and NADPH, as well as biosynthetic precursors for multiple other pathways, including synthesis of several amino acids. In particular, α-ketoglutarate can be converted to glutamate, which is a precursor for glutamine, arginine, and proline synthesis, and oxaloacetate can be converted to aspartate, which serves as a precursor for the synthesis of several amino acids including asparagine, methionine, lysine, threonine, and isoleucine. Mutants that are auxotrophic for one or more of these amino acids, including glutamine (Lee et al., 2006), arginine (Gordhan et al., 2002), aspartate (Jansen et al., 2020), methionine (Berney et al., 2015; Hasenoehrl et al., 2019), lysine (Pavelka et al., 2003), and threonine (Hasenoehrl et al., 2019) are severely attenuated during infection, demonstrating that the ability to synthesize these amino acids from TCA cycle intermediates is critical for *Mtb* to establish and maintain infection in the host. The essentiality of de novo amino acid biosynthesis during infection is particularly surprising because *Mtb* can assimilate nitrogen from asparagine, aspartate, glutamate, glutamine, leucine, alanine, and glycine during growth in macrophages *in vitro* (Gouzy et al., 2014; Borah et al., 2019). *Mtb* can also divert carbon from the CO2-generating steps of the TCA cycle *via* the glyoxylate shunt pathway (Muñoz-Elías and Mckinney, 2005). The glyoxylate shunt enables growth on fatty acids as a sole carbon source because it prevents loss of carbon *via* CO2, allowing for net gain of carbon from acetyl-CoA. This carbon can then be routed to other essential biosynthetic pathways such as amino acid synthesis or gluconeogenesis to generate cell wall precursors. In contrast, carbon sources that feed into glycolysis can be used to re-generate TCA cycle intermediates, allowing for carbon to leave the TCA cycle for biosynthesis and also be replenished independent of the glyoxylate shunt.

As opposed to *in vitro* cultures where *Mtb* can utilize multiple different carbon sources, *Mtb* isolated directly from infected mouse lungs was found to preferentially metabolize fatty acids over other carbon sources such as glucose or glycerol (Segal and Bloch, 1956). In humans, direct RNA-sequencing of *Mtb* from patient sputum revealed up-regulation of transcripts encoding enzymes required for cholesterol degradation (Lai et al., 2021). Furthermore, the Mtb-specific cholesterol byproduct 4-cholesten-3-one is increased in patients with active tuberculosis, suggesting that *Mtb* actively metabolizes

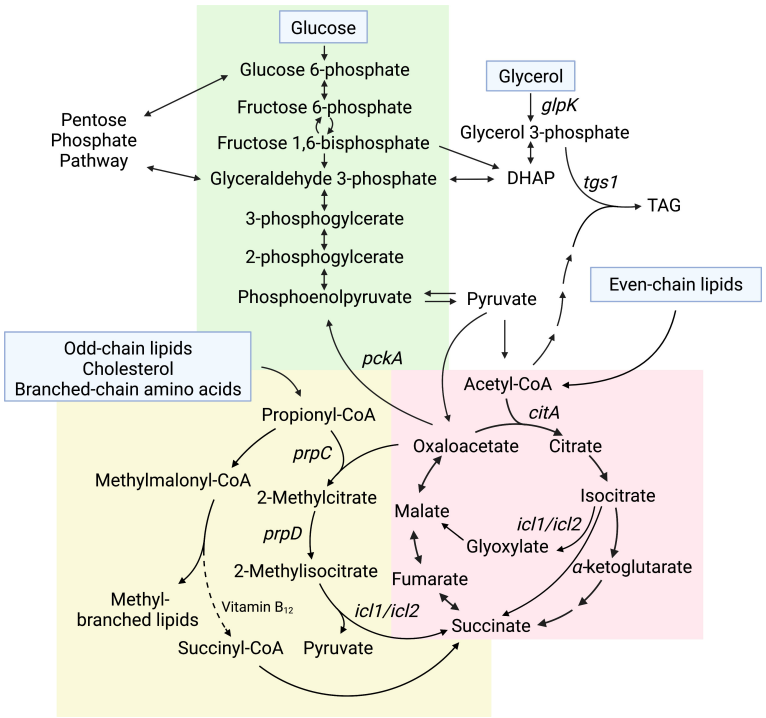


FIGURE 1
Core central carbon metabolism pathways that impact *Mtb* drug sensitivity. Carbon sources that feed into *Mtb* central carbon metabolism are listed in blue boxes. Carbon flowing down the pathway from glucose toward pyruvate, indicated by the downward pointing arrows, is glycolysis, whereas the reverse pathway, indicated by upward pointing arrows, is gluconeogenesis (green background). Even-chain lipids feed into the TCA cycle (red background). Odd-chain lipids, cholesterol, and branched-chain amino acids feed into the MCC and methylmalonyl-CoA pathways (yellow background). Genes discussed in the text that impact antibiotic sensitivity are indicated on the pathway.

cholesterol during infection (Chandra et al., 2022). These data indicate that *Mtb* carbon metabolism is shifted in the host to preferentially rely on lipids over carbohydrate carbon sources.

The preferential use of lipids by *Mtb* during infection is further supported by experiments using *Mtb* mutants in metabolic pathways, which demonstrate that *Mtb* requires the glyoxylate shunt to colonize mice and requires cholesterol uptake and catabolism to maintain infection (Muñoz-Elías and Mckinney, 2005; Pandey and Sassetti, 2008; Nesbitt et al., 2010). This is consistent with data showing that gluconeogenesis, which allows TCA cycle intermediates to be used to generate essential cell wall precursors, is more important than glycolysis for *Mtb* growth in the host. While a mutant that lacks hexose kinase activity, the first step of glycolysis, is only slightly attenuated later during infection (Marrero et al., 2013), mutants lacking enzymes required for gluconeogenesis are unable to grow in mice at all (Marrero et al., 2013; Puckett et al., 2014; Trujillo et al., 2014; Ganapathy et al., 2015). These findings demonstrate that *Mtb* relies on gluconeogenic substrates, such as lipids, for growth during infection, rather than sugars or glycerol. Therefore, the host environment, which induces a higher proportion of drug tolerant *Mtb*, also leads to a shift in *Mtb* metabolic requirements compared to unstressed *in vitro* culturing conditions.

The impact of lipid metabolism in *Mtb* on drug tolerance

Triacylglycerols (TAG) and cholesterol are abundant lipid carbon sources available to *Mtb* during infection (Kim et al., 2010). *Mtb* liberates free fatty acids from TAG Deb et al.,2006, which are oxidized to acetyl-CoA, and degrades cholesterol through a series of reactions to pyruvate, acetyl-CoA, succinyl-CoA, and propionyl-CoA (Wilburn et al., 2018). The majority of cholesterol degradation products can directly feed into the TCA cycle or serve as substrates for gluconeogenesis. The exception is propionyl-CoA, which is toxic to the bacteria if it is not metabolized further (Munoz-Elias et al., 2006; Eoh and Rhee, 2014). Propionyl-CoA can be coupled with oxaloacetate through the methylcitrate cycle (MCC) to be detoxified to succinate and

pyruvate (Figure 1) (Munoz-Elias et al., 2006; Eoh and Rhee, 2014). However, the MCC is dispensable for infection (Munoz-Elias et al., 2006), which may be because the environment encountered in the host enables propionyl-CoA detoxification through two alternative pathways. Specifically, the presence of exogenous even-chain fatty acids would enable *Mtb* to detoxify propionyl-CoA through incorporation into methyl-branched lipids, and access to vitamin B12 would enable detoxification of propionyl-CoA to succinyl-CoA (Jain et al., 2007; Savvi et al., 2008; Lee et al., 2013). Therefore, access to lipids or to vitamin B12 may obviate the need for the MCC during growth in the host even though the bacteria are catabolizing cholesterol.

Metabolism of cholesterol and the production of propionyl-CoA are associated with increased *Mtb* drug tolerance (Figure 2). *Mtb* grown in media containing cholesterol as a sole carbon source or containing mixed carbon sources including propionate exhibits decreased sensitivity to rifampicin (Koh et al., 2022). Exposure to propionate also activates PrpR, a regulator that induces expression of the *prpDC* operon, which encodes MCC enzymes PrpD and PrpC (Figure 1) (Masiewicz et al., 2012). Mutants with reduced or no PrpR activity, which are presumed to accumulate propionyl-CoA due to decreased expression of *prpDC*, exhibit slower growth in media containing propionate and increased tolerance to isoniazid, rifampicin, and ofloxacin (Hicks et al., 2018). Supplementing the *prpR* mutants with vitamin B12 enables shunting of propionyl-CoA to succinyl-CoA *via* methylmalonyl-CoA and is sufficient to rescue the growth defect in propionate media and reverse the drug tolerance of the mutants, supporting that accumulation of MCC intermediates contributes to drug tolerance (Hicks et al., 2018). The prpR mutants are similarly less sensitive to killing by antibiotics during *in vitro* infection of human macrophages (Hicks et al., 2018). Consistent with a role for PrpR-mediated regulation of the MCC in drug tolerance, mutations in prpR were enriched in drug resistant clinical isolates (Hicks et al., 2018). Since prpR mutations are associated with but do not confer drug resistance, it is possible these mutations promote a drug tolerance phenotype during infection, allowing the bacteria to survive and subsequently acquire drug resistance mutations. Consistent with decreased MCC activity promoting drug tolerance, knocking down expression of Icl1, which performs the final enzymatic step in the MCC, leads to accumulation of MCC intermediates in *Mtb* cultured in propionate media and causes nearly 10-fold less killing by isoniazid (Quinonez et al., 2022). In addition, exposure of *Mtb* to exogenous methylisocitrate, an MCC intermediate, is sufficient to

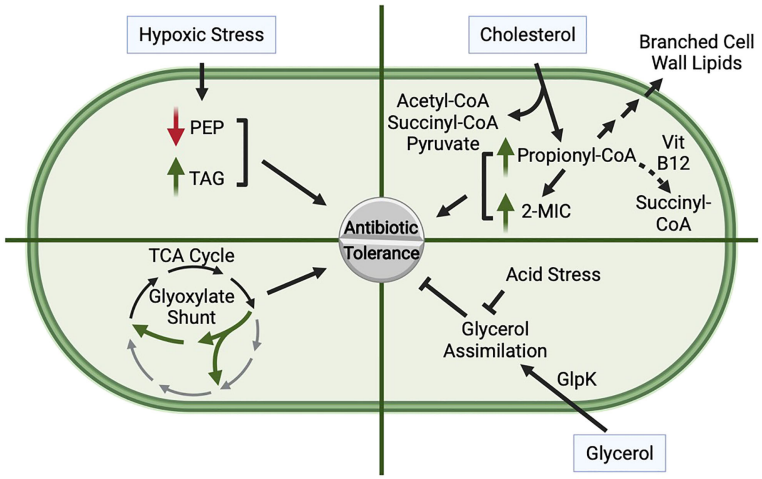


FIGURE 2
Role of Central Carbon Metabolism in Promoting Antibiotic Tolerance. Hypoxic stress, cholesterol metabolism, glycerol assimilation, low pH, and shunting of the TCA cycle *via* the glyoxylate shunt can each impact antibiotic tolerance of *Mtb*. Hypoxia: Exposure of *Mtb* to hypoxia leads to decreased levels of phosphoenolpyruvate (PEP) and an accumulation of triacylglycerol (TAG), both of which lead to an increase in drug tolerance. Cholesterol: Cholesterol is catabolized to acetyl-CoA, succinyl-CoA, pyruvate, and propionyl-CoA. Propionyl-CoA is detoxified through multiple pathways, including assimilation into branched chain lipids, conversion to succinyl-CoA through a vitamin B12-dependent pathway, or through the methylcitrate cycle (MCC) in which methylisocitrate (2-MIC) is an intermediate. Supplementation with cholesterol, propionate, or 2-MIC promotes antibiotic tolerance, and mutant strains that accumulate elevated levels of propionyl-CoA or 2-MIC are more tolerant to antibiotics. Glycerol Assimilation: Glycerol is assimilated into glycolysis and gluconeogenesis through phosphorylation by GlpK. Loss of glycerol catabolism leads to increased drug tolerance, suggesting that glycerol assimilation antagonizes antibiotic tolerance. Furthermore, *Mtb* in low pH is unable to efficiently catabolize glycerol, likely due to defects in glycolysis, resulting in increased antibiotic tolerance. Glyoxylate Shunt Activity: Mutants that lack the glyoxylate shunt are more sensitive to antibiotics, suggesting that rerouting carbon through the glyoxylate shunt promotes antibiotic tolerance.

decrease killing by isoniazid (Quinonez et al., 2022). Collectively, these studies suggest that accumulation of propionyl-CoA or MCC intermediates leads to antibiotic tolerance.

Propionyl-CoA accumulation slows *Mtb* growth and slow growth rate has been associated with drug tolerance (Wayne and Hayes, 1996; Baek et al., 2011). Therefore, reduced growth rate could explain how cholesterol metabolism decreases antibiotic sensitivity. Another possible contributor to cholesterol-induced drug tolerance could be the changes to cell wall lipids that occur during propionyl-CoA metabolism. Growth on propionyl-CoA-generating carbon sources causes *Mtb* to synthesize branched lipids such as phthiocerol dimycocerosate (PDIM) and sulfolipid-1 with increased chain lengths (Jain et al., 2007; Yang et al., 2009; Griffin et al., 2012; Borah et al., 2021; Koh et al., 2022). PDIM is a major structural lipid intercalated in the outer *Mtb* envelope and has been shown to create a barrier that is particularly impenetrable to polar molecules (Wang et al., 2020). Thus, it is possible that alterations to PDIM chain length during growth on cholesterol or propionate may impact the permeability of the *Mtb* cell envelope, which could explain the altered antibiotic sensitivity.

Mtb metabolism during hypoxia and the association with drug tolerance

During exposure to hypoxia, *Mtb* exhibits decreased levels of phosphoenolpyruvate (PEP) (Figure 2), an intermediate in glycolysis and gluconeogenesis, which is likely caused by decreased synthesis of PEP from oxaloacetate (Lim et al., 2021) (Figure 1). Supplementing hypoxic *Mtb* with exogenous PEP enhances killing by isoniazid (Lim et al., 2021), suggesting that the decrease in PEP during hypoxia contributes to hypoxia-induced drug tolerance. Notably, supplementation with pyruvate does not have the same effect, suggesting that this effect is specific for PEP, and access to additional carbon alone is not sufficient to sensitize *Mtb* to isoniazid. PEP supplementation also promotes *Mtb* sensitivity to D-cycloserine, a cell wall biosynthesis inhibitor, in aerated conditions, suggesting that the effect of PEP on drug tolerance is not specific for hypoxia (Lim et al., 2021). In addition to feeding into glycolysis and/or gluconeogenesis, PEP can also feed into the TCA cycle by conversion into oxaloacetate, can serve as a substrate for synthesis of the peptidoglycan precursor N-acetylmuramic acid, and is a substrate for the shikimate pathway (Lim et al., 2021). Which of these pathways contributes to the PEP-dependent drug sensitivity is still unknown.

During hypoxia, there is also decreased flux through several NAD(P)H-generating steps of the TCA cycle, likely to prevent production of NAD(P)H in conditions that these cofactors cannot be re-oxidized. This altered flux is caused by re-routing of acetyl-CoA to fatty acid and subsequent TAG biosynthesis (Baek et al., 2011), increased glyoxylate shunt activation (Eoh and Rhee, 2013), and reversal of several steps in the TCA cycle to generate succinate from oxaloacetate (Watanabe et al., 2011; Zimmermann et al., 2015). Deletion of the TAG biosynthesis gene *tgs1* or overexpression of the citrate synthase gene *citA* prevented re-rerouting of acetyl-CoA to fatty acid and TAG biosynthesis during hypoxia and iron starvation (Baek et al., 2011). These mutants failed to arrest growth and exhibited enhanced sensitivity to isoniazid, streptomycin, ciprofloxacin, and ethambutol in hypoxic and iron starvation conditions (Baek et al., 2011). The Δ*tgs1* and *citA*-overexpressing strains were also significantly more sensitive to killing by isoniazid in a mouse model of infection, supporting that the re-routing of acetyl-CoA to fatty acid biosynthesis promotes drug tolerance (Figure 2) (Baek et al., 2011). Redirecting carbon away from the TCA cycle can also promote drug tolerance in aerated conditions. The glyoxylate shunt enables bypassing of two NAD(P)H- and CO2-generating steps of the TCA cycle. In addition to promoting growth on lipids by conserving carbon, the glyoxylate shunt may also decrease the generation of NAD(P)H during growth on glucose, preventing oxidative stress caused by electron transport chain activity (Nandakumar et al., 2014). A Δ*icl1/icl2* double mutant, which lacks the first step of the glyoxylate shunt, exhibits >10-fold enhanced killing by isoniazid, rifampicin, or streptomycin compared to the wild-type strain during aerobic growth on glucose (Nandakumar et al., 2014). Therefore, diverting carbon through the glyoxylate shunt can promote *Mtb* drug tolerance (Figure 2), likely by alleviating oxidative stress caused by TCA cycle and downstream electron transport chain activity. These studies demonstrate that by re-routing carbon away from the TCA cycle, *Mtb* becomes more tolerant to antibiotics.

Glycerol metabolism and low pH-induced drug tolerance

Mtb is unable to grow on glycerol as the sole carbon source in low pH (Baker et al., 2014). This is likely due to inefficient assimilation of glycerol into lower glycolysis caused by decreased glyceraldehyde-3-phosphate dehydrogenase activity in low pH (Gouzy et al., 2021) (Figure 1). This nonpermissive growth condition results in acidic pH-induced drug tolerance (Figure 2), whereas growth on pyruvate, which enables *Mtb* growth in low pH, prevents the pH-induced drug tolerance (Baker and Abramovitch, 2018). Glycerol is assimilated into glycolysis and gluconeogenesis through phosphorylation by GlpK and subsequent conversion to DHAP (Figure 1). In media containing glycerol and other carbon sources, a Δ*glpK* mutant exhibited decreased sensitivity to isoniazid and rifampicin compared to wild-type *Mtb*, further supporting

that decreasing glycerol metabolism increases drug tolerance (Bellerose et al., 2019; Safi et al., 2019). *glpK* is dispensable in the mouse model of *Mtb* infection, suggesting that glycerol is not a primary carbon source in mice (Pethe et al., 2010). However, free glycerol is detectable in infected mouse lungs, suggesting *Mtb* would have access to glycerol in the host (Safi et al., 2019). Furthermore, a Δ*glpK* mutant survived better than wild-type *Mtb* during treatment with pyrazinamide or any drug combination involving pyrazinamide, but not during isoniazid or rifampicin monotherapy, in a mouse model of infection (Bellerose et al., 2019). Therefore, the inability to metabolize glycerol during infection in mice promotes *Mtb* drug tolerance specifically to pyrazinamide. Since the *glpk* mutant does not exhibit increased tolerance to pyrazinamide *in vitro*, the pyrazinamide-specific tolerance in the *glpK* mutant is dependent upon the microenvironment within the host (Bellerose et al., 2019). Multiple groups have also identified *glpK* mutations in *Mtb* clinical isolates (Bellerose et al., 2019; Safi et al., 2019; Vargas and Farhat, 2020), and in some datasets these mutants are more commonly found in drug resistant isolates than in drug sensitive isolates (Bellerose et al., 2019; Safi et al., 2019). The *glpK* mutations are associated with but do not confer drug resistance themselves. However, if these mutations promote drug tolerance, they may enable *Mtb* to survive during antibiotic therapy, extending the time wherein *Mtb* may acquire a drug resistance mutation.

Drug tolerance is often conditional

In this review, we have highlighted studies demonstrating that *Mtb* undergoes changes in carbon metabolism in response to host derived stresses that render *Mtb* more tolerant to antibiotics. However, the observed drug tolerance is rarely pan-antibiotic. For example, in hypoxic conditions, while *Mtb* becomes extremely tolerant to some antibiotics, including isoniazid, rifampicin, and streptomycin, it remains susceptible to antibiotics that target ATP synthase, and in some cases becomes more sensitive to killing by ATP synthase inhibitors and other antibiotics that target the electron transport chain (Koul et al., 2008; Rao et al., 2008; Gengenbacher et al., 2010; Sarathy et al., 2018; Lee et al., 2021). Furthermore, in an experiment to identify *Mtb* mutants with altered sensitivity to either isoniazid, rifampicin, pyrazinamide, or ethambutol during mouse infection, the majority of mutants identified only exhibited significantly altered susceptibility to a single antibiotic (Bellerose et al., 2020). Therefore, antibiotic tolerance can be conditional and specific to individual antibiotics.

Metabolic changes in *Mtb* that result in increased drug tolerance are often correlated with growth arrest, including toxicity mediated by propionyl-CoA or MCC intermediate accumulation (Hicks et al., 2018; Quinonez et al., 2022), hypoxia-induced TAG accumulation (Baek et al., 2011), and decreased glycerol metabolism in low pH conditions (Baker et al., 2014). However, there is also data, particularly in infection models, that suggests growth arrest is not universally associated with antibiotic tolerance (Raffetseder et al., 2014; Bellerose et al., 2019, Bellerose et al., 2020). Specifically, the Δ*glpK* mutant had no fitness defect in mice yet had altered antibiotic susceptibility to pyrazinamide (Bellerose et al., 2019). In addition, although there is a correlation between mutants that were less sensitive to isoniazid and mutants that had a fitness defect in mice, this association was not observed with mutants that were less sensitive to the other antibiotics (Bellerose et al., 2020).

Conclusion

Understanding the mechanisms by which *Mtb* metabolism impacts tolerance to specific antibiotics, particularly in the host environment, could lead to novel therapeutic approaches. We have highlighted several studies that demonstrate it is possible to manipulate metabolic pathways to reverse tolerance to a number of frontline antibiotics. For example, the *Mtb* Δ*tgs1* mutant is more susceptible to killing by isoniazid, rifampicin, or streptomycin during stress and in mice (Baek et al., 2011). This suggests that developing inhibitors of TAG biosynthesis could be a viable approach to enhance the efficacy of these antibiotics in the clinic. Additionally, supplementation with exogenous PEP was sufficient to enhance killing of hypoxic *Mtb* by isoniazid (Lim et al., 2021). Thus, changing the metabolic state of *Mtb* can potentiate killing by frontline antibiotics. Our review focused on central carbon metabolism, however these pathways are intricately connected to other metabolic networks, such as amino acid biosynthesis. De novo biosynthesis of amino acids from the TCA cycle and other pathways is essential for *Mtb* virulence and recent studies have shown that inhibiting amino acid biosynthesis is a promising approach for therapeutic development (Wellington et al., 2017). Although some metabolic enzymes may not be druggable targets due to shared structural homology with the mammalian homolog or difficulty in identifiying small molecules that effectively inhibit the enzyme, further elucidation of which metabolic pathways are essential during infection and how specific

pathways contribute to drug tolerance will provide new opportunities for exploration.

Author contribution

All authors participated in the conceptualization and writing of this mini-review.

Funding

CS is supported by NIH grant AI134847 as well as a Burroughs Wellcome Fund Investigators in the Pathogenesis of Infectious Disease Award. GH is supported by National Science Foundation graduate research fellowship DGE-1745038 and NIGMS cell and molecular biology training grant GM007067. EW is supported by NIAID award T32AI007172. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Acknowledgments

We are grateful to H. Eoh for kindly sharing his unpublished manuscript for this issue while we prepared our final versions.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Aldridge, B. B., Fernandez-Suarez, M., Heller, D., Ambravaneswaran, V., Irimia, D., Toner, M., et al. (2012). Asymmetry and aging of mycobacterial cells lead to variable growth and antibiotic susceptibility. *Science* 335, 100–104. doi: 10.1126/science.1216166

Baek, S.-H., Li, A. H., and Sassetti, C. M. (2011). Metabolic regulation of mycobacterial growth and antibiotic sensitivity. *PloS Biol.* 9, 1–10. doi: 10.1371/journal.pbio.1001065

Baker, J. J., and Abramovitch, R. B. (2018). Genetic and metabolic regulation of mycobacterium tuberculosis acid growth arrest. *Sci. Rep.* 8, 1–16. doi: 10.1038/s41598-018-22343-4

Baker, J. J., Johnson, B. K., and Abramovitch, R. B. (2014). Slow growth of mycobacterium tuberculosis at acidic pH is regulated by phoPR and host-associated carbon sources. *Mol. Microbiol.* 94, 56–69. doi: 10.1111/mmi.12688

Bellerose, M. M., Baek, S.-H., Huang, C.-C., Moss, C. E., Koh, E.-I., Proulx, M. K., et al. (2019). Common variants in the glycerol kinase gene reduce tuberculosis drug efficacy. *mBio* 10, 1–15. doi: 10.1128/mBio.00663-19

Bellerose, M. M., Proulx, M. K., Smith, C. M., Baker, R. E., Ioerger, T. R., and Sassetti, C. M. (2020). Distinct bacterial pathways influence the efficacy of antibiotics against mycobacterium tuberculosis. *mSystems* 5, 1–18. doi: 10.1128/mSystems00396-20

Berney, M., Berney-Meyer, L., Wong, K. W., Chen, B., Chen, M., Kim, J., et al. (2015). Essential roles of methionine and s-adenosylmethionine in the autarkic lifestyle of mycobacterium tuberculosis. *PNAS* 112, 10008–10013. doi: 10.1073/pnas.1513033112

Betts, J. C., Lukey, P. T., Robb, L. C., McAdam, R. A., and Duncan, K. (2002). Evaluation of a nutrient starvation model of mycobacterium tuberculosis persistence by gene and protein expression profiling. *Mol. Microbiol.* 43, 717–731. doi: 10.1046/j.1365-2958.2002.02779.x

Borah, K., Beyß, M., Theorell, A., Wu, H., Basu, P., Mendum, T. A., et al. (2019). Intracellular mycobacterium tuberculosis exploits multiple host nitrogen sources during growth in human macrophages. *Cell Rep.* 29, 3580–3591. doi: 10.1016/j.celrep.2019.11.037

Borah, K., Mendem, T. A., Hawkins, D. D., Ward, J. L., Beale, M. H., Larrouy-Maumus, G., et al. (2021). Metabolic fluxes for nutritional flexibility of mycobacterium tuberculosis. *Mol. Syst. Biol.* 17, 1–17. doi: 10.15252/msb.202110280

Bryant, J. M., Harris, S. R., Parkhill, J., Dawson, R., Diacon, A. H., van Helden, P., et al. (2013). Whole-genome sequencing to establish relapse or re-infection with mycobacterium tuberculosis: a retrospective observational study. *Lancet Respir. Med.* 1, 786–792. doi: 10.1016/S2213-2600(13)70231-5

Chandra, P., Coullon, H., Agarwal, M., Goss, C. W., and Philips, J. A. (2022). Macrophage global metabolomics identifies cholestenone as host/pathogen cometabolite present in humn mycobacterium tuberculosis infection. *J. Clin. Invest.* 132, 1–15. doi: 10.1172/JCI152509

Chen, X., He, G., Lin, S., Wang, S., Sun, F., Chen, J., et al. (2020). Analysis of serial multidrug-resistant tuberculosis strains causing treatment failure and within-host evolution by whole-genome sequencing. *mSphere* 5, e00884–e00820. doi: 10.1128/mSphere

Deb, C., Daniel, J., Sirokova, T. D., Abomoelak, B., Dubey, V. S., and Kolattukudy, P. E. A novel lipase belonging to the hormon-sensitive lipase family induced under starvation to utilize stored triacylglycerol in mycobacterium tuberculosis. (2006) 7, 3866–3875. doi: 10.1074/jbc.M505556200

Deb, C., Lee, C.-M., Dubey, V. S., Daniel, J., Abomoelak, B., Sirakova, T. D., et al. (2009). A novel *In vitro* multiple-stress dormancy model for mycobacterium tuberculosis generates a lipid-loaded, drug-tolerant, dormant pathogen. *PloS One* 4, 1–15. doi: 10.1371/journal.pone.0006077

de Carvalho, L. P. S., Fischer, S. M., Marrero, J., Nathan, C., Ehrt, S., and Rhee, K. Y. (2010). Metabolomics of mycobacterium tuberculosis reveals compartmentalized co-catabolism of carbon substrates. *Chem. Biol.* 17, 1122–1131. doi: 10.1016/j.chembiol.2010.08.009

Ehlers, S., and Schaible, U. E. (2013). The granuloma in tuberculosis: dynamics of a host–pathogen collusion. *Front. Immunol.* 3. doi: 10.3389/fimmu.2012.00411

Eoh, H., and Rhee, K. Y. (2013). Multifunctional essentiality of succinate metabolism in adaptation to hypoxia in mycobacterium tuberculosis. *PNAS* 110, 6554–6559. doi: 10.1073/pnas.1219375110

Eoh, H., and Rhee, K. Y. (2014). Methylcitrate cycle defines the bactericidal essentiality of isocitrate lyase for survival of mycobacterium tuberculosis on fatty acids. *PNAS* 111, 4976–4981. doi: 10.1073/pnas.1400390111

Ganapathy, U., Marrero, J., Calhoun, S., Eoh, H., Pedro Sorio de Carvalho, L., Rhee, K., et al. (2015). Two enzymes with redundant fructose bisphosphatase activity sustain gluconeogenesis and virulence in mycobacterium tuberculosis. *Nat. Commun.* 6, 1–12. doi: 10.1038/ncomms8912

Gengenbacher, M., Rao, S. P. S., Pethe, K., and Dick, T. (2010). Nutrient-starved, non-replicating mycobacterium tuberculosis requires respiration, ATP synthase and isocitrate lyase for maintenance of ATP homeostasis and viability. *Microbiol. (N Y)* 156, 81–87. doi: 10.1099/mic.0.033084-0

Ghazy, R. M., el Saeh, H. M., Abdulaziz, S., Hammouda, E. A., Elzorkany, A. M., Khidr, H., et al. (2022). A systematic review and meta-analysis of the catastrophic costs incurred by tuberculosis patients. *Sci. Rep.* 12, 558–574. doi: 10.1038/s41598-021-04345-x

Gillespie, S. H., Crook, A. M., McHugh, T. D., Mendel, C. M., Meredith, S. K., Murray, S. R., et al. (2014). Four-month moxifloxacin-based regimens for drug-sensitive tuberculosis. *New Engl. J. Med.* 371, 1577–1587. doi: 10.1056/nejmoa1407426

Gordhan, B. G., Smith, D. A., Alderton, H., McAdam, R. A., Bancroft, G. J., and Mizrahi, V. (2002). Construction and phenotypic characterization of an auxotrophic mutant of mycobacterium tuberculosis defective in l-arginine biosynthesis. *Infection Immun.* 70, 3080–3084. doi: 10.1128/IAI.70.6.3080-3084.2002

Gouzy, A., Healy, C., Black, K. A., Rhee, K. Y., and Ehrt, S. (2021). Growth of mycobacterium tuberculosis at acidic pH depends on lipid assimilation and is accompanied by reduced GAPDH activity. *PNAS* 118, 1–9. doi: 10.1073/pnas.2024571118/-/DCSupplemental

Gouzy, A., Larrouy-Maumus, G., Bottai, D., Levillain, F., Dumas, A., Wallach, J. B., et al. (2014). Mycobacterium tuberculosis exploits asparagine to assimilate nitrogen and resist acid stress during infection. *PloS Pathog.* 10, 1–14. doi: 10.1371/journal.ppat.1003928

Griffin, J. E., Pandey, A. K., Gilmore, S. A., Mizrahi, V., McKinney, J. D., Bertozzi, C. R., et al. (2012). Cholesterol catabolism by mycobacterium tuberculosis requires transcriptional and metabolic adaptations. *Chem. Biol.* 19, 218–227. doi: 10.1016/j.chembiol.2011.12.016

Hasenoehrl, E. J., Sajorda, D. R., Berney-Meyer, L., Johnson, S., Tufariello, J. M., Fuhrer, T., et al. (2019). Derailing the aspartate pathway of mycobacterium tuberculosis to eradicate persistent infection. *Nat. Commun.* 10, 1–12. doi: 10.1038/s41467-019-12224-3

Hasenoehrl, E. J., Wiggins, T. J., and Berney, M. (2021). Bioenergetic inhibitors: Antibiotic efficacy and mechanisms of action in mycobacterium tuberculosis. *Front. Cell. Infection Microbiol.* 10. doi: 10.3389/fcimb.2020.611683

Hesper Rego, E., Audette, R. E., and Rubin, E. J. (2017). Deletion of a mycobacterial divisome factor collapses single-cell phenotypic heterogeneity. *Nature* 546, 153–157. doi: 10.1038/nature22361

Hicks, N. D., Yang, J., Zhang, X., Zhao, B., Grad, Y. H., Liu, L., et al. (2018). Clinically prevalent mutations in mycobacterium tuberculosis alter propionate metabolism and mediate multidrug tolerance. *Nat. Microbiol.* 3, 1032–1042. doi: 10.1038/s41564-018-0218-3

Hobby, G. L. (1955). The fate of tubercle bacilli *In vitro*. *Am. J. Med.* 18, 753–763. doi: 10.1016/0002-9343(55)90189-3

Jain, M., Petzold, C., Schelle, M. W., Leavell, M. D., Mougous Joseph, D., Bertozzi, C. R., et al. (2007). Lipidomics reveals control of mycobacterium tuberculosis virulence lipids *via* metabolic coupling. *PNAS* 104, 5133–5138. doi: 10.1073/pnas.0610634104

Jain, P., Weinrick, B. C., Kalivoda, E. J., Yang, H., Munsamy, V., Vilcheze, C., et al. (2016). Dual-reporter mycobacteriophages (Φ2DRMS) reveal preexisting mycobacterium tuberculosis persistent cells in human sputum. *mBio* 7, 1–22. doi: 10.1128/mBio.01023-16

Jansen, R. S., Mandyoli, L., Hughes, R., Wakabayashi, S., Pinkham, J. T., Selbach, B., et al. (2020). Aspartate aminotransferase Rv3722c governs aspartate-dependent nitrogen metabolism in mycobacterium tuberculosis. *Nat. Commun.* 11, 1–13. doi: 10.1038/s41467-020-15876-8

Jindani, A., Harrison, T. S., Nunn, A. J., Phillips, P. P. J., Churchyard, G. J., Charalambous, S., et al. (2014). High-dose rifapentine with moxifloxacin for pulmonary tuberculosis. *New Engl. J. Med.* 371, 1599–1608. doi: 10.1056/nejmoa1314210

Kim, M. J., Wainwright, H. C., Locketz, M., Bekker, L. G., Walther, G. B., Dittrich, C., et al. (2010). Caseation of human tuberculosis granulomas correlates with elevated host lipid metabolism. *EMBO Mol. Med.* 2, 258–274. doi: 10.1002/emmm.201000079

Kjellsson, M. C., Via, L. E., Goh, A., Weiner, D., Low, K. M., Kern, S., et al. (2012). Pharmacokinetic evaluation of the penetration of antituberculosis agents in rabbit pulmonary lesions. *Antimicrob Agents Chemother* 56, 446–457. doi: 10.1128/AAC.05208-11

Koh, E.-I., Oluoch, P. O., Ruecker, N., Proulx, M. K., Soni, V., Murphy, K. C., et al. (2022). Chemical-genetic interaction mapping links carbon metabolism and cell wall structure to tuberculosis drug efficacy. *PNAS* 119, 1–12. doi: 10.1073/pnas.2201632119

Koul, A., Vranckx, L., Dendouga, N., Balemans, W., van den Wyngaert, I., Vergauwen, K., et al. (2008). Diarylquinolines are bactericidal for dormant mycobacteria as a result of disturbed ATP homeostasis. *J. Biol. Chem.* 283, 25273–25280. doi: 10.1074/jbc.M803899200

Kundu, M., and Basu, J. (2021). Applications of transcriptomics and proteomics for understanding dormancy and resuscitation in mycobacterium tuberculosis. *Front. Microbiol.* 12. doi: 10.3389/fmicb.2021.642487

Kurthkoti, K., Amin, H., Marakalala, M. J., Ghanny, S., Subbian, S., Sakatos, A., et al. (2017). The capacity of mycobacterium tuberculosis to survive iron starvation might enable it to persist in iron- deprived microenvironments of human granulomas. *mBio* 8, 1–17. doi: 10.1128/mBio.01092-17

Lai, R. P. J., Cortes, T., Marais, S., Rockwood, N., Burke, M. L., Garza-Garcia, A., et al. (2021). Transcriptomic characterization of tuberculous sputum reveals a host warburg effect and microbial cholesterol catabolism. *mBio* 12, 1–13. doi: 10.1128/mBio.01766-21

Larrouy-Maumus, G., Marino, L. B., Madduri, A. V. R., Ragan, T. J., Hunt, D. M., Bassano, L., et al. (2016). Cell-envelope remodeling as a determinant of phenotypic antibacterial tolerance in mycobacterium tuberculosis. *ACS Infect. Dis.* 2, 352–360. doi: 10.1021/acsinfecdis.5b00148

Larsen, M. H., Biermann, K., Tandberg, S., Hsu, T., and Jacobs, W. R. (2007). Genetic manipulation of mycobacterium tuberculosis. *Curr. Protoc. Microbiol.* 6, 1–21. doi: 10.1002/9780471729259.mc10a02s6

Lee, B. S., Hards, K., Engelhart, C. A., Hasenoehrl, E. J., Kalia, N. P., Mackenzie, J. S., et al. (2021). Dual inhibition of the terminal oxidases eradicates antibiotic-tolerant mycobacterium tuberculosis. *EMBO Mol. Med.* 13, 1–16. doi: 10.15252/emmm.202013207

Lee, S., Jeon, B. Y., Bardarov, S., Chen, M., Morris, S. L., and Jacobs, W. R. (2006). Protection elicited by two glutamine auxotrophs of mycobacterium tuberculosis and *in vivo* growth phenotypes of the four unique glutamine synthetase mutants in a murine model. *Infection Immun.* 74, 6491–6495. doi: 10.1128/IAI.00531-06

Lee, W., VanderVen, B. C., Fahey, R. J., and Russell, D. G. (2013). Intracellular mycobacterium tuberculosis exploits host-derived fatty acids to limit metabolic stress. *J. Biol. Chem.* 288, 6788–6800. doi: 10.1074/jbc.M112.445056

Lim, J., Lee, J. J., Lee, S., Kim, S., Eum, S.-Y., and Eoh, H. (2021). Phosphoenolpyruvate depletion mediates both growth arrest and drug tolerance of mycobacterium tuberculosis in hypoxia. *PNAS* 118, 1–12. doi: 10.1073/pnas.2105800118

Loebel, R. O., Shorr, E., and Richardson, H. B. (1933). The influence of foodstuffs upon the respiratory metabolism and growth of human tubercle bacilli. *J. Bacteriol* 2, 139–166. doi: 10.1128/jb.26.2.139-166

Manina, G., Neeraj, D., and McKinney, J. D. (2015). Stress and host immunity amplify mycobacterium tuberculosis phenotypic heterogeneity and induce nongrowing metabolically active forms. *Cell Host Microbe* 17, 32–46. doi: 10.1016/j.chom.2014.11.016

Marakalala, M. J., Raju, R. M., Sharma, K., Zhang, Y. J., Eugenin, E. A., Prideaux, B., et al. (2016). Inflammatory signaling in human tuberculosis granulomas is spatially organized. *Nat. Med.* 22, 531–538. doi: 10.1038/nm.4073

Marrero, J., Trujillo, C., Rhee, K. Y., and Ehrt, S. (2013). Glucose phosphorylation is required for mycobacterium tuberculosis persistence in mice. *PloS Pathog.* 9, 1–11. doi: 10.1371/journal.ppat.1003116

Masiewicz, P., Brzostek, A., Wolanski, M., Dziadek, J., and Zakrzewska-Czerwinska, J. (2012). A novel role of the PrpR as a transcription factor involved in the regulation of methylcitrate pathway in mycobacterium tuberculosis. *PloS One* 7, 1–14. doi: 10.1371/journal.pone.0043651

Muñoz-Elías, E. J., and Mckinney, J. D. (2005). Mycobacterium tuberculosis isocitrate lyases 1 and 2 are jointly required for *in vivo* growth and virulence. *Nat. Med.* 11, 638–644. doi: 10.1038/nm1252

Munoz-Elias, E. J., Upton, A. M., Cherian, J., and McKinney, J. D. (2006). Role of the methylcitrate cycle in mycobacterium tuberculosis metabolism, intracellular growth, and virulence. *Mol. Microbiol.* 60, 1109–1122. doi: 10.1111/j.1365-2958.2006.05155.x

Nahid, P., Mase, S. R., Migliori, G. B., Sotgiu, G., Bothamley, G. H., Brozek, J. L., et al. (2019). Treatment of drug-resistant tuberculosis an official ATS/CDC/ERS/IDSA clinical practice guideline. *Am. J. Respir. Crit. Care Med.* 200, E93–E142. doi: 10.1164/rccm.201909-1874ST

Nandakumar, M., Nathan, C., and Rhee, K. Y. (2014). Isocitrate lyase mediates broad antibiotic tolerance in mycobacterium tuberculosis. *Nat. Commun.* 5, 1–10. doi: 10.1038/ncomms5306

Nesbitt, N. M., Yang, X., Fontán, P., Kolesnikova, I., Smith, I., Sampson, N. S., et al. (2010). A thiolase of mycobacterium tuberculosis is required for virulence and production of androstenedione and androstadienedione from cholesterol. *Infection Immun.* 78, 275–282. doi: 10.1128/IAI.00893-09

Pandey, A. K., and Sassetti, C. M. (2008). Mycobacterial persistence requires the utilization of host cholesterol. *PNAS* 105, 4376–4380. doi: 10.1073/pnas.0711159105

Pavelka, M. S., Chen, B., Kelley, C. L., Collins, F. M., and Jacobs, W. R. (2003). Vaccine efficacy of a lysine auxotroph of mycobacterium tuberculosis. *Infection Immun.* 71, 4190–4192. doi: 10.1128/IAI.71.7.4190-4192.2003

Pethe, K., Sequeira, P. C., Agarwalla, S., Rhee, K., Kuhen, K., Phong, W. Y., et al. (2010). A chemical genetic screen in mycobacterium tuberculosis identifies carbon-source-dependent growth inhibitors devoid of *in vivo* efficacy. *Nat. Commun.* 1, 1–8. doi: 10.1038/ncomms1060

Pisu, D., Huang, L., Grenier, J. K., and Russell, D. G. (2020). Dual RNA-seq of mtb-infected macrophages *In vivo* reveals ontologically distinct host-pathogen interactions. *Cell Rep.* 30, 335–350. doi: 10.1016/j.celrep.2019.12.033

Prideaux, B., Via, L. E., Zimmerman, M. D., Eum, S., Sarathy, J., O'Brien, P., et al. (2015). The association between sterilizing activity and drug distribution into tuberculosis lesions. *Nat. Med.* 21, 1223–1227. doi: 10.1038/nm.3937

Puckett, S., Trujillo, C., Eoh, H., Marrero, J., Spencer, J., Jackson, M., et al. (2014). Inactivation of fructose-1,6-Bisphosphate aldolase prevents optimal Co-catabolism of glycolytic and gluconeogenic carbon substrates in mycobacterium tuberculosis. *PLosPathogens* 10, 1–11. doi: 10.1371/journal.ppat.1004144

Quinonez, C. G., Jin Lee, J., Lim, J., Odell, M., Lawson, C. P., Anyogu, A., et al. (2022). The role of fatty acid metabolism in drug tolerance of mycobacterium tuberculosis. *mBio* 13, 1–16. doi: 10.1128/mbio.03559-21

Raffetseder, J., Pienaar, E., Blomgran, R., Eklund, D., Brodin, V. P., Andersson, H., et al. (2014). Replication rates of mycobacterium tuberculosis in human macrophages do not correlate with mycobacterial antibiotic susceptibility. *PloS One* 9, e112426. doi: 10.1371/JOURNAL.PONE.0112426

Rao, S. P. S., Alonso, S., Rand, L., Dick, T., and Pethe, K. (2008). The proton motive force is required for maintaining ATP homeostasis and viability of hypoxic, nonreplicating mycobacterium tuberculosis. *PNAS* 105, 11945–11950. doi: 10.1073/pnas.0711697105

Remm, S., Earp, J. C., Dick, T., Dartois, V., and Seeger, M. A. (2021). Critical discussion on drug efflux in mycobacterium tuberculosis. *FEMS Microbiol. Rev.* 46, 1–15. doi: 10.1093/femsre/fuab050

Safi, H., Gopal, P., Lingaraju, S., Ma, S., Levine, C., Dartois, V., et al. (2019). Phase variation in mycobacterium tuberculosis glpK produces transiently heritable drug tolerance. *PNAS* 116, 19665–19674. doi: 10.1073/pnas.1907631116

Santucci, P., Aylan, B., Botella, L., Bernard, E. M., Bussi, C., Pellegrino, E., et al. (2022). Visualizing pyrazinamide action by live single-cell imaging of phagosome acidification and mycobacterium tuberculosis pH homeostasis. *mBio* 13, 1–21. doi: 10.1128/mbio.00117-22

Santucci, P., Greenwood, D. J., Fearns, A., Chen, K., Jiang, H., and Gutierrez, M. G. (2021). Intracellular localisation of mycobacterium tuberculosis affects efficacy of the antibiotic pyrazinamide. *Nat. Commun.* 12, 1–16. doi: 10.1038/s41467-021-24127-3

Sarathy, J. P., Zuccotto, F., Hsinpin, H., Sandberg, L., Via, L. E., Marriner, G. A., et al. (2018). Prediction of drug penetration in tuberculosis lesions. *ACS Infect. Dis.* 2, 552–563. doi: 10.1021/acsinfecdis.6b00051

Savvi, S., Warner, D. F., Kana, B. D., McKinney, J. D., Mizrahi, V., and Dawes, S. S. (2008). Functional characterization of a vitamin B12-dependent methylmalonyl pathway in mycobacterium tuberculosis: Implications for propionate metabolism during growth on fatty acids. *J. Bacteriol* 190, 3886–3895. doi: 10.1128/JB.01767-07

Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., et al. (2003). Transcriptional adaptation of mycobacterium tuberculosis within macrophages: Insights into the phagosomal environment. *J. Exp. Med.* 198, 693–704. doi: 10.1084/jem.20030846

Segal, W., and Bloch, H. (1956). BIOCHEMICAL DIFFERENTIATION OF MYCOBACTERIUM TUBERCULOSIS GROWN *IN VIVO* AND *IN VITRO*. *J. Biochem.* 72, 132–141. doi: 10.1128/jb.72.2.132-141

Sharma, S., Briffotaux, J., Khan, A., Karakousis, P. C., Danchik, C., and Wang, S. (2021). Targeting the mycobacterium tuberculosis stringent response as a strategy for shortening tuberculosis treatment. *Front. Microbiol.* 12. doi: 10.3389/fmicb.2021.744167

Sukheja, P., Kumar, P., Mittal, N., Li, S. G., Singleton, E., Russo, R., et al. (2017). A novel small-molecule inhibitor of the mycobacterium tuberculosis demethylmenaquinone methyltransferase meng is bactericidal to both growing and nutritionally deprived persister cells. *mBio* 8, e02022–16. doi: 10.1128/mBio.02022-16

Tan, S., Sukumar, N., Abramovitch, R. B., Parish, T., and Russell, D. G. (2013). Mycobacterium tuberculosis responds to chloride and pH as synergistic cues to the immune status of its host cell. *PloS Pathog.* 9, e1003282. doi: 10.1371/journal.ppat.1003282

Trujillo, C., Blumenthal, A., Marrero, J., Rhee, K. Y., Schnappinger, D., and Ehrt, S. (2014). Triosephosphate isomerase is dispensable *in vitro* yet essential for mycobacterium tuberculosis to establish infection. *mBio* 5, e00085. doi: 10.1128/mBio.00085-14

Turapov, O., O'Connor, B. D., Sarybaeva, A. A., Williams, C., Patel, H., Kadyrov, A. S., et al. (2016). Phenotypically adapted mycobacterium tuberculosis populations from sputum are tolerant to first-line drugs. *Antimicrob Agents Chemother* 60, 2476–2483. doi: 10.1128/AAC.01380-15

Vargas, R., and Farhat, M. R. (2020). Antibiotic treatment and selection for glpK mutations in patients with active tuberculosis disease. *PNAS* 117, 3910–3912. doi: 10.1073/pnas.1920788117

Via, L. E., Lin, P. L., Ray, S. M., Carrillo, J., Allen, S. S., Seok, Y. E., et al. (2008). Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates. *Infection Immun.* 76, 2333–2340. doi: 10.1128/IAI.01515-07

Vilcheze, C., Hartman, T., Weinrick, B., Jain, P., Weisbrod, T. R., Leung, L. W., et al. (2017). Enhanced respiration prevents drug tolerance and drug resistance in mycobacterium tuberculosis. *PNAS* 114, 4495–4500. doi: 10.1073/pnas.1704376114

Voskuil, M. I., Bartek, I. L., Visconti, K., and Schoolnik, G. K. (2011). The response of mycobacterium tuberculosis to reactive oxygen and nitrogen species. *Front. Microbiol.* 2. doi: 10.3389/fmicb.2011.00105

Voskuil, M. I., Schnappinger, D., Visconti, K. C., Harrell, M. I., Dolganov, G. M., Sherman, D. R., et al. (2003). Inhibition of respiration by nitric oxide induces a mycobacterium tuberculosis dormancy program. *J. Exp. Med.* 198, 705–713. doi: 10.1084/jem.20030205

Wakamoto, Y., Dhar, N., Chait, R., Schneider, K., Signorino-Gelo, F., Leibler, S., et al. (2013). Dynamic persistence of antibiotic-stressed mycobacteria. *Science* 339, 91–95. doi: 10.1126/science.1229858

Wallace, F., and Sutherland, I. (1955). THE CLINICAL SIGNIFICANCE OF POSITIVE CULTURES AND OF ISONIAZID-RESISTANT TUBERCLE BACILLI DURING THE TREATMENT OF PULMONARY TUBERCULOSIS. *Thorax* 10, 85–98. doi: 10.1136/thx.10.2.85

Wang, Q., Boshoff, H. I. M., Harrison, J. R., Ray, P. C., Green, S. R., Wyatt, P. G., et al. (2020). PE/PPE proteins mediate nutrient transport across the outer membrane of mycobacterium tuberculosis. *Science* 367, 1147–1151. doi: 10.1126/science.aav5912

Watanabe, S., Zimmermann, M., Goodwin, M. B., Sauer, U., Barry, C. E., and Boshoff, H. I. (2011). Fumarate reductase activity maintains an energized membrane in anaerobic mycobacterium tuberculosis. *PloS Pathog.* 7, 1002287. doi: 10.1371/journal.ppat.1002287

Wayne, L. G., and Hayes, L. G. (1996). An *In vitro* model for sequential study of shiftdown of mycobacterium tuberculosis through two stages of nonreplicating persistence. *Infection Immun.* 64, 2062–2069. doi: 10.1128/iai.64.6.2062-2069

Wellington, S., Nag, P. P., Michalska, K., Johnston, S., Jedrzejczak, R., Kaushik, V. K., et al. (2017). A small-molecule allosteric inhibitor of mycobacterium tuberculosis tryptophan synthase. *Nat. Chem. Biol.* 13, 943–955. doi: 10.1038/nCHeMBIO.2420

WHO (2017) *Guidelines for treatment of drug-susceptible tuberculosis and patient care*. Available at: https://www.who.int/publications/i/item/9789241550000.

WHO (2021) *Global tuberculosis report 2021*. Available at: https://www.who.int/publications/i/item/9789240037021.

Wilburn, K. M., Fieweger, R. A., and VanderVen, B. C. (2018). Cholesterol and fatty acids grease the wheels of mycobacterium tuberculosis pathogenesis. *Pathog. Dis.* 76, 1–14. doi: 10.1093/femspd/fty021

Xie, Z., Siddiqi, N., and Rubin, E. J. (2005). Differential antibiotic susceptibilities of starved mycobacterium tuberculosis isolates. *Antimicrob. Agents Chemother.* 49, 4778–4780. doi: 10.1128/AAC.49.11.4778-4780.2005

Yang, X., Nesbitt, N. M., Dubnau, E., Smith, I., and Sampson, N. S. (2009). Cholesterol metabolism increases the metabolic pool of propionate in mycobacterium tuberculosis. *Biochemistry* 48, 3819–3821. doi: 10.1021/bi9005418

Zimmermann, M., Kuehne, A., Boshoff, H. I., Barry, C. E., Zamboni, N., and Sauer, U. (2015). Dynamic exometabolome analysis reveals active metabolic pathways in non-replicating mycobacteria. *Environ. Microbiol.* 17, 4802–4815. doi: 10.1111/1462-2920.13056

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 24 August 2022
DOI 10.3389/fcimb.2022.980844

OPEN ACCESS

EDITED BY
Helena Ingrid Boshoff,
National Institutes of Health (NIH),
United States

REVIEWED BY
Bavesh Kana,
University of the Witwatersrand,
South Africa
Kyle Rohde,
University of Central Florida,
United States

*CORRESPONDENCE
Matthew B. McNeil
matthew.mcneil@otago.ac.nz
Gregory M. Cook
greg.cook@otago.ac.nz

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 29 June 2022
ACCEPTED 03 August 2022
PUBLISHED 24 August 2022

CITATION
McNeil MB, Cheung C-Y, Waller NJE, Adolph C, Chapman CL, Seeto NEJ, Jowsey W, Li Z, Hameed HMA, Zhang T and Cook GM (2022) Uncovering interactions between mycobacterial respiratory complexes to target drug-resistant *Mycobacterium tuberculosis*. *Front. Cell. Infect. Microbiol.* 12:980844. doi: 10.3389/fcimb.2022.980844

Uncovering interactions between mycobacterial respiratory complexes to target drug-resistant *Mycobacterium tuberculosis*

Matthew B. McNeil[1,2*], Chen-Yi Cheung[1], Natalie J. E. Waller[1], Cara Adolph[1], Cassandra L. Chapman[1], Noon E. J. Seeto[1], William Jowsey[1], Zhengqiu Li[3], H. M. Adnan Hameed[4,5,6,7], Tianyu Zhang[4,5,6,7] and Gregory M. Cook[1,2*]

[1]Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand, [2]Maurice Wilkins, Centre for Molecular Biodiscovery, The University of Auckland, Auckland, New Zealand, [3]School of Pharmacy, Jinan University, Guangzhou, China, [4]State Key Laboratory of Respiratory Disease, Guangzhou Institutes of Biomedicine and Health (GIBH), Chinese Academy of Sciences (CAS), Guangzhou, China, [5]China-New Zealand Joint Laboratory of Biomedicine and Health, Guangzhou Institutes of Biomedicine and Health (GIBH), Chinese Academy of Sciences (CAS), Guangzhou, China, [6]Guangdong-Hong Kong-Macau Joint Laboratory of Respiratory Infectious Diseases, Guangzhou, China, [7]University of Chinese Academy of Sciences (UCAS), Beijing, China

Mycobacterium tuberculosis remains a leading cause of infectious disease morbidity and mortality for which new drug combination therapies are needed. Mycobacterial bioenergetics has emerged as a promising space for the development of novel therapeutics. Further to this, unique combinations of respiratory inhibitors have been shown to have synergistic or synthetic lethal interactions, suggesting that combinations of bioenergetic inhibitors could drastically shorten treatment times. Realizing the full potential of this unique target space requires an understanding of which combinations of respiratory complexes, when inhibited, have the strongest interactions and potential in a clinical setting. In this review, we discuss (i) chemical-interaction, (ii) genetic-interaction and (iii) chemical-genetic interaction studies to explore the consequences of inhibiting multiple mycobacterial respiratory components. We provide potential mechanisms to describe the basis for the strongest interactions. Finally, whilst we place an emphasis on interactions that occur with existing bioenergetic inhibitors, by highlighting interactions that occur with alternative respiratory components we envision that this information will provide a rational to further explore alternative proteins as potential drug targets and as part of unique drug combinations.

KEYWORDS

respiration, synthetic lethality, drug combinations, antibiotics, synergy

Introduction

M. tuberculosis, the causative agent of tuberculosis (TB), is an obligate human pathogen and significant cause of infectious disease morbidity and mortality, being responsible for an estimated 5.8 million new infections and 1.3 million deaths among HIV-negative people and an additional 214 000 among HIV-positive people (WHO, 2021). Combination drug regimens are favored for the treatment of TB due to their ability to increase efficacy, delay the development of resistance and reduce toxic side effects. Favorable drug interactions are typically the result of either (I) therapeutic synergy, when the effect (i.e. growth inhibition or bacterial killing) of drug combinations is greater than the sum of the individual drugs, or (II) synthetic lethal interactions, when the individual drugs alone are non-lethal, whilst the combination results in killing. Alternatively, some drug combinations serve to prevent the emergence of drug resistance, with additive drugs preventing the isolation of resistant mutants despite not increasing the killing of the partner compound. The need for combination therapies for the treatment of *M. tuberculosis* is also necessitated by additional factors including (I) the unique cellular structure of *M. tuberculosis* that makes it inherently tolerant of many antibiotics, (II) the ability of *M. tuberculosis* to switch into a metabolically inactive state that is phenotypically tolerant to many antibiotics and host-immune responses, and (III) unequal drug penetration at the site of infection (Kerantzas and Jacobs, 2017). As a result, drug-susceptible strains of *M. tuberculosis* are treated with a frontline combination regimen of four antibiotics for two months followed by four months of isoniazid and rifampicin. Typically the frontline regimen achieves cure rates of 85%. Unfortunately, the prolonged treatment time of available regimens can lead to patient non-compliance, which ultimately drives the acquisition of drug resistance. Consequently, despite the extensive use of combination therapies, there is clinical resistance to all available antibiotics. Treatment options for drug resistant strains are limited, often highly toxic and require even longer treatment times, typically >9 months (Lange et al., 2018), with much lower cure rates. Whilst new regimens, including the BPaL regimen, can reduce treatment length of drug-resistant strains to 6-months, there remains an urgent need for new drug classes and new approaches to the design of combination therapies to prevent drug resistance and further reduce treatment times.

The discovery and subsequent FDA approval of the F_1F_o-ATP synthase inhibitor bedaquiline (BDQ) has shifted the focus of many drug-discovery programs towards the development of bioenergetic inhibitors i.e., compounds that target the mycobacterial electron transport chain (ETC) (Andries et al., 2005; Diacon et al., 2014; Cook et al., 2017). The mycobacterial ETC is a series of membrane-bound or membrane-associated enzymes that are responsible for coupling the oxidation of electron donors that are generated from central carbon metabolism to the reduction of oxygen as a terminal electron acceptor. Several of these enzymes are proton pumping, allowing for the establishment of a proton motive force (PMF) that is used to generate ATP *via* oxidative phosphorylation (Cook et al., 2017; Hards et al., 2020). Several recent studies have highlighted the benefit of combining bioenergetic inhibitors to accelerate the rate of bacterial clearance from various infection models. More importantly, the clinical efficacy of multiple bioenergetic inhibitors is highlighted by the FDA approval of the BPaL combination therapy that consists of two bioenergetic inhibitors and one translational inhibitor (Conradie et al., 2020).

The focus of this review is to highlight recent advances in our understanding of interactions between bioenergetic components and their associated inhibitors. We discuss interactions identified (either synergistic or antagonistic) from chemical, genetic and chemical-genetic and interaction studies. Whilst each strategy has drawbacks, each approach has contributed novel insights.

Respiratory components as targets for bioenergetic inhibitors in *M. tuberculosis*

Here for context, we provide a brief description of each component that forms the mycobacterial ETC. Whilst the majority of this context is based on results from *M. tuberculosis*, we do highlight results obtained from the fast-growing model organism *Mycobacterium smegmatis*. We acknowledge that there may be differences between these species and have highlighted this when it may be a confounding factor. Prior reviews should be consulted for a more detailed discussion of each component and their functions (Cook et al., 2017; Hards and Cook, 2018; Hards et al., 2020; Hasenoehrl et al., 2020).

Mycobacterial electron donating dehydrogenases

The mycobacterial ETC has several primary dehydrogenases that couple the oxidation of respiratory substrates or recycling of redox cofactors from the citric acid cycle to the reduction of the electron carrier menaquinone. Dehydrogenases that donate electrons to the ETC, including glycerol-3-phosphate dehydrogenase, carbon-monoxide dehydrogenase, proline dehydrogenase or L-lactate dehydrogenase are not directly linked to the citric acid cycle and are not discussed in this review, although they are expertly

reviewed elsewhere (Rhee et al., 2011; Cook et al., 2014; Cook et al., 2017; Hasenoehrl et al., 2020).

M. tuberculosis has two classes of NADH: menaquinone oxidoreductases that couple the oxidation of NADH to the reduction of menaquinone (Weinstein et al., 2005; Yano et al., 2006). This includes a proton-pumping type I NADH dehydrogenase (NDH-1) and a non-proton pumping type II NADH dehydrogenase (NDH-2) (Figure 1). Prior work has demonstrated that NDH-1, is analogous to mitochondrial Complex-1 and is not required for the growth of *M. tuberculosis* (Rao et al., 2008; Heikal et al., 2014; Heikal et al., 2018; Petri et al., 2018; Vilcheze et al., 2018; Beites et al., 2019). *M. tuberculosis* has two copies of NDH-2, encoded by *ndh* and *ndhA* (Vilcheze et al., 2018). Genetic studies have demonstrated that *ndh* and *ndhA* can be individually deleted, but cannot be simultaneously deleted when grown in the presence of fatty acids (Vilcheze et al., 2018; Beites et al., 2019). Growth in the absence of fatty acids, restores this conditional essentiality (Beites et al., 2019). Somewhat unexpectedly, and in contrast to prior chromosomal deletion mutants, the use of CRISPR interference demonstrated that the transcriptional repression of *ndh*, but not *ndhA*, is sufficient to impair the growth of *M. tuberculosis* (Bosch et al., 2021; McNeil et al., 2021). Furthermore, the transcriptional repression of *ndh* resulted in bacterial killing in both *M. tuberculosis* and *Mycobacterium smegmatis*, a fast-growing non-pathogenic mycobacterial species (McNeil et al., 2022). The discrepancies in phenotypes associated with the genetic inhibition of *ndh* are likely to be a result of differences in experimental approaches with chromosomal deletion mutants selecting for metabolically adapted strains that are able to grow in the absence of *ndh*, whilst transcriptional inhibition may observe the immediate effects of inhibiting *ndh* prior to metabolic adaptation (Weinstein et al., 2005; Yano et al., 2006; Heikal et al., 2016).

Two mycobacterial succinate dehydrogenase enzymes (i.e., SDH-1 and SDH-2) directly link the citric acid cycle to the ETC, by coupling the oxidation of succinate to the reduction of menaquinone in mycobacterial species (Berney and Cook, 2010; Pecsi et al., 2014; Heikal et al., 2016; Hards et al., 2019; Hards et al., 2020). Prior genetic strategies in *M. tuberculosis* have demonstrated that each complex can be individually deleted and are functionally redundant as a SDH-1 and SDH-2 double mutant was non-viable (Hartman et al., 2014). Interestingly, deletion of SDH-1 in *M. tuberculosis* disrupted cellular respiration, accelerated cell death during stationary phase and had a survival defect during chronic infection (Hartman et al., 2014). SDH-2 was essential in *M. smegmatis* (Pecsi et al., 2014), further supporting the hypothesis that these enzymes perform unique, yet overlapping functions. Definitive evidence that SDH enzymes are indeed important for survival and persistence come from the study of the succinate dehydrogenase suicide inhibitor 3-nitropropionate (3NP) (Eoh and Rhee, 2013). 3NP is a valuable tool for *in vitro* studies of SDH, but cannot be used to assess the chemical inhibition of SDH in an infection model because of its associated toxicities. Inhibiting *M. tuberculosis* SDH (SDH-1 or 2 or both) using 3NP resulted in a time-dependent killing of *M. tuberculosis* during adaptation to hypoxia/persistence (Eoh and Rhee, 2013). Re-aeration of these cultures in the presence of 3NP also inhibited growth. Treatment of non-replicating/hypoxia cultures of *M. smegmatis* with 3NP leads to cell death *via* a mechanism that dissipates the membrane potential (Pecsi et al., 2014). Taken

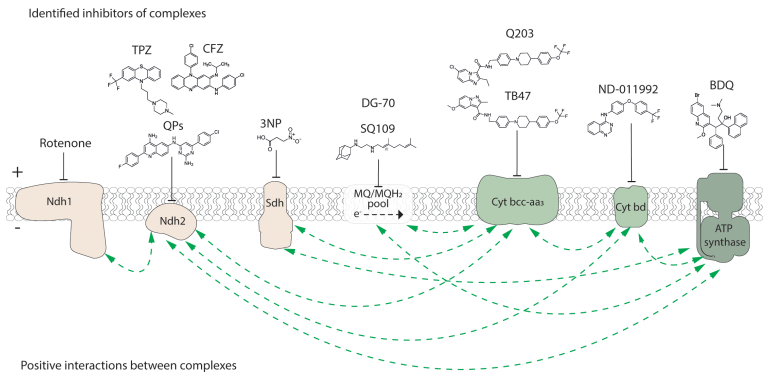


FIGURE 1
Inhibitors of the electron transport chain and ATP synthase of M. tuberculosis. Known inhibitors of respiratory components, example structures and their targets are illustrated with flathead arrows. Flathead arrow placement does not reflect the specific site of inhibition. The dashed arrow in the MQ/MQH2 pool represents the direction of electron flow. Electron transport chain complexes and the ATP synthase are coloured as: election donors, beige; electron acceptors, green. Plus (+) and minus (-) symbols represent high and low proton concentration gradient across the cytoplasmic membrane, respectively. QPs, quinolinyl pyrimidines; TPZ, trifluoperazine; CFZ, clofazimine; 3NP, 3-nitropropionate; BDQ, bedaquiline. Positive interactions (i.e. synergy or synthetic lethality) between bioenergetic components as reported from either a chemical, genetic or chemical-genetic interaction study and as described in the body of the manuscript are shown with dashed green arrows.

together these studies establish that sustained metabolism of succinate through SDH is an essential component of *M. tuberculosis* metabolic adaptation to hypoxia/persistence and that SDH inhibitors will target these cells. Resolving the mechanisms of functional redundancy and specific functions of each SDH enzyme in combination with recently published structures will assist in the design of chemical inhibitors (Gong et al., 2020; Zhou X. et al., 2021).

Like SDH enzymes, mycobacterial malate:quinone oxidoreductase (MQO) couples the oxidation of malate as part of the citric acid cycle to the reduction of menaquinone (Harold et al., 2022). *M. tuberculosis* also encodes a malate-dehydrogenases (MDH) that couples the oxidation of malate to the recycling of NAD^+ to NADH. CRISPR interference of both *mdh* and *mqo* expression in *M. tuberculosis* impairs growth more severely than either gene knockdown alone (Harold et al., 2022). It has been proposed that exergonic MQO activity powers mycobacterial growth under non-energy limiting conditions, and that endergonic MDH activity complements MQO activity, but at an energetic cost for mycobacterial growth (Harold et al., 2022). *M. tuberculosis* utilises MDH to facilitate a reductive TCA cycle to produce fumarate (oxaloacetate reduction) for use as a terminal electron acceptor (Watanabe et al., 2011). This provides an alternative electron acceptor for *M. tuberculosis* in conditions of hypoxia thereby providing a mechanism to maintain the membrane potential and recycle reducing agents. *M. tuberculosis* can also use Mqo and Mdh in combination to form a futile cycle to provide a pseudo NDH-2-type enzyme that is able to complement the growth of *ndh2* mutants (Miesel et al., 1998; Vilcheze et al., 2005). Rittershaus et al. (2018) demonstrated that MDH is required for the metabolism and survival of *M. tuberculosis in vitro* and *in vivo*. Moreover, chemical inhibitors of *M. tuberculosis* MDH had the ability to inhibit growth and rapidly kill hypoxic quiescent cells (non-replicating) *in vitro* and during infection of the murine lung (Rittershaus et al., 2018). We propose that the addition of an inhibitor of MQO, in combination with MDH inhibitors, may enhance the growth inhibition of *M. tuberculosis* further while still retaining potent activity against non-replicating populations. In this regard, MQO is not found in mammalian cells and potent inhibitors have been reported that target the mitochondrial malate:quinone oxidoreductase of the malarial parasite *Plasmodium falciparum* (Hartuti et al., 2018). The significant structural differences reported between the human and mycobacterial MDH enzymes (Rittershaus et al., 2018) suggest a combination therapy of MQO and MDH inhibitors may be attractive for TB drug development.

Mycobacterial electron carriers

Menaquinone is the primary electron carrying lipoquinone in mycobacteria that is responsible for shuttling electrons between respiratory dehydrogenases and terminal oxidases (Cook et al., 2017). Menaquione biosynthesis occurs *via* a series of enzymes that are similar to the *E. coli* synthesizing genes *menA-J* (Cook et al., 2017). Despite the importance of menaquinone biosynthesis in ETC function, not all menaquinone synthesizing genes are designated as essential genes (Dhiman et al., 2009; Griffin et al., 2011; Cook et al., 2017; Bosch et al., 2021; McNeil et al., 2021). Interestingly, targeted transcriptional repression of *menA* and *menD* with CRISPR interference had no effect on mycobacterial growth (McNeil et al., 2021). This is in contrast to transposon mutagenesis and whole genome CRISPR-interference studies, in which both genes were designated as being necessary for mycobacterial growth (Griffin et al., 2011; Bosch et al., 2021). Prior studies have demonstrated that the consequences of CRISPRi mediated inhibition of individual metabolic genes can be mitigated by metabolic buffering, in which multiple cell divisions are needed to deplete enzymes or enzyme products below levels needed to produce a bacterial phenotype (Donati et al., 2021). Whether this is the case for menaquione biosynthesis genes in *M. tuberculosis* requires further investigation. Nevertheless, the absence of menaquinone from humans, makes it an attractive drug target. Multiple small molecules have been identified as inhibitors of various menaqinone biosynthetic enzymes including MenA and MenD (Dhiman et al., 2009; Fang et al., 2010; Debnath et al., 2012; Berube et al., 2019; Bashiri et al., 2020). Further to this, recent metagenomic guided drug discovery efforts recently identified novel antibiotic classes that directly bind menaquinone, several of which had *in vitro* and *ex vivo* activity against *M. tuberculosis* (Li et al., 2022).

Electron accepting terminal oxidases

Under aerobic conditions, a supercomplex of cytochrome bc_1 (Complex III; *qcrBCD*) and an aa_3-type cytochrome *c* oxidase (Complex IV; *ctaBCDE*), herein referred to as Cyt-bc_1aa_3, couples the transfer of (i) electrons from reduced menaquinol to cytochrome *c* to (ii) proton translocation and the generation of a PMF (Gong et al., 2018; Wiseman et al., 2018). Both complexes are essential for mycobacterial growth, yet the deletion or transcriptional inhibition of either complex in *M. tuberculosis* results in a bacteriostatic phenotype (Beites et al., 2019; Bosch et al., 2021; McNeil et al., 2021; McNeil et al., 2022). Numerous small molecule inhibitors of cytochrome *bc1* have been identified (Kang et al., 2014; Lu et al., 2019; Yu et al., 2021; Zeng et al., 2021), of which Q203 (Telacebec) is the most advanced through clinical trials (Pethe et al., 2013). Structural studies have demonstrated that Q203 binds to the menaquinone binding site, effectively blocking menaquinone oxidation (Zhou S. et al., 2021). This mechanism of action is supported by resistance against Q203 and the majority of other Cyt-bc_1aa_3

inhibitors being at residues near the menaquinone-binding site (Liu et al., 2019; Lu et al., 2019; Yanofsky et al., 2021; Zhou S. et al., 2021).

Cytochrome bd-type menaquinol oxidase (Cyt-*bd*) is an alternative non-proton pumping high-affinity terminal oxidase that is only found in Prokaryotes (Borisov et al., 2021). From an energetic point of view, Cyt-*bd* is considered less efficient as it does not translocate protons. Despite this, Cyt-*bd* is able to generate a PMF by transmembrane charge separation with an H+/e ratio of 0.94 ± 0.18 (Borisov et al., 2011). Cyt-*bd* is encoded by *cydAB* as part of the *cydABDC* operon, with both genes being non-essential for mycobacterial growth (McNeil et al., 2021). Because of the reduced efficiency but higher affinity, Cyt-*bd* functions predominantly under low oxygen conditions *in vitro* (Kana et al., 2001; Berney and Cook, 2010; Aung et al., 2014) and *in vivo* (Shi et al., 2005; Cai et al., 2021). Cyt-*bd* becomes essential for mycobacterial growth when the Cyt-bc_1aa_3 supercomplex is deleted or expression inhibited (Matsoso et al., 2005; Arora et al., 2014; Lu et al., 2015; Kalia et al., 2017; Lu et al., 2018; Beites et al., 2019; Lee et al., 2019; Bosch et al., 2021; Hards et al., 2022).

Mycobacterial F_1F_o ATP synthase

The PMF generated by the mycobacterial ETC is used by the F_1F_o-ATP synthase to generate ATP from ADP and inorganic phosphate. The mycobacterial F_1F_o-ATP synthase is encoded by the of *atpBEFHAGDC* operon, with all genes being essential for mycobacterial growth (Griffin et al., 2011; Zhang et al., 2012; Bosch et al., 2021; McNeil et al., 2021) even on fermentable carbon sources like glucose (Tran and Cook, 2005). Furthermore, the transcriptional repression of *atpE* and *atpB* not only inhibited mycobacterial growth, but resulted in rapid bacterial killing (McNeil et al., 2020). BDQ (Sirturo®) was approved by the US FDA in 2012 for the treatment of adults with pulmonary MDR-TB (Diacon et al., 2012; Cohen, 2013; Diacon et al., 2014). BDQ is generally bactericidal and can kill drug-resistant mycobacterial species and dormant bacilli (Diacon et al., 2012; Diacon et al., 2014). It acts quickly compared to most TB drugs, but still requires many weeks of therapy and BDQ-resistance has been reported, including in treatment-naïve populations (Andries et al., 2014; Villellas et al., 2017). BDQ targets the F_1F_o-ATP synthase of *M. tuberculosis* and binds to the *c*-subunit rotor in the membrane-embedded part of the F_1F_o-ATP synthase (Andries et al., 2005a; Preiss et al., 2015) decreasing intracellular ATP levels (Koul et al., 2007; Koul et al., 2008; Koul et al., 2014). Structural analysis demonstrated that BDQ binds to ATP synthase 'leading' and 'lagging' sites of subunit *c* and to a lesser degree subunit *a*, resulting in major conformational changes in mycobacterial ATP synthase (Guo et al., 2021; Montgomery et al., 2021). BDQ mediated inhibition of ATP synthesis also has downstream effects on a number of metabolic processes including the inhibition of glutamine synthetase (Wang et al., 2019) and the rerouting of mycobacterial metabolism that places an increased reliance on glycolysis and substrate level phosphorylation to generate cellular ATP (Mackenzie et al., 2020). BDQ also dissipates the ΔpH component of the PMF in mycobacteria (Hards et al., 2015; Hards et al., 2018). This depends on target-based accumulation of BDQ and leads to an uncoupled microenvironment around the F_1F_o-ATP synthase (Hards et al., 2018). Several new inhibitors of the mycobacterial F_1F_o-ATP synthase have been identified (Singh et al., 2015; Tantry et al., 2017; Narang et al., 2019; Kamariah et al., 2020; Hards et al., 2022). However, further studies are needed to determine their clinical safety and efficacy.

Interactions with mycobacterial respiratory components

Mycobacterial respiratory components have emerged as a promising target space, not only for the development of novel therapeutics, but also as part of unique combination therapies. This is due to the observation that the inhibition of many mycobacterial respiratory components have positive interactions, not only with other respiratory components, but other cellular processes in general. For the remainder of this review, we describe each of these interactions and provide possible mechanistic explanations. We present interactions identified from either chemical, genetic and chemical-genetic interaction studies. We consider both (i) synergistic interactions (i.e. when the sum of the outcome is greater than the individual components) and (ii) synthetic lethal interactions, (i.e. when the loss/inhibition of neither component is lethal but combined results in cell death) as being "positive interactions". We also discuss examples of antagonism, as identifying the basis for these unfavorable interactions will aid in the development of improved combination therapeutics. Whilst the following sections place an emphasis on interactions with clinically advanced inhibitors and their targets, i.e. BDQ, Q203 and pretomanid, we also discuss possible interactions between other respiratory components in the hope that this will highlight priority targets for future drug development.

Interactions with the F_1F_o-ATP synthase and the inhibitor BDQ

Numerous inhibitors of the mycobacterial F_1F_o-ATP synthase have been identified (Singh et al., 2015; Tantry et al., 2017; Narang et al., 2019; Hotra et al., 2020; Kamariah et al., 2020; Denny, 2021; Choi et al., 2022; Hards et al., 2022), of which

BDQ is the most clinically advanced and was FDA approved for the treatment of multi-drug resistant *M. tuberculosis* in 2012 (Diacon et al., 2012; Cohen, 2013; Diacon et al., 2014). The bioenergetic consequences of inhibiting the F_1F_O-ATP synthase have resulted in ATP synthase inhibitors, including BDQ, having favorable interactions with other respiratory inhibitors. For example, several studies have demonstrated interactions between simultaneous inhibition of menaquinone biosynthesis and ATP synthase function (Sukheja et al., 2017; Berube et al., 2019). Inhibitors of MenA, which catalyzes the penultimate step in menaquinone biosynthesis had a synergistic killing interaction with BDQ, resulting in complete sterilization of *M. tuberculosis* cultures within 14 – 21 days of treatment (Berube et al., 2019). The synergy between inhibition of menaquinone biosynthesis and the ATP synthase was not observed in genetic studies, although this may be attributable to variations in model organism (i.e. *M. smegmatis* vs *M. tuberculosis*) or experimental approach (i.e. genetic-genetic interactions vs chemical-chemical interactions) (Sukheja et al., 2017; Berube et al., 2019; McNeil et al., 2022).

The non-proton pumping type II NADH dehydrogenase (NDH-2) is essential for mycobacterial growth. Phenothiazine-like compounds including CFZ have long been thought to kill through a redox cycle involving reduction by NDH-2 and spontaneous re-oxidation by O_2, producing toxic levels of ROS (Yano et al., 2006; Yano et al., 2011; Heikal et al., 2016). As BDQ stimulates the respiration rate of *M. tuberculosis* (Hards et al., 2015; Lamprecht et al., 2016; Hards and Cook, 2018; Hards et al., 2018), this results in an accumulation of reducing equivalents that potentiates CFZ reduction, increasing ROS production. This drives the rapid sterilization of *M. tuberculosis in vitro* and in macrophages when BDQ and CFZ are used in combination (Lamprecht et al., 2016; Berube and Parish, 2018). Genetic studies have also reported synergistic interactions between the mycobacterial NDH-2 and the ATP synthase (McNeil et al., 2022). CRISPRi knockdown of *ndh* and *atpE* in *M. smegmatis* is bactericidal by itself and thus prevents the identification of synergistic interactions (McNeil et al., 2022). McNeil et al. (2022) overcame this by engineering sub-optimal sgRNAs that produced a bacteriostatic phenotype. This allowed for the identification of interactions between genes that when knocked down in combination, resulted in cell death (McNeil et al., 2022). This multiplexed approach identified a synthetic lethal interaction between NDH-2 and the ATP synthase that killed *M. smegmatis* (McNeil et al., 2022). Further to this, an NDH-2 conditional mutant in *M tuberculosis* was also more sensitive to growth inhibition by BDQ (Beites et al., 2019).

Chemical and genetic interaction studies have failed to show an interaction between inhibition of the ATP synthase and primary terminal oxidase Cyt-*bcc*-aa_3 (Lamprecht et al., 2016; Beites et al., 2019; McNeil et al., 2022). Despite this, deletion of the alternative terminal oxidase Cyt-*bd* has been shown to increase sensitivity of *M. tuberculosis* to killing by BDQ (Berney et al., 2014; Kalia et al., 2017). This interaction wasn't reproduced in genetic studies, again possibly due to differences in experimental approach or model system (McNeil et al., 2022).

Given the cellular importance of ATP, it is surprising that following BDQ exposure metabolic remodeling allows mycobacterial to initially tolerate BDQ, with minimal killing observed in the first five days of exposure (Koul et al., 2014; Hards et al., 2015). However, disrupting the metabolic response to BDQ can remove this initial tolerance and sensitize *M. tuberculosis* to rapid killing. For example, substrate level phosphorylation is prioritized for the generation of ATP production following BDQ exposure (Mackenzie et al., 2020). As such genetic inhibition of glycolysis by deleting pyruvate kinase *pykA* increased BDQ lethality, sterilizing cultures within 5-6 days. Interestingly, this synergy between glycolysis and ATP synthase inhibition only occurred at specific glycolytic nodes, with deletion of the first rate-limiting site in glycolysis, i.e. phosphofructokinase not increasing BDQ lethality. BDQ exposure also has downstream effects on cellular glutamine levels, with the depletion of ATP limiting the ability of glutamine synthetase to convert glutamate into glutamine (Wang et al., 2019). Reduced glutamine levels suggested that BDQ treated cells would have increased susceptibility to the targeted inhibition of glutamine synthetase. Consequently, BDQ synergized with methionine sulfoximine, an inhibitor of glutamine synthetase resulting in increased BDQ lethality (Wang et al., 2019). BDQ has also been shown to synergize with inhibitors of mycobacterial stress response and cell wall synthesis (Lechartier et al., 2012; Wang et al., 2019; Yang et al., 2019).

The most promising combination therapy involving BDQ, is the BPaL regime, consisting of BDQ, pretomanid and linezolid. BPaL is an all oral regime, that when utilized over a 6 month period achieve 90% favorable outcomes in patients with extensively drug-resistant or non-responsive multi-drug resistant *M. tuberculosis* (Conradie et al., 2020). Whilst the BPaL regimen has since been FDA approved, BDQ has significant toxicity issues including hepatotoxicity, irregular cardiac rhythm, and increases in blood bilirubin (Moodliar et al., 2021; Yao et al., 2022). There are also concerns associated with BDQ resistance, which is the result of (i) mutations in the transcriptional regulator *Rv0678* that up-regulate the expression of the efflux pump MmpL5 or (ii) in residues of ATP synthase that prevent BDQ binding. Clinical resistance to BDQ *via* mutations in the transcriptional regulator *Rv0678* have already been reported, even in treatment naïve populations prior to the inclusion of BDQ (Huitric et al., 2010; Andries et al., 2014; Hartkoorn et al., 2014; Villellas et al., 2017).

Inhibitors of the mycobacterial F_1F_o-ATP synthase are promising anti-tubercular agents, particularly given their ability to synergize with a range of other inhibitors suggesting that they could form the backbone of novel therapeutic regimens. Whilst BDQ highlights the potential for this class of

compound, issues with toxicity and resistance highlight the need for improved derivatives or completely new chemical classes of ATP synthase inhibitors.

Interactions with cytochrome oxidase bcc-aa$_3$ oxidase and the inhibitor Q203

M. tuberculosis respires oxygen *via* two terminal respiratory oxidases, Cyt-*bcc_aa*$_3$ and Cyt-*bd* (Cook et al., 2017). Numerous small molecule inhibitors of the cytochrome bc_1 complex have been identified (Abrahams et al., 2012; Pethe et al., 2013; Kang et al., 2014; Lu et al., 2019; Yanofsky et al., 2021; Zhou S. et al., 2021), of which Q203 (aka Telacebec) is the most advanced through clinical trials whilst TB47 is in preclinical development (Pethe et al., 2013; Kang et al., 2014; Lu et al., 2019). *M. tuberculosis* is able to control the flow of electrons to either oxidase providing metabolic flexibility when either oxidase is deleted or inhibited (Lamprecht et al., 2016). This has been demonstrated by the ability to obtain gene deletions of Cyt-*bcc_aa*$_3$ or Cyt-*bd* independently, whilst the chemical or genetic inhibition of the Cyt-*bcc_aa*$_3$ stops growth but the cells remain viable (bacteriostatic) (Beites et al., 2019; Bosch et al., 2021; McNeil et al., 2021; McNeil et al., 2022). Despite this, various studies have demonstrated that blocking this functional redundancy results in improved inhibitory outcomes. For example, chemical-genetic studies demonstrated that the deletion of one terminal oxidase, increases susceptibility to inhibition of the alternative, with mutants lacking Cyt-*bcc_aa*$_3$ having increased susceptibility to inhibitors of Cyt-*bd* (Lu et al., 2018; Lee et al., 2021), and mutants lacking Cyt-*bd* having increased susceptibility to inhibitors of Cyt-*bcc_aa*$_3$ (Arora et al., 2014; Lu et al., 2015; Kalia et al., 2017; Moosa et al., 2017; Liu et al., 2019; Lu et al., 2019; Chong et al., 2020). These positive interactions are supported by the observation that Q203 completely inhibits respiration in the Cyt-*bd* mutant (Lamprecht et al., 2016). Further to this, the simultaneous inhibition of both terminal oxidases has been shown to have synthetic lethal phenotypes, resulting in cell death (Kalia et al., 2017; Lu et al., 2018; Liu et al., 2019; Lee et al., 2021). Importantly, this bactericidal outcome of dual terminal oxidase inhibition is conserved when using diverse carbon sources under replicating conditions, under non-replicating conditions and in murine models of infection (Kalia et al., 2017; Lee et al., 2021; McNeil et al., 2022). Subsequently, this has spurred interest in identifying and developing improved inhibitors of Cyt-*bd*. Recent examples include ND-011992 (Lee et al., 2021), MQL-H2 (Harikishore et al., 2020) and HM2-16F (Hards et al., 2022), all of which have synthetic lethal interactions with Q203. The less-than-optimal pharmacokinetic properties of ND-011992 make it unsuitable for development and alternative chemical scaffolds targeting cytochrome *bd* are required (Lee et al., 2019). The recent high-resolution structures of cytochrome bd from *M. tuberculosis* and *M. smegmatis* now provide a detailed molecular framework for the discovery of new bd inhibitors (Safarian et al., 2021; Wang et al., 2021). Interestingly, in *Mycobacterium ulcerans*, which has a non-functional Cyt-bd and relies exclusively on Cyt-*bcc_aa*$_3$ as a terminal oxidase, inhibitors of Cyt-*bcc_aa*$_3$ are bactericidal (Scherr et al., 2018; Liu et al., 2019). Alternatively, naturally occurring polymorphisms in the Cyt-*bcc_aa*$_3$ of *Mycobacterium abscessus* provide a high level of resistance to Q203 (Sorayah et al., 2019). Combined, this data demonstrates that starving *M. tuberculosis*, and potentially other mycobacterial species, of their ability to use oxygen as a terminal electron acceptor can result in bactericidal outcomes and is a vulnerable target for drug discovery (Bajeli et al., 2020; Lee B. S. et al., 2020; Sviriaeva et al., 2020).

Menaquinone is the primary electron carrier in *M. tuberculosis* (Dhiman et al., 2009; Debnath et al., 2012; Cook et al., 2017). Interestingly, the majority of Cyt-bc1aa3 inhibitors bind to the menaquinone binding site to block menaquinone oxidation (Lu et al., 2019; Yanofsky et al., 2021). Studies have demonstrated interactions between simultaneous inhibition of menaquinone biosynthesis and inhibition of Cyt-*bcc_aa*$_3$ (Berube et al., 2019). Inhibitors of MenA, which catalyzes the penultimate step in menaquinone biosynthesis, synergized with a cytochrome bc_1-aa_3 inhibitor (Berube et al., 2019). The synergy between inhibition of menaquinone biosynthesis and the cytochrome bc_1-aa_3 terminal oxidase was further demonstrated in genetic studies, where the simultaneous transcriptional repression of *menD* and *qcrB* resulted in stronger growth inhibition than the single knockdown of either gene alone (McNeil et al., 2022). The inhibition of menaquinone biosynthesis not only reduces available menaquinone, but it is also likely to reduce the levels of intracellular ATP needed to sustain growth and survival (Dhiman et al., 2009; Sukheja et al., 2017). This coupled with the bioenergetic effects of Cyt-*bcc_aa*$_3$ inhibitors that ultimately lead to a reduction in the intracellular levels of ATP would result in target synergy and bacterial cell death.

Several studies have also reported synergistic interactions between the mycobacterial NADH dehydrogenase type II enzymes (NDH-2) and the Cyt-*bcc_aa*$_3$ terminal oxidase in mycobacteria (Beites et al., 2019; McNeil et al., 2022). CRISPRi knockdowns have uncovered a synthetic lethal interaction between NDH-2 and the Cyt-*bcc_aa*$_3$ that killed *M. smegmatis* faster than the dual knockdown of *qcrB* and *cydB* on both fermentable and non-fermentable carbon sources (McNeil et al., 2022). Studies have also reported synergistic killing between CFZ, a proposed inhibitor of NDH-2 and inhibitors of Cyt-*bcc_aa*$_3$ including Q203 and phenoxy alkyl benzimidazoles (PABs) under both replicating and non-replicating conditions (Lamprecht et al., 2016) (Berube and

Parish, 2018). However, there are conflicting reports regarding this interaction as the deletion of *ctaE-qcrCAB* did not alter sensitivity to growth inhibition by NDH-2 inhibitors, including CFZ (Beites et al., 2019). It should be noted, that the same authors conclude that CFZ does not require NDH-2 to inhibit the growth of *M. tuberculosis* as conditional inactivation of NDH-2 (i.e. *ndhA* + *ndh* double mutant) in *M. tuberculosis* had no effect on the growth inhibitory properties of CFZ, (Beites et al., 2019). Between these various studies, differences in (i) biological readouts, i.e. bacterial viability vs growth inhibition, (ii) experimental approaches of gene deletion compared to gene knockdown and (iii) experimental model i.e. *M. tuberculosis* or *M. smegmatis* may go some way to explaining these reported differences. Despite this, we conclude that there is sufficient evidence to suggest that whilst targeting NDH-2 may not synergize with the growth inhibitory properties of Cyt-*bcc_aa*$_3$ inhibition, they may offer another potential avenue to enhance the bactericidal activity of the normally bacteriostatic Cyt-*bcc_aa*$_3$ inhibitors.

Cyt-*bcc_aa*$_3$ inhibition also synergizes with the first line *M. tuberculosis* drugs PZA (PZA) and rifampicin (RIF) in a mouse model (Lu et al., 2019). Specifically, the Cyt-*bcc_aa*$_3$ TB47 inhibitor exhibited a potent synergy with subtherapeutic doses of PZA and RIF, causing a 4- and 5-fold reduction in lung CFU compared to the respective monotherapy (Lu et al., 2019). Here, it was proposed that a disruption to the NADH/NAD+ ratio by TB47 may explain the synergy of TB47 with RIF and PZA. TB47 also exhibited a highly unique synergistic activity with clarithromycin *in vitro* and in mouse models against different mycobacterial species (Liu et al., 2020; Yu et al., 2020). TB47-containing 3-drug regimens cured Buruli ulcer *in vivo* in ≤ 2 weeks when dosed daily or in ≤ 3 weeks when dose twice per week (6 doses in total) compared to the current first-line treatment of RIF and streptomycin which required 8 weeks and had 26.67% mice relapsing post treatment (Gao et al., 2021).

The bioenergetic consequences of Cyt-*bcc_aa*$_3$ inhibition, that lead to reduced ATP levels and severely impaired bacterial growth have also been shown to antagonise the bactericidal activities of the anti-tubercular drugs isoniazid (INH) and moxifloxacin (Lee et al., 2019). Specifically, the metabolic downregulation following Q203 exposure dissipated the transient increase in intracellular respiration that is associated with cell death and was observed following INH or moxifloxacin exposure alone (Lee et al., 2019). This is consistent with the antagonism frequently seen between bactericidal and bacteriostatic antibiotics (Ocampo et al., 2014). Interestingly, a known efflux pump inhibitor verapamil increased the potency of Q203 against *M. tuberculosis*, indicating that the upregulation of efflux pumps may be associated with low level resistance to Q203 (Jang et al., 2017). This further highlights how an improved understanding of the mechanisms of antibiotic mediated mycobacterial death are essential to the improved design of optimized combination therapies.

Interactions between other mycobacterial respiratory components

There are many bioenergetic components in *M. tuberculosis* for which there are no bona-fide small molecule inhibitor with favorable pharmacological properties. Given the resource investment required to advance small molecule through the drug development pipeline, it is essential that high priority targets with the greatest chance of clinical success when used in combination therapies are identified and validated. Here we discuss interactions between other mycobacterial respiratory components, specifically NADH dehydrogenase, succinate dehydrogenase, malate dehydrogenase and menaquinone biosynthesis. We acknowledge that there may be is overlap with prior sections.

NADH dehydrogenase inhibitor interactions

A synthetically lethal interaction exists between the two different NADH dehydrogenase enzymes (NDH-1 and NDH-2) in *M. tuberculosis*, with the deletion of NDH-2 (Δ*ndh*Δ*ndhA*) rendering *M. tuberculosis* susceptible to killing by NDH-1 inhibitors such as rotenone, which normally has no effect on the growth of *M. tuberculosis* (Beites et al., 2019). Of the two enzymes, NDH-2 is being actively pursued as a drug target as it is the primary mycobacterial NADH dehydrogenase (Weinstein et al., 2005; Shirude et al., 2012; Dunn et al., 2014; Harbut et al., 2018; Vilcheze et al., 2018; Beites et al., 2019) and is absent from mammalian mitochondria. To date, two main classes of compound interact with the mycobacterial NDH-2 enzymes: the phenothiazines [e.g., thioridazine (THZ), chlorpromazine (CPZ)] and CFZ, and both compounds have a range of positive interactions reported both *in vitro* and *in vivo*. For example, THZ and CPZ have been shown to enhance the activity of TB antibiotics including RIF and streptomycin (Crowle et al., 1992; Viveiros and Amaral, 2001).

While not technically an inhibitor of NDH-2, the mechanism of action of CFZ is linked to NDH-2 activity (Yano et al., 2011). CFZ is thought to kill through *via* a NDH-2 recycling mechanism that produces toxic levels of ROS (Yano

et al., 2011). Accordingly, inhibitors that potentiate CFZ reduction and thus ROS production are synergistic with CFZ. Has highlighted from prior sections, this includes both BDQ and Q203 (Lamprecht et al., 2016). Inhibition of the Cytbc_1-aa_3 oxidase with phenoxy alkyl benzimidazoles (PABs) synergized with CFZ under replicating and non-replicating conditions (Berube and Parish, 2018). These interactions are consistent with genetic studies, showing that the inactivation of *ndh* with either *atpE* or *qcrB* having a bactericidal phenotype (McNeil et al., 2022). Moreover, CFZ has been previously shown to directly compete with menaquinone for reduction by NDH-2 and, as such, menaquinone biosynthesis inhibitors synergize strongly with CFZ to kill *M. tuberculosis* (Berube et al., 2019). Interestingly, deletion of cytochrome *bd* synergized with NDH-2 inhibition in *M. tuberculosis* (Beites et al., 2019) and the deletion of cytochrome *bd* sensitized *M. smegmatis* to killing by CFZ at concentrations that were bacteriostatic against wild-type (Lu et al., 2015). This is despite no interaction being observed between the dual knockdown of *cydB* and *ndh* or *nuoD* in *M. smegmatis* (McNeil et al., 2022). These differences may be partly explained by choice of model system, experimental approach, or recent evidence suggesting that CFZ doesn't require NDH-2 for inhibitory activity against *M. tuberculosis* (Beites et al., 2019). Further, studies in murine infection models have shown that CFZ synergizes with RIF, INH and PZA to halve the treatment period for drug-sensitive *M. tuberculosis* (Li et al., 2017) as well as potentiating the activity of second-line drug regimens against drug-resistant *M. tuberculosis* (Grosset et al., 2013). This suggests that targeting NDH-2 offers another potential avenue to enhance the activity of the normally bacteriostatic Cytbc_1-aa_3 inhibitors although further work is required to reconcile some experimental differences.

While several promising synergies have been identified between inhibiting the mycobacterial NADH dehydrogenases and other respiratory complexes, it is also worth noting that there is also the potential for antagonistic interactions to occur, particularly between INH and CFZ. Mutations in *ndh* have previously been shown to confer INH resistance in *M. tuberculosis*, *M. smegmatis*, and *M. bovis* BCG (Miesel et al., 1998; Lee et al., 2001; Vilcheze et al., 2005), and both targeted protein depletion and chemical inhibition of NDH-2 attenuate INH activity in *M. tuberculosis* (Harbut et al., 2018; Koh et al., 2022). This antagonism is postulated to be due to alterations in the NADH/NAD^+ ratio preventing the formation of the active INH-NAD adduct or competing with its binding to the NADH-dependent enoyl-ACP reductase, InhA (Miesel et al., 1998; Lee et al., 2001; Vilcheze et al., 2005; Koh et al., 2022). Despite this, genetic deletion of *ndh* and *ndhA* alone or in combination did not alter INH susceptibility in *M. tuberculosis*, which was attributed to insufficient NADH accumulation in these mutants to affect INH activity (Vilcheze et al., 2018; Beites et al., 2019). Nevertheless, future NDH-2 inhibitors should be investigated for their potential interactions with INH. Likewise, as CFZ is activated by NDH-2, interactions between NDH-2 inhibitors and CFZ should be evaluated. Importantly, two recent studies demonstrated that CFZ retains bactericidal activity against *M. tuberculosis* in the absence of NDH enzymes (Vilcheze et al., 2018; Beites et al., 2019), suggesting that antagonism with NDH-2 inhibitors is unlikely as other mechanisms of CFZ activation or activity exist independently of NDH-2.

SDH and MQO/MDH inhibitor interactions

Whereas the NADH dehydrogenases inhibitor interactions have been well studied, the remaining electron-donating complexes are comparatively under studied and under explored as drug targets. A recent study showed that inhibition of SDH activity using CRISPRi sensitizes *M. tuberculosis* to growth inhibition and killing by BDQ and the cytochrome bcc-aa_3 inhibitors, Q203 and TB47 (Adolph et al., 2022). Likewise, knockdown of *qcrB* or *ctaC* with *sdhA2* synergized in *M. smegmatis* (McNeil et al., 2022). Moreover, when gene knockdown was induced simultaneously with drug treatment, impaired succinate oxidation prevented the emergence of INH and PA-824(pretomanid) resistance, suggesting that SDH inhibitors may also be effective anti-resistance agents when used in combination with INH or pretomanid (Adolph et al., 2022). However, when SDH enzymes were pre-depleted prior to antibiotic challenge an antagonistic interaction was observed with both INH and pretomanid and the other cell wall inhibitors tested; ethionamide (ETH), EMB and SQ109 (Adolph et al., 2022). This is similar to the observations that BDQ and Q203 can attenuate the bactericidal activity of INH, EMB and ETH by preventing a drug-induced lethal ATP burst (Shetty and Dick, 2018; Lee et al., 2019; Zeng et al., 2021). Given the absence of specific SDH inhibitors, it remains to be determined which of these interactions are observed at the chemical level.

Inhibition of the two malate oxidizing enzymes, MDH and MQO, using CRISPRi synergizes to significantly impair the growth of *M. tuberculosis* compared to the knockdown of either gene alone (Harold et al., 2022). Interactions beyond this have yet to be investigated. However, it is possible that MDH or MQO inhibitors would synergize with NDH inhibitors as the net reaction of MQO and MDH acting in concert is the same as the NADH dehydrogenase reaction (Blaza et al., 2017), suggesting these three enzymes may be functionally redundant (Molenaar et al., 2000). Consistent with this, MDH was shown to be able to compensate for *ndh* mutants in *M. smegmatis* (Miesel

et al., 1998; Vilcheze et al., 2005) and CRISPRi studies showed a weakly additive interaction between the knockdown of *ndh* and *mqo* in *M. smegmatis* (McNeil et al., 2022). However, these interactions require further investigation in *M. tuberculosis*.

Menaquinone biosynthesis inhibitor interactions

Several studies have demonstrated that chemical or genetic inhibition of menaquinone biosynthesis sensitizes mycobacteria to further inhibition of various other respiratory complexes (Sukheja et al., 2017; Berube et al., 2019; McNeil et al., 2022). Specifically, inhibition of MenA, which catalyzes the penultimate step in menaquinone biosynthesis, synergized with sub-bactericidal concentrations of BDQ, CFZ and the cytochrome *bcc-aa*$_3$ inhibitor, ND-10885, resulting in complete sterilization of *M. tuberculosis* cultures within 14 - 21 days of treatment (Berube et al., 2019). Likewise, inhibition of the terminal biosynthetic enzyme, MenG, with the biphenyl amide DG70 also synergized with BDQ, again sterilizing cultures within 21 days (Sukheja et al., 2017). DG70 displayed further synergy with pretomanid, sterilizing cultures of *M. bovis* BCG within 10 days, as well as having an additive interaction with the NDH-2 inhibitor, THZ (Sukheja et al., 2017).

As previously highlighted, the synergy between inhibition of menaquinone biosynthesis and the cytochrome *bc*$_1$-*aa*$_3$ terminal oxidase was genetically validated (McNeil et al., 2022). Interestingly, CRISPRi did not recreate the synergy between BDQ (*atpE* knockdown) and menaquinone biosynthesis inhibition (*menD* knockdown), and the synthetic lethality between *ndh* knockdown (representing CFZ and THZ interactions) and *menD* was only observed when glycerol was used as the primary carbon source (McNeil et al., 2022). This is likely due to insufficient gene knockdown using CRISPRi or metabolic buffering of residual menaquinone preventing an observable interaction (Donati et al., 2021; McNeil et al., 2022). Nevertheless, disruption of menaquinone biosynthesis appears to be broadly synergistic with other bioenergetic inhibitors in *M. tuberculosis*. Mechanistically, this may be explained by the fact that MenA and MenG inhibitors completely block mycobacterial oxygen consumption (Dhiman et al., 2009; Sukheja et al., 2017) and reduce intracellular ATP levels by > 50%, which may sensitize *M. tuberculosis* to further perturbations of ETC activity, resulting in lethal disruptions to ATP synthesis and PMF generation.

Beyond the respiratory chain, inhibition of MenG with DG70 has also been shown to synergize with RIF, as well as significantly enhancing the activity INH (Sukheja et al., 2017). The dual treatment of log phase cultures of *M. bovis* BCG with INH and DG70 rapidly increased the rate of killing compared to either drug alone and sterilized cultures within 10 days (Sukheja et al., 2017). As previously highlighted, inhibiting respiration by targeting the ATP synthase or cytochrome *bcc-aa*$_3$ terminal oxidase prevents INH killing of *M. tuberculosis* and *M. bovis* BCG (Shetty and Dick, 2018; Lee et al., 2019; Zeng et al., 2021), raising concerns that bioenergetic inhibitors may have unintended antagonistic interactions with conventional TB drugs. However, the potent synergy between DG70 and INH demonstrates that this is not always the case and provides an alternative approach to retaining the benefits of bioenergetic inhibitors (i.e., bactericidal activity under replicating and non-replicating conditions) while avoiding antagonistic interactions with current TB drugs. Defining the mechanisms for these different interactions will allow for further improvements in the design of combination therapies involving bioenergetic inhibitors.

In addition to specific inhibitors of the menaquinone biosynthesis pathway, SQ109, which is undergoing clinical development for the treatment of drug-resistant *M. tuberculosis*, has also reported to target both MenA and MenG (Li et al., 2014), in addition to functioning as an uncoupler and MmpL3 inhibitor (Feng et al., 2015). SQ109 synergizes with INH and RIF *in vitro* and in chronic mice models of infection (Chen et al., 2006; Nikonenko et al., 2007) providing another example of potential synergies that can be achieved by targeting menaquinone biosynthesis. However, given the multiple mechanisms of action of SQ109, it is unclear to what degree inhibition of menaquinone biosynthesis contributes to this synergy.

Overall, menaquinone biosynthesis inhibitors offer a broad range of synergies and avoids antagonistic interactions seen by some bioenergetic inhibitors suggesting they could be efficacious in a broad range of regimens. Particularly promising is the ability to enhance the activity of currently available bioenergetic inhibitors, namely BDQ, pretomanid and terminal oxidase inhibitors Q203.

Interactions with the F_{420}-dependent respiratory inhibitor pretomanid

Pretomanid is a bicyclic nitroimidazole derivative that was discovered when testing 3-substituted bicyclic nitroimidazole-containing compounds for antitubercular activity (Stover et al., 2000; Manjunatha et al., 2006a; Manjunatha et al., 2009). Treatment with pretomanid *in vitro* is bactericidal at sub-micromolar concentrations in *M. tuberculosis* and it shows a narrow spectrum of activity that ranges from low to no activity in non-tubercular *Mycobacteria* (Stover et al., 2000; Manjunatha et al., 2006a; Manjunatha et al., 2006b; Manjunatha et al., 2009; Zheng et al., 2022). Whilst not a strict bioenergetic inhibitor, pretomanid kills both replicating as well as hypoxic non-

replicating bacilli by targeting both energy production and cell wall synthesis of *M. tuberculosis* (Singh et al., 2008; Manjunatha et al., 2009). Catalysis of pretomanid from a prodrug to an active form is carried out by the F_{420}-dependent nitroreductase Ddn (Manjunatha et al., 2006a; Manjunatha et al., 2009; Lee B. M. et al., 2020). Ddn is suggested to protect against oxidative stress by reduction of quinones to dihydroquinones, which avoids the cytotoxic effects of semiquinones (Gurumurthy et al., 2012; Gurumurthy et al., 2013). Resistance to pretomanid is through mutations in genes essential for F_{420} synthesis or recycling pathways including *ddn*, *fgd1*, or the *fbi* gene products (Lee B. M. et al., 2020; Gomez-Gonzalez et al., 2021), all of which are non-essential under replicating conditions (Griffin et al., 2011). No cross-resistance to bicyclic nitroimidazole has been observed with any other class of anti-tubercular drug (Stover et al., 2000; Matsumoto et al., 2006).

The bactericidal activity of pretomanid is driven by the respiratory poisoning and inhibition of mycolic acid synthesis (Stover et al., 2000; Singh et al., 2008). Transcriptional profiling of pretomanid-treated *M. tuberculosis* under aerobic conditions showed dysregulation of genes controlling cell wall synthesis such as the *iniBAC* operon, as well as genes that respond to respiratory poisoning such as the *cydABDC* operon (Boshoff et al., 2004). This respiratory poisoning has differing effects on replicating and non-replicating cells: non-replicating cells under hypoxic conditions are unable to exit dormancy and replicating cells are unable to synthesize the cell wall. Similar mechanisms of killing of pretomanid have been observed in studies of the FDA approved drug delamanid that is also a bicyclic nitroimidazole (Matsumoto et al., 2006; Xavier and Lakshmanan, 2014). Metabolomics of pretomanid-treated *M. smegmatis* showed the additional accumulation of phosphate sugars, consistent with reduced FGD1 activity in the pentose-phosphate pathway, leading to cell arrest through accumulation of the toxic metabolite methylglyoxal that modifies peptides and DNA (Murata-Kamiya and Kamiya, 2001; Thornalley et al., 2010; Baptista et al., 2018). This multi-target phenotype of pretomanid suggests that it has the potential to synergize with bioenergetic inhibitors in addition to inhibitors that target cell wall synthesis.

Pretomanid was FDA approved in August 2019 as part of the BPaL regime, an all oral treatment for extensively drug-resistant or treatment non-responsive multi-drug resistant tuberculosis (Keam, 2019). BPaL consists of BDQ, PMD, and linezolid, and in clinical trials showed a favorable results, with 90% of patients achieving a culture negative status (Conradie et al., 2020). The positive results of this combination suggests that pretomanid has positive interactions with either BDQ or linezolid. Potential synergy between BDQ and pretomanid has been identified in part through pretomanid-led repression of the transcription factor Rv0880 that activates a tolerance response and leads to survival against BDQ (Peterson et al., 2016). Multiple links between pretomanid-induced disruption of the stress response appear to underpin further synergistic interactions. This includes pretomanid-mediated reduction in the expression of dormancy genes, contributing to a reduced oxygen consumption and ATP levels of the bacilli leading to a synergistic interaction between the Cyt-bc1aa3 inhibitor TB47 (Zeng et al., 2021). The biphenyl amide DG70 that targets MenG required for menaquinone biosynthesis also showed enhanced bactericidal activity with pretomanid against *in vitro* cultures of *M. tuberculosis* (Sukheja et al., 2017).

Synergistic drug interactions with pretomanid have also been observed in mice with addition of PZA or moxifloxacin to combination of BDQ plus pretomanid showing reduced time to relapse compared to BDQ + pretomanid alone, and the combination of all four drugs showed a further decrease in relapse (Tasneen et al., 2011; Li et al., 2017). The addition of linezolid to BDQ plus pretomanid treatment of *M. tuberculosis* HN878 in mice showed lower efficacy compared to BDQ plus pretomanid alone but greater efficacy for the treatment of *M. tuberculosis* H37Rv. This highlights that differences between geographic lineages of *M. tuberculosis* may confound the efficacy of not only individual drugs but unique drug combinations (Bigelow et al., 2020).

Review Conclusion

Inhibitors of mycobacterial bioenergetics have been shown to have significant clinical potential, in combating both the spread of drug resistance and in reducing treatment times for drug susceptible strains of *M. tuberculosis*. Much of this potential relies on the ability of metabolic inhibitors to disrupt mycobacterial metabolism and potentiate the activity of other anti-tuberculosis agents, in particular other inhibitors of mycobacterial bioenergetics or metabolism. Achieving the full clinical potential of bioenergetic combinations requires a thorough understanding of the consequences of inhibiting specific bioenergetic nodes, both in isolation and in combination with other metabolic dysregulation. Whilst significant insights have been gained from studying the effects of on-target chemical inhibitors, many potential drug targets lack bona-fide chemical inhibitors. Genetic tools to deplete essential genes at a transcriptional and/or a translation level have filled this gap and provided significant insights into the consequences of inhibiting mycobacterial bioenergetics as well as the potential for highly efficacious combination therapies. The continued advancement and application of the mycobacterial genetics should allow for the continued investigation and prioritization of drug targets and unique combination therapies centered around bioenergetic inhibitors. There is also a need for an improved understanding of the mechanisms that provide resistance to bioenergetic inhibitors, including those in pre-existing clinical isolates, as they will allow for the development

of companion diagnostic tools to further the clinical efficacy and lifespan of these novel agents and combinations.

Author contributions

All authors contributed to the writing and editing of this review.

Funding

This work was supported by the Joint Research Health Research Council of New Zealand (HRC)-NSFC Collaboration grant numbers 20/1211 (to GC), 82061128001 (to TZ), and partially by the Chinese Academy of Sciences (154144KYSB20190005, YJKYYQ20210026). This work was also supported by Joint funding from the Maurice Wilkins Centre and the Chinese Academy of Sciences (awarded to MM, GC, and ZL). The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Abrahams, K. A., Cox, J. A. G., Spivey, V. L., Loman, N. J., Pallen, M. J., Constantinidou, C., et al. (2012). Identification of novel imidazo[1,2-a]pyridine inhibitors targeting m. tuberculosis QcrB. *PloS One* 7 (12), e52951. doi: 10.1371/journal.pone.0052951.

Adolph, C., McNeil, M. B., and Cook, G. M. (2022). Impaired succinate oxidation prevents growth and influences drug susceptibility in *Mycobacterium tuberculosis*. *mBio*, e0167222. doi: 10.1128/mbio.01672-22

Andries, K., Verhasselt, P., Guillemont, J., Gohlmann, H. W., Neefs, J. M., Winkler, H., et al. (2005). A diarylquinoline drug active on the ATP synthase of mycobacterium tuberculosis. *Science* 307, 223–227. doi: 10.1126/science.1106753

Andries, K., Villellas, C., Coeck, N., Thys, K., Gevers, T., Vranckx, L., et al. (2014). Acquired resistance of mycobacterium tuberculosis to bedaquiline. *PloS One* 9 (7), e102135. doi: 10.1371/journal.pone.0102135

Arora, K., Ochoa-Montano, B., Tsang, P. S., Blundell, T. L., Dawes, S. S., Mizrahi, V., et al. (2014). Respiratory flexibility in response to inhibition of cytochrome c oxidase in mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 58 (11), 6962–6965. doi: 10.1128/AAC.03486-14

Aung, H. L., Berney, M., and Cook, G. M. (2014). Hypoxia-activated cytochrome bd expression in mycobacterium smegmatis is cyclic AMP receptor protein dependent. *J. Bacteriol.* 196 (17), 3091–3097. doi: 10.1128/JB.01771-14

Bajeli, S., Baid, N., Kaur, M., Pawar, G. P., Chaudhari, V. D., and Kumar, A. (2020). Terminal respiratory oxidases: A targetables vulnerability of mycobacterial bioenergetics? *Front. Cell Infect. Microbiol.* 10. doi: 10.3389/fcimb.2020.589318

Baptista, R., Fazakerley, D. M., Beckmann, M., Baillie, L., and Mur, L. A. J. (2018). Untargeted metabolomics reveals a new mode of action of pretomanid (PA-824). *Sci. Rep.* 8 (1), 5084. doi: 10.1038/s41598-018-23110-1

Bashiri, G., Nigon, L. V., Jirgis, E. N. M., Ho, N. A. T., Stanborough, T., Dawes, S. S., et al. (2020). Allosteric regulation of menaquinone (vitamin K2) biosynthesis in the human pathogen mycobacterium tuberculosis. *J. Biol. Chem.* 295 (12), 3759–3770. doi: 10.1074/jbc.RA119.012158

Beites, T., O'Brien, K., Tiwari, D., Engelhart, C. A., Walters, S., Andrews, J., et al. (2019). Plasticity of the mycobacterium tuberculosis respiratory chain and its impact on tuberculosis drug development. *Nat. Commun.* 10 (1), 4970. doi: 10.1038/s41467-019-12956-2

Berney, M., and Cook, G. M. (2010). Unique flexibility in energy metabolism allows mycobacteria to combat starvation and hypoxia. *PloS One* 5 (1), e8614. doi: 10.1371/journal.pone.0008614

Berney, M., Hartman, T. E., and Jacobs, W. R.Jr. (2014). A mycobacterium tuberculosis cytochrome bd oxidase mutant is hypersensitive to bedaquiline. *MBio* 5 (4), e01275–e01214. doi: 10.1128/mBio.01275-14

Berube, B. J., and Parish, T. (2018). Combinations of respiratory chain inhibitors have enhanced bactericidal activity against mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 62 (1), e01677-17. doi: 10.1128/AAC.01677-17

Berube, B. J., Russell, D., Castro, L., Choi, S. R., Narayanasamy, P., and Parish, T. (2019). Novel MenA inhibitors are bactericidal against mycobacterium tuberculosis and synergize with electron transport chain inhibitors. *Antimicrob. Agents Chemother.* 63 (6), e02661-18. doi: 10.1128/AAC.02661-18

Bigelow, K. M., Tasneen, R., Chang, Y. S., Dooley, K. E., and Nuermberger, E. L. (2020). Preserved efficacy and reduced toxicity with intermittent linezolid dosing in combination with bedaquiline and pretomanid in a murine tuberculosis model. *Antimicrob. Agents Chemother.* 64 (10), e01178-20. doi: 10.1128/AAC.01178-20

Blaza, J. N., Bridges, H. R., Aragao, D., Dunn, E. A., Heikal, A., Cook, G. M., et al. (2017). The mechanism of catalysis by type-II NADH: Quinone oxidoreductases. *Sci. Rep.* 7, 40165. doi: 10.1038/srep40165

Borisov, V. B., Murali, R., Verkhovskaya, M. L., Bloch, D. A., Han, H., Gennis, R. B., et al. (2011). Aerobic respiratory chain of escherichia coli is not allowed to work in fully uncoupled mode. *Proc. Natl. Acad. Sci. U.S.A.* 108 (42), 17320–17324. doi: 10.1073/pnas.1108217108

Borisov, V. B., Siletsky, S. A., Paiardini, A., Hoogewijs, D., Forte, E., Giuffre, A., et al. (2021). Bacterial oxidases of the cytochrome bd family: Redox enzymes of unique structure, function, and utility as drug targets. *Antioxid. Redox Signal* 34 (16), 1280–1318. doi: 10.1089/ars.2020.8039

Bosch, B., DeJesus, M. A., Poulton, N. C., Zhang, W., Engelhart, C. A., Zaveri, A., et al. (2021). Genome-wide gene expression tuning reveals diverse vulnerabilities of m. *Tuberc. Cell* 184 (17), 4579–4592 e4524. doi: 10.1016/j.cell.2021.06.033

Boshoff, H. I., Myers, T. G., Copp, B. R., McNeil, M. R., Wilson, M. A., and Barry, C. (2004). The transcriptional responses of mycobacterium tuberculosis to inhibitors of metabolism: Novel insights into drug mechanisms of action. *J. Biol. Chem* 279 (38), 40174–84. doi: 10.1074/jbc.M406796200

Cai, Y., Jaecklein, E., Mackenzie, J. S., Papavinasasundaram, K., Olive, A. J., Chen, X., et al. (2021). Host immunity increases mycobacterium tuberculosis reliance on cytochrome bd oxidase. *PloS Pathog.* 17 (7), e1008911. doi: 10.1371/journal.ppat.1008911

Chen, P., Gearhart, J., Protopopova, M., Einck, L., and Nacy, C. A. (2006). Synergistic interactions of SQ109, a new ethylene diamine, with front-line antitubercular drugs *in vitro*. *J. Antimicrob. Chemother.* 58 (2), 332–337. doi: 10.1093/jac/dkl227

Choi, P. J., Lu, G. L., Sutherland, H. S., Giddens, A. C., Franzblau, S. G., Cooper, C. B., et al. (2022). Synthetic studies towards isomeric pyrazolopyrimidines as potential ATP synthesis inhibitors of mycobacterium tuberculosis. structural correction of reported n-(6-(2-(dimethylamino)ethoxy)-5-fluoropyridin-3-yl)-2-

(4-fluorophenyl)-5-(triflu oromethyl)pyrazolo[1,5-alpha]pyrimidin-7-amine. *Tetrahedron Lett.* 90. doi: 10.1016/j.tetlet.2021.153611

Chong, S. M. S., Manimekalai, M. S. S., Sarathy, J. P., Williams, Z. C., Harold, L. K., Cook, G. M., et al. (2020). Antituberculosis activity of the antimalaria cytochrome bcc oxidase inhibitor SCR0911. *ACS Infect. Dis.* 6 (4), 725–737. doi: 10.1021/acsinfecdis.9b00408

Cohen, J. (2013). Infectious disease. approval of novel TB drug celebrated–with restraint. *Science* 339 (6116), 130. doi: 10.1126/science.339.6116.130

Conradie, F., Diacon, A. H., Ngubane, N., Howell, P., Everitt, D., Crook, A. M., et al. (2020). Treatment of highly drug-resistant pulmonary tuberculosis. *N. Engl. J. Med.* 382 (10), 893–902. doi: 10.1056/NEJMoa1901814

Cook, G. M., Greening, C., Hards, K., and Berney, M. (2014). Energetics of pathogenic bacteria and opportunities for drug development. *Adv. Microb. Physiol.* 65, 1–62. doi: 10.1016/bs.ampbs.2014.08.001

Cook, G. M., Hards, K., Dunn, E., Heikal, A., Nakatani, Y., Greening, C., et al. (2017). Oxidative phosphorylation as a target space for tuberculosis: Success, caution, and future directions. *Microbiol. Spectr.* 5 (3), TBTB2-0014-2016. doi: 10.1128/microbiolspec.TBTB2-0014-2016

Crowle, A. J., Douvas, G. S., and May, M. H. (1992). Chlorpromazine: A drug potentially useful for treating mycobacterial infections. *Chemotherapy* 38 (6), 410–9. doi: 10.1159/000239036

Debnath, J., Siricilla, S., Wan, B., Crick, D. C., Lenaerts, A. J., Franzblau, S. G., et al. (2012). Discovery of selective menaquinone biosynthesis inhibitors against mycobacterium tuberculosis. *J. Med. Chem.* 55 (8), 3739–3755. doi: 10.1021/jm201608g

Denny, W. A. (2021). Inhibitors of F1F0-ATP synthase enzymes for the treatment of tuberculosis and cancer. *Future Med. Chem.* 13 (10), 911–926. doi: 10.4155/fmc-2021-0010

Dhiman, R. K., Mahapatra, S., Slayden, R. A., Boyne, M. E., Lenaerts, A., Hinshaw, J. C., et al. (2009). Menaquinone synthesis is critical for maintaining mycobacterial viability during exponential growth and recovery from non-replicating persistence. *Mol. Microbiol.* 72 (1), 85–97. doi: 10.1111/j.1365-2958.2009.06625.x

Diacon, A. H., Donald, P. R., Pym, A., Grobusch, M., Patientia, R. F., Mahanyele, R., et al. (2012). Randomized pilot trial of eight weeks of bedaquiline (TMC207) treatment for multidrug-resistant tuberculosis: Long-term outcome, tolerability, and effect on emergence of drug resistance. *Antimicrob. Agents Chemother.* 56 (6), 3271–3276. doi: 10.1128/AAC.06126-11

Diacon, A. H., Pym, A., Grobusch, M. P., de los Rios, J. M., Gotuzzo, E., Vasilyeva, I., et al. (2014). Multidrug-resistant tuberculosis and culture conversion with bedaquiline. *N. Engl. J. Med.* 371 (8), 723–732. doi: 10.1056/NEJMoa1313865

Donati, S., Kuntz, M., Pahl, V., Farke, N., Beuter, D., Glatter, T., et al. (2021). Multi-omics analysis of CRISPRi-knockdowns identifies mechanisms that buffer decreases of enzymes in e. coli metabolism. *Cell Syst.* 12 (1), 56–67 e56. doi: 10.1016/j.cels.2020.10.011

Dunn, E. A., Roxburgh, M., Larsen, L., Smith, R. A., McLellan, A. D., Heikal, A., et al. (2014). Incorporation of triphenylphosphonium functionality improves the inhibitory properties of phenothiazine derivatives in mycobacterium tuberculosis. *Bioorganic Medicinal Chem.* 22 (19), 5320–5328. doi: 10.1016/j.bmc.2014.07.050

Eoh, H., and Rhee, K. Y. (2013). Multifunctional essentiality of succinate metabolism in adaptation to hypoxia in mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. U.S.A.* 110 (16), 6554–6559. doi: 10.1073/pnas.1219375110

Fang, M., Toogood, R. D., Macova, A., Ho, K., Franzblau, S. G., McNeil, M. R., et al. (2010). Succinylphosphonate esters are competitive inhibitors of MenD that show active-site discrimination between homologous alpha-ketoglutarate-decarboxylating enzymes. *Biochemistry* 49 (12), 2672–2679. doi: 10.1021/bi901432d

Feng, X., Zhu, W., Schurig-Briccio, L. A., Lindert, S., Shoen, C., Hitchings, R., et al. (2015). Antiinfectives targeting enzymes and the proton motive force. *Proc. Natl. Acad. Sci. U.S.A.* 112 (51), E7073–E7082. doi: 10.1073/pnas.1521988112

Gao, Y., Hameed, H. M. A., Liu, Y., Guo, L., Fang, C., Tian, X., et al. (2021). Ultra-short-course and intermittent TB47-containing oral regimens produce stable cure against buruli ulcer in a murine model and prevent the emergence of resistance for mycobacterium ulcerans. *Acta Pharm. Sin. B* 11 (3), 738–749. doi: 10.1016/j.apsb.2020.11.007

Gomez-Gonzalez, P. J., Perdigao, J., Gomes, P., Puyen, Z. M., Santos-Lazaro, D., Napier, G., et al. (2021). Genetic diversity of candidate loci linked to mycobacterium tuberculosis resistance to bedaquiline, delamanid and pretomanid. *Sci. Rep.* 11 (1), 19431. doi: 10.1038/s41598-021-98862-4

Gong, H., Gao, Y., Zhou, X., Xiao, Y., Wang, W., Tang, Y., et al. (2020). Cryo-EM structure of trimeric mycobacterium smegmatis succinate dehydrogenase with a membrane-anchor SdhF. *Nat. Commun.* 11 (1), 4245. doi: 10.1038/s41467-020-18011-9

Gong, H., Li, J., Xu, A., Tang, Y., Ji, W., Gao, R., et al. (2018). An electron transfer path connects subunits of a mycobacterial respiratory supercomplex. *Science* 362 (6418). doi: 10.1126/science.aat8923

Griffin, J. E., Gawronski, J. D., Dejesus, M. A., Ioerger, T. R., Akerley, B. J., and Sassetti, C. M. (2011). High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. *PloS Pathog.* 7 (9), e1002251. doi: 10.1371/journal.ppat.1002251

Grosset, J. H., Tyagi, S., Almeida, D. V., Converse, P. J., Li, S. Y., Ammerman, N. C., et al. (2013). Assessment of clofazimine activity in a second-line regimen for tuberculosis in mice. *Am. J. Respir. Crit. Care Med.* 188 (5), 608–612. doi: 10.1164/rccm.201304-0753OC

Guo, H., Courbon, G. M., Bueler, S. A., Mai, J., Liu, J., and Rubinstein, J. L. (2021). Structure of mycobacterial ATP synthase bound to the tuberculosis drug bedaquiline. *Nature* 589 (7840), 143–147. doi: 10.1038/s41586-020-3004-3

Gurumurthy, M., Mukherjee, T., Dowd, C. S., Singh, R., Niyomrattanakit, P., Tay, J. A., et al. (2012). Substrate specificity of the deazaflavin-dependent nitroreductase from mycobacterium tuberculosis responsible for the bioreductive activation of bicyclic nitroimidazoles. *FEBS J.* 279 (1), 113–125. doi: 10.1111/j.1742-4658.2011.08404.x

Gurumurthy, M., Rao, M., Mukherjee, T., Rao, S. P., Boshoff, H. I., Dick, T., et al. (2013). A novel F(420) -dependent anti-oxidant mechanism protects mycobacterium tuberculosis against oxidative stress and bactericidal agents. *Mol. Microbiol.* 87 (4), 744–755. doi: 10.1111/mmi.12127

Harbut, M. B., Liu, R., Yang, B., Yano, T., Vilcheze, C., Lockner, J., et al. (2018). Small molecules targeting mycobacterium tuberculosis type II NADH dehydrogenase with antimycobacterial activity. *Angew Chem. Int. Ed Engl* 57 (13), 3478–3482 doi: 10.1002/anie.201800260

Hards, K., Adolph, C., Harold, L. K., McNeil, M. B., Cheung, C. Y., Jinich, A., et al. (2020). Two for the price of one: Attacking the energetic-metabolic hub of mycobacteria to produce new chemotherapeutic agents. *Prog. Biophys. Mol. Biol.* 152, 35–44. doi: 10.1016/j.pbiomolbio.2019.11.003

Hards, K., Cheung, C. Y., Waller, N., Adolph, C., Keighley, L., Tee, Z. S., et al. (2022). An amiloride derivative is active against the F1Fo-ATP synthase and cytochrome bd oxidase of mycobacterium tuberculosis. *Commun. Biol.* 5 (1), 166. doi: 10.1038/s42003-022-03110-8

Hards, K., and Cook, G. M. (2018). Targeting bacterial energetics to produce new antimicrobials. *Drug Resist. Update* 36, 1–12. doi: 10.1016/j.drup.2017.11.001

Hards, K., McMillan, D. G. G., Schurig-Briccio, L. A., Gennis, R. B., Lill, H., Bald, D., et al. (2018). Ionophoric effects of the antitubercular drug bedaquiline. *Proc. Natl. Acad. Sci. U.S.A.* 115 (28), 7326–7331. doi: 10.1073/pnas.1803723115

Hards, K., Robson, J. R., Berney, M., Shaw, L., Bald, D., Koul, A., et al. (2015). Bactericidal mode of action of bedaquiline. *J. Antimicrob. Chemother.* 70 (7), 2028–2037. doi: 10.1093/jac/dkv054

Hards, K., Rodriguez, S. M., Cairns, C., and Cook, G. M. (2019). Alternate quinone coupling in a new class of succinate dehydrogenase may potentiate mycobacterial respiratory control. *FEBS Lett.* 593 (5), 475–486. doi: 10.1002/1873-3468.13330

Harikishore, A., Chong, S. S. M., Ragunathan, P., Bates, R. W., and Gruber, G. (2020). Targeting the menaquinol binding loop of mycobacterial cytochrome bd oxidase. *Mol. Divers* 25 (1), 517–524. doi: 10.1007/s11030-020-10034-0.

Harold, L. K., Jinich, A., Hards, K., Cordeiro, A., Keighley, L. M., Cross, A., et al. (2022). Deciphering functional redundancy and energetics of malate oxidation in mycobacteria. *J. Biol. Chem.* 298 (5), 101859. doi: 10.1016/j.jbc.2022.101859

Hartkoorn, R. C., Uplekar, S., and Cole, S. T. (2014). Cross-resistance between clofazimine and bedaquiline through upregulation of MmpL5 in mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 58 (5), 2979–2981. doi: 10.1128/AAC.00037-14

Hartman, T., Weinrick, B., Vilcheze, C., Berney, M., Tufariello, J., Cook, G. M., et al. (2014). Succinate dehydrogenase is the regulator of respiration in mycobacterium tuberculosis. *PloS Pathog.* 10 (11), e1004510. doi: 10.1371/journal.ppat.1004510

Hartuti, E. D., Inaoka, D. K., Komatsuya, K., Miyazaki, Y., Miller, R. J., Xinying, W., et al. (2018). Biochemical studies of membrane bound plasmodium falciparum mitochondrial l-malate:quinone oxidoreductase, a potential drug target. *Biochim. Biophys. Acta Bioenerg.* 1859 (3), 191–200. doi: 10.1016/j.bbabio.2017.12.004

Hasenoehrl, E. J., Wiggins, T. J., and Berney, M. (2020). Bioenergetic inhibitors: Antibiotic efficacy and mechanisms of action in mycobacterium tuberculosis. *Front. Cell Infect. Microbiol.* 10. doi: 10.3389/fcimb.2020.611683

Heikal, A., Hards, K., Cheung, C. Y., Menorca, A., Timmer, M. S., Stocker, B. L., et al. (2016). Activation of type II NADH dehydrogenase by quinolinequinones mediates antitubercular cell death. *J. Antimicrob. Chemother.* 71 (10), 2840–2847. doi: 10.1093/jac/dkw244

Heikal, A., Nakatani, Y., Dunn, E., Weimar, M. R., Day, C. L., Baker, E. N., et al. (2014). Structure of the bacterial type II NADH dehydrogenase: A monotopic membrane protein with an essential role in energy generation. *Mol. Microbiol.* 91 (5), 950–964. doi: 10.1111/mmi.12507

Heikal, A., Nakatani, Y., Jiao, W., Wilson, C., Rennison, D., Weimar, M. R., et al. (2018). 'Tethering' fragment-based drug discovery to identify inhibitors of the

essential respiratory membrane protein type II NADH dehydrogenase. *Bioorg. Med. Chem. Lett.* 28 (13), 2239–2243. doi: 10.1016/j.bmcl.2018.05.048

Hotra, A., Ragunathan, P., Ng, P. S., Seankongsuk, P., Harikishore, A., Sarathy, J. P., et al. (2020). Discovery of a novel mycobacterial f-ATP synthase inhibitor and its potency in combination with diarylquinolines. *Angew Chem. Int. Ed Engl* 59 (32), 13295–13304. doi: 10.1002/anie.202002546

Huitric, E., Verhasselt, P., Koul, A., Andries, K., Hoffner, S., and Andersson, D. I. (2010). Rates and mechanisms of resistance development in mycobacterium tuberculosis to a novel diarylquinoline ATP synthase inhibitor. *Antimicrob. Agents Chemother.* 54 (3), 1022–1028. doi: 10.1128/AAC.01611-09

Jang, J., Kim, R., Woo, M., Jeong, J., Park, D. E., Kim, G., et al. (2017). Efflux attenuates the antibacterial activity of Q203 in mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 61 (7), e02637-16. doi: 10.1128/AAC.02637-16

Kalia, N. P., Hasenoehrl, E. J., Ab Rahman, N. B., Koh, V. H., Ang, M. L. T., Sajorda, D. R., et al. (2017). Exploiting the synthetic lethality between terminal respiratory oxidases to kill mycobacterium tuberculosis and clear host infection. *Proc. Natl. Acad. Sci. U.S.A* 114 (28), 7426–7431 doi: 10.1073/pnas.1706139114

Kamariah, N., Ragunathan, P., Shin, J., Saw, W. G., Wong, C. F., Dick, T., et al. (2020). Unique structural and mechanistic properties of mycobacterial f-ATP synthases: Implications for drug design. *Prog. Biophys. Mol. Biol.* 152, 64–73. doi: 10.1016/j.pbiomolbio.2019.11.006

Kana, B. D., Weinstein, E. A., Avarbock, D., Dawes, S. S., Rubin, H., and Mizrahi, V. (2001). Characterization of the cydAB-encoded cytochrome bd oxidase from mycobacterium smegmatis. *J. Bacteriol.* 183 (24), 7076–7086. doi: 10.1128/JB.183.24.7076-7086.2001

Kang, S., Kim, R. Y., Seo, M. J., Lee, S., Kim, Y. M., Seo, M., et al. (2014). Lead optimization of a novel series of Imidazo[1,2-a]pyridine amides leading to a clinical candidate (Q203) as a multi- and extensively-drug resistant antituberculosis agent. *J. Medicinal Chem* 57 (12), 5293–305 doi: 10.1021/jm5003606

Keam, S. J. (2019). Pretomanid: First approval. *Drugs* 79 (16), 1797–1803. doi: 10.1007/s40265-019-01207-9

Kerantzas, C. A., and Jacobs, W. R.Jr (2017). Origins of combination therapy for tuberculosis: Lessons for future antimicrobial development and application. *mBio* 8 (2), e01586–e01516. doi: 10.1128/mBio.01586-16

Koh, E. I., Oluoch, P. O., Ruecker, N., Proulx, M. K., Soni, V., Murphy, K. C., et al. (2022). Chemical-genetic interaction mapping links carbon metabolism and cell wall structure to tuberculosis drug efficacy. *Proc. Natl. Acad. Sci. U.S.A.* 119 (15), e2201632119. doi: 10.1073/pnas.2201632119

Koul, A., Dendouga, N., Vergauwen, K., Molenberghs, B., Vranckx, L., Willebrords, R., et al. (2007). Diarylquinolines target subunit c of mycobacterial ATP synthase. *Nat. Chem. Biol.* 3 (6), 323–324. doi: 10.1038/nchembio884

Koul, A., Vranckx, L., Dendouga, N., Balemans, W., Van den Wyngaert, I., Vergauwen, K., et al. (2008). Diarylquinolines are bactericidal for dormant mycobacteria as a result of disturbed ATP homeostasis. *J. Biol. Chem.* 283 (37), 25273–25280. doi: 10.1074/jbc.M803899200

Koul, A., Vranckx, L., Dhar, N., Gohlmann, H. W., Ozdemir, E., Neefs, J. M., et al. (2014). Delayed bactericidal response of mycobacterium tuberculosis to bedaquiline involves remodelling of bacterial metabolism. *Nat. Commun.* 5, 3369. doi: 10.1038/ncomms4369

Lamprecht, D. A., Finin, P. M., Rahman, M. A., Cumming, B. M., Russell, S. L., Jonnala, S. R., et al. (2016). Turning the respiratory flexibility of mycobacterium tuberculosis against itself. *Nat. Commun.* 7, 12393. doi: 10.1038/ncomms12393

Lange, C., Chesov, D., Heyckendorf, J., Leung, C. C., Udwadia, Z., and Dheda, K. (2018). Drug-resistant tuberculosis: An update on disease burden, diagnosis and treatment. *Respirology* 23 (7), 656–673. doi: 10.1111/resp.13304

Lechartier, B., Hartkoorn, R. C., and Cole, S. T. (2012). *In vitro* combination studies of benzothiazinone lead compound BTZ043 against mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 56 (11), 5790–5793. doi: 10.1128/AAC.01476-12

Lee, B. S., Hards, K., Engelhart, C. A., Hasenoehrl, E. J., Kalia, N. P., Mackenzie, J. S., et al. (2021). Dual inhibition of the terminal oxidases eradicates antibiotic-tolerant mycobacterium tuberculosis. *EMBO Mol. Med.* 13 (1), e13207. doi: 10.15252/emmm.202013207

Lee, B. M., Harold, L. K., Almeida, D. V., Afriat-Jurnou, L., Aung, H. L., Forde, B. M., et al. (2020). Predicting nitroimidazole antibiotic resistance mutations in mycobacterium tuberculosis with protein engineering. *PloS Pathog.* 16 (2), e1008287. doi: 10.1371/journal.ppat.1008287

Lee, B. S., Kalia, N. P., Jin, X. E. F., Hasenoehrl, E. J., Berney, M., and Pethe, K. (2019). Inhibitors of energy metabolism interfere with antibiotic-induced death in mycobacteria. *J. Biol. Chem.* 294 (6), 1936–1943. doi: 10.1074/jbc.RA118.005732

Lee, B. S., Sviriaeva, E., and Pethe, K. (2020). Targeting the cytochrome oxidases for drug development in mycobacteria. *Prog. Biophys. Mol. Biol.* 152, 45–54. doi: 10.1016/j.pbiomolbio.2020.02.001

Lee, A. S., Teo, A. S., and Wong, S. Y. (2001). Novel mutations in ndh in isoniazid-resistant mycobacterium tuberculosis isolates. *Antimicrob. Agents Chemother* 45 (7), 2157–2159. doi: 10.1128/AAC.45.7.2157-2159.2001

Li, L., Koirala, B., Hernandez, Y., MacIntyre, L. W., Ternei, M. A., Russo, R., et al. (2022). Identification of structurally diverse menaquinone-binding antibiotics with *in vivo* activity against multidrug-resistant pathogens. *Nat. Microbiol.* 7 (1), 120–131. doi: 10.1038/s41564-021-01013-8

Li, K., Schurig-Briccio, L. A., Feng, X., Upadhyay, A., Pujari, V., Lechartier, B., et al. (2014). Multi-target drug discovery for tuberculosis and other infectious diseases. *J. Med. Chem* 57 (7), 3126–3139. doi: 10.1021/jm500131s

Li, S. Y., Tasneen, R., Tyagi, S., Soni, H., Converse, P. J., Mdluli, K., et al. (2017). Bactericidal and sterilizing activity of a novel regimen with bedaquiline, pretomanid, moxifloxacin, and pyrazinamide in a murine model of tuberculosis. *Antimicrob. Agents Chemother.* 61 (9), e00913-17. doi: 10.1128/AAC.00913-17

Liu, Y., Gao, Y., Liu, J., Tan, Y., Liu, Z., Chhotaray, C., et al. (2019). The compound TB47 is highly bactericidal against mycobacterium ulcerans in a buruli ulcer mouse model. *Nat. Commun.* 10 (1), 524. doi: 10.1038/s41467-019-08464-y

Liu, Y., Tan, Y., Islam, M. M., Cao, Y., Lu, X., Zeng, S., et al. (2020). Assessment of clofazimine and TB47 combination activity against mycobacterium abscessus using a bioluminescent approach. *Antimicrob. Agents Chemother.* 64 (3), e01881-19. doi: 10.1128/AAC.01881-19

Lu, P., Asseri, A. H., Kremer, M., Maaskant, J., Ummels, R., Lill, H., et al. (2018). The anti-mycobacterial activity of the cytochrome bcc inhibitor Q203 can be enhanced by small-molecule inhibition of cytochrome bd. *Sci. Rep.* 8 (1), 2625. doi: 10.1038/s41598-018-20989-8

Lu, P., Heineke, M. H., Koul, A., Andries, K., Cook, G. M., Lill, H., et al. (2015). The cytochrome bd-type quinol oxidase is important for survival of mycobacterium smegmatis under peroxide and antibiotic-induced stress. *Sci. Rep.* 5, 10333. doi: 10.1038/srep10333

Lu, X., Williams, Z., Hards, K., Tang, J., Cheung, C.-Y., Aung, H. L., et al. (2019). Pyrazolo [1, 5-a] pyridine inhibitor of the respiratory cytochrome bcc complex for the treatment of drug-resistant tuberculosis. *ACS Infect. Dis.* 5 (2), 239–249. doi: 10.1021/acsinfecdis.8b00225

Mackenzie, J. S., Lamprecht, D. A., Asmal, R., Adamson, J. H., Borah, K., Beste, D. J. V., et al. (2020). Bedaquiline reprograms central metabolism to reveal glycolytic vulnerability in mycobacterium tuberculosis. *Nat. Commun.* 11 (1), 6092. doi: 10.1038/s41467-020-19959-4

Manjunatha, U., Boshoff, H. I., and Barry, C. E. (2009). The mechanism of action of PA-824: Novel insights from transcriptional profiling. *Commun. Integr. Biol.* 2 (3), 215–218. doi: 10.4161/cib.2.3.7926

Manjunatha, U. H., Boshoff, H., Dowd, C. S., Zhang, L., Albert, T. J., Norton, J. E., et al. (2006a). Identification of a nitroimidazo-oxazine-specific protein involved in PA-824 resistance in mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. U.S.A.* 103 (2), 431–436. doi: 10.1073/pnas.0508392103

Manjunatha, U. H., Lahiri, R., Randhawa, B., Dowd, C. S., Krahenbuhl, J. L., and Barry, C. E.3rd (2006b). Mycobacterium leprae is naturally resistant to PA-824. *Antimicrob. Agents Chemother.* 50 (10), 3350–3354. doi: 10.1128/AAC.00488-06

Matsoso, L. G., Kana, B. D., Crellin, P. K., Lea-Smith, D. J., Pelosi, A., Powell, D., et al. (2005). Function of the cytochrome bc1-aa3 branch of the respiratory network in mycobacteria and network adaptation occurring in response to its disruption. *J. Bacteriol.* 187 (18), 6300–6308. doi: 10.1128/JB.187.18.6300-6308.2005

Matsumoto, M., Hashizume, H., Tomishige, T., Kawasaki, M., Tsubouchi, H., Sasaki, H., et al. (2006). OPC-67683, a nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis *in vitro* and in mice. *PloS Med.* 3 (11), e466. doi: 10.1371/journal.pmed.0030466

McNeil, M. B., Keighley, L. M., Cook, J. R., Cheung, C. Y., and Cook, G. M. (2021). CRISPR interference identifies vulnerable cellular pathways with bactericidal phenotypes in mycobacterium tuberculosis. *Mol. Microbiol.* 116 (4), 1033–1043. doi: 10.1111/mmi.14790

McNeil, M. B., Ryburn, H. W. K., Harold, L. K., Tirados, J. F., and Cook, G. M. (2020). Transcriptional inhibition of the F1F0-type ATP synthase has bactericidal consequences on the viability of mycobacteria. *Antimicrob. Agents Chemother.* 64 (8), e00492-20. doi: 10.1128/AAC.00492-20

McNeil, M. B., Ryburn, H. W., Tirados, J., Cheung, C. Y., and Cook, G. M. (2022). Multiplexed transcriptional repression identifies a network of bactericidal interactions between mycobacterial respiratory complexes. *iScience* 25 (1), 103573. doi: 10.1016/j.isci.2021.103573

Miesel, L., Weisbrod, T. R., Marcinkeviciene, J. A., Bittman, R., and Jacobs, W. R. (1998). NADH dehydrogenase defects confer isoniazid resistance and conditional lethality in mycobacterium smegmatis. *J. Bacteriol.* 80 (9), 2459–2467. doi: 10.1128/JB.180.9.2459-2467.1998

Molenaar, D., van der Rest, M. E., Drysch, A., and Yucel, R. (2000). Functions of the membrane-associated and cytoplasmic malate dehydrogenases in the citric acid cycle of corynebacterium glutamicum. *J. Bacteriol.* 182 (24), 6884–6891. doi: 10.1128/JB.182.24.6884-6891.2000

Montgomery, M. G., Petri, J., Spikes, T. E., and Walker, J. E. (2021). Structure of the ATP synthase from mycobacterium smegmatis provides targets for treating tuberculosis. *Proc. Natl. Acad. Sci. U.S.A.* 118 (47), e2111899118. doi: 10.1073/pnas.2111899118

Moodliar, R., Aksenova, V., Frias, M. V. G., van de Logt, J., Rossenu, S., Birmingham, E., et al. (2021). Bedaquiline for multidrug-resistant TB in paediatric patients. *Int. J. Tuberc. Lung Dis.* 25 (9), 716–724. doi: 10.5588/ijtld.21.0022

Moosa, A., Lamprecht, D. A., Arora, K., Barry, C. E.3rd, Boshoff, H. I. M., Ioerger, T. R., et al. (2017). Susceptibility of mycobacterium tuberculosis cytochrome bd oxidase mutants to compounds targeting the terminal respiratory oxidase, cytochrome c. *Antimicrob. Agents Chemother.* 61 (10), e01338-17. doi: 10.1128/AAC.01338-17

Murata-Kamiya, N., and Kamiya, H. (2001). Methylglyoxal, an endogenous aldehyde, crosslinks DNA polymerase and the substrate DNA. *Nucleic Acids Res.* 29 (16), 3433–3438. doi: 10.1093/nar/29.16.3433

Narang, R., Kumar, R., Kalra, S., Nayak, S. K., Khatik, G. L., Kumar, G. N., et al. (2019). Recent advancements in mechanistic studies and structure activity relationship of FoF1 ATP synthase inhibitor as antimicrobial agent. *Eur. J. Med. Chem.* 182, 111644. doi: 10.1016/j.ejmech.2019.111644

Nikonenko, B. V., Protopopova, M., Samala, R., Einck, L., and Nacy, C. A. (2007). Drug therapy of experimental tuberculosis (TB): Improved outcome by combining SQ109, a new diamine antibiotic, with existing TB drugs. *Antimicrob. Agents Chemother.* 51 (4), 1563–1565. doi: 10.1128/AAC.01326-06

Ocampo, P. S., Lazar, V., Papp, B., Arnoldini, M., Abel zur Wiesch, P., Busa-Fekete, R., et al. (2014). Antagonism between bacteriostatic and bactericidal antibiotics is prevalent. *Antimicrob. Agents Chemother.* 58 (8), 4573–4582. doi: 10.1128/AAC.02463-14

Pecsi, I., Hards, K., Ekanayaka, N., Berney, M., Hartman, T., Jacobs, W. R.Jr., et al. (2014). Essentiality of succinate dehydrogenase in mycobacterium smegmatis and its role in the generation of the membrane potential under hypoxia. *MBio* 5 (4), e01093-14. doi: 10.1128/mBio.01093-14

Peterson, E. J. R., Ma, S., Sherman, D. R., and Baliga, N. S. (2016). Network analysis identifies Rv0324 and Rv0880 as regulators of bedaquiline tolerance in mycobacterium tuberculosis. *Nat. Microbiol.* 1 (8), 16078. doi: 10.1038/nmicrobiol.2016.78

Pethe, K., Bifani, P., Jang, J., Kang, S., Park, S., Ahn, S., et al. (2013). Discovery of Q203, a potent clinical candidate for the treatment of tuberculosis. *Nat. Med.* 19 (9), 1157–1160. doi: 10.1038/nm.3262

Petri, J., Shimaki, Y., Jiao, W., Bridges, H. R., Russell, E. R., Parker, E. J., et al. (2018). Structure of the NDH-2 - HQNO inhibited complex provides molecular insight into quinone-binding site inhibitors. *Biochim. Biophys. Acta Bioenerg.* 1859 (7), 482–490. doi: 10.1016/j.bbabio.2018.03.014

Preiss, L., Langer, J. D., Yildiz, O., Eckhardt-Strelau, L., Guillemont, J. E., Koul, A., et al. (2015). Structure of the mycobacterial ATP synthase fo rotor ring in complex with the anti-TB drug bedaquiline. *Sci. Adv.* 1 (4), e1500106. doi: 10.1126/sciadv.1500106

Rao, S. P., Alonso, S., Rand, L., Dick, T., and Pethe, K. (2008). The protonmotive force is required for maintaining ATP homeostasis and viability of hypoxic, nonreplicating *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 105 (33), 11945–11950. doi: 10.1073/pnas.0711697105

Rhee, K. Y., Carvalho, L. P., Bryk, R., Ehrt, S., Marrero, J., Park, S. W., et al. (2011). Central carbon metabolism in mycobacterium tuberculosis: a unexpected frontier. *Trends Microbiol* 19 (7), 307–314 doi: 10.1016/j.tim.2011.03.008

Rittershaus, E. S. C., Baek, S. H., Krieger, I. V., Nelson, S. J., Cheng, Y. S., Nambi, S., et al. (2018). A lysine acetyltransferase contributes to the metabolic adaptation to hypoxia in *Mycobacterium tuberculosis*. *Cell Chem. Biol.* 25 (12), 1495–1505 e1493. doi: 10.1016/j.chembiol.2018.09.009

Safarian, S., Opel-Reading, H. K., Wu, D., Mehdipour, A. R., Hards, K., Harold, L. K., et al. (2021). The cryo-EM structure of the bd oxidase from m. tuberculosis reveals a unique structural framework and enables rational drug design to combat TB. *Nat. Commun.* 12 (1), 5236. doi: 10.1038/s41467-021-25537-z

Scherr, N., Bieri, R., Thomas, S. S., Chauffour, A., Kalia, N. P., Schneide, P., et al. (2018). Targeting the mycobacterium ulcerans cytochrome bc1:aa3 for the treatment of buruli ulcer. *Nat. Commun.* 9 (1), 5370. doi: 10.1038/s41467-018-07804-8

Shetty, A., and Dick, T. (2018). Mycobacterial cell wall synthesis inhibitors cause lethal ATP burst. *Front. Microbiol.* 9. doi: 10.3389/fmicb.2018.01898

Shirude, P. S., Paul, P., Choudhury, N. R., Kedari, C., Bandodkar, B., and Ugarkar, B. G. (2012). Quinolinyl pyrimidines: Potent inhibitors of NDH-2 as a novel class of anti-TB agents. *ACS Medicinal Chem. Lett.* 3 (9), 736–740. doi: 10.1021/ml300134b

Shi, L., Sohaskey, C. D., Kana, B. D., Dawes, S., North, R. J., Mizrahi, V., et al. (2005). Changes in energy metabolism of mycobacterium tuberculosis in mouse lung and under *in vitro* conditions affecting aerobic respiration. *Proc. Natl. Acad. Sci. U.S.A.* 102 (43), 15629–15634. doi: 10.1073/pnas.0507850102

Singh, R., Manjunatha, U., Boshoff, H. I., Ha, Y. H., Niyomrattanakit, P., Ledwidge, R., et al. (2008). PA-824 kills nonreplicating mycobacterium tuberculosis by intracellular NO release. *Science* 322 (5906), 1392–1395. doi: 10.1126/science.1164571

Singh, S., Roy, K. K., Khan, S. R., Kashyap, V. K., Sharma, A., Jaiswal, S., et al. (2015). Novel, potent, orally bioavailable and selective mycobacterial ATP synthase inhibitors that demonstrated activity against both replicating and non-replicating m. tuberculosis. *Bioorg. Med. Chem.* 23 (4), 742–752. doi: 10.1016/j.bmc.2014.12.060

Sorayah, R., Manimekalai, M. S. S., Shin, S. J., Koh, W. J., Gruber, G., and Pethe, K. (2019). Naturally-occurring polymorphisms in QcrB are responsible for resistance to telacebec in mycobacterium abscessus. *ACS Infect. Dis.* 5 (12), 2055–2060. doi: 10.1021/acsinfecdis.9b00322

Stover, C. K., Warrener, P., VanDevanter, D. R., Sherman, D. R., Arain, T. M., Langhorne, M. H., et al. (2000). A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. *Nature* 405 (6789), 962–966. doi: 10.1038/35016103

Sukheja, P., Kumar, P., Mittal, N., Li, S. G., Singleton, E., Russo, R., et al. (2017). A novel small-molecule inhibitor of the mycobacterium tuberculosis demethylmenaquinone methyltransferase MenG is bactericidal to both growing and nutritionally deprived persister cells. *MBio* 8 (1), e02022-16. doi: 10.1128/mBio.02022-16

Sviriaeva, E., Subramanian Manimekalai, M. S., Gruber, G., and Pethe, K. (2020). Features and functional importance of key residues of the mycobacterium tuberculosis cytochrome bd oxidase. *ACS Infect. Dis* 6 (7), 1697–1707 doi: 10.1021/acsinfecdis.9b00449

Tantry, S. J., Markad, S. D., Shinde, V., Bhat, J., Balakrishnan, G., Gupta, A. K., et al. (2017). Discovery of Imidazo[1,2-a]pyridine ethers and squaramides as selective and potent inhibitors of mycobacterial adenosine triphosphate (ATP) synthesis. *J. Med. Chem.* 60 (4), 1379–1399. doi: 10.1021/acs.jmedchem.6b01358

Tasneen, R., Li, S. Y., Peloquin, C. A., Taylor, D., Williams, K. N., Andries, K., et al. (2011). Sterilizing activity of novel TMC207- and PA-824-containing regimens in a murine model of tuberculosis. *Antimicrob. Agents Chemother.* 55 (12), 5485–5492. doi: 10.1128/AAC.05293-11

Thornalley, P. J., Waris, S., Fleming, T., Santarius, T., Larkin, S. J., Winklhofer-Roob, B. M., et al. (2010). Imidazopurinones are markers of physiological genomic damage linked to DNA instability and glyoxalase 1-associated tumour multidrug resistance. *Nucleic Acids Res.* 38 (16), 5432–5442. doi: 10.1093/nar/gkq306

Tran, S. L., and Cook, G. M. (2005). The F1Fo-ATP synthase of *Mycobacterium smegmatis* is essential for growth. *J. Bacteriol.* 187 (14), 5023–5028. doi: 10.1128/JB.187.14.5023-5028.2005

Vilcheze, C., Weinrick, B., Leung, L. W., and Jacobs, W. R.Jr. (2018). Plasticity of mycobacterium tuberculosis NADH dehydrogenases and their role in virulence. *Proc. Natl. Acad. Sci. U. S. A.* 115 (7), 1599–1604 doi: 10.1073/pnas.1721545115

Vilcheze, C., Weisbrod, T. R., Chen, B., Kremer, L., Hazbon, M. H., Wang, F., et al. (2005). Altered NADH/NAD+ ratio mediates coresistance to isoniazid and ethionamide in mycobacteria. *Antimicrob. Agents Chemother.* 49 (2), 708–720. doi: 10.1128/AAC.49.2.708-720.2005

Villellas, C., Coeck, N., Meehan, C. J., Lounis, N., de Jong, B., Rigouts, L., et al. (2017). Unexpected high prevalence of resistance-associated Rv0678 variants in MDR-TB patients without documented prior use of clofazimine or bedaquiline. *J. Antimicrob. Chemother.* 72 (3), 684–690. doi: 10.1093/jac/dkw502

Viveiros, M., and Amaral, L. (2001). Enhancement of antibiotic activity against poly-drug resistant mycobacterium tuberculosis by phenothiazines. *Int. J. Antimicrob. Agents* 17 (3), 225–228. doi: 10.1016/s0924-8579(00)00343-5

Wang, W., Gao, Y., Tang, Y., Zhou, X., Lai, Y., Zhou, S., et al. (2021). Cryo-EM structure of mycobacterial cytochrome bd reveals two oxygen access channels. *Nat. Commun.* 12 (1), 4621. doi: 10.1038/s41467-021-24924-w

Wang, Z., Soni, V., Marriner, G., Kaneko, T., Boshoff, H. I. M., Barry, C. E.3rd, et al. (2019). Mode-of-action profiling reveals glutamine synthetase as a collateral metabolic vulnerability of m. tuberculosis to bedaquiline. *Proc. Natl. Acad. Sci. U.S.A.* 116 (39), 19646–19651. doi: 10.1073/pnas.1907946116

Watanabe, S., Zimmermann, M., Goodwin, M. B., Sauer, U., Barry, C. E.3rd, and Boshoff, H. I. (2011). Fumarate reductase activity maintains an energized membrane in anaerobic mycobacterium tuberculosis. *PloS Pathog.* 7 (10), e1002287. doi: 10.1371/journal.ppat.1002287

Weinstein, E. A., Yano, T., Li, L. S., Avarbock, D., Avarbock, A., Helm, D., et al. (2005). Inhibitors of type II NADH:menaquinone oxidoreductase represent a class of antitubercular drugs. *Proc. Natl. Acad. Sci. U.S.A.* 102 (12), 4548–4553. doi: 10.1073/pnas.0500469102

WHO (2021). *Global tuberculosis report. licence: CC BY-NC-SA 3.0 IGO*. World Health Organisation.

Wiseman, B., Nitharwal, R. G., Fedotovskaya, O., Schafer, J., Guo, H., Kuang, Q., et al. (2018). Structure of a functional obligate complex III2IV2 respiratory supercomplex from mycobacterium smegmatis. *Nat. Struct. Mol. Biol.* 25 (12), 1128–1136. doi: 10.1038/s41594-018-0160-3

Xavier, A. S., and Lakshmanan, M. (2014). Delamanid: A new armor in combating drug-resistant tuberculosis. *J. Pharmacol. Pharmacother.* 5 (3), 222–224. doi: 10.4103/0976-500X.136121

Yang, X., Yuan, T., Ma, R., Chacko, K. I., Smith, M., Deikus, G., et al. (2019). Mce3R stress-resistance pathway is vulnerable to small-molecule targeting that improves tuberculosis drug activities. *ACS Infect. Dis.* 5 (7), 1239–1251. doi: 10.1021/acsinfecdis.9b00099

Yanofsky, D. J., Di Trani, J. M., Krol, S., Abdelaziz, R., Bueler, S. A., Imming, P., et al. (2021). Structure of mycobacterial CIII2CIV2 respiratory supercomplex bound to the tuberculosis drug candidate telacebec (Q203). *Elife* 10, e71959. doi: 10.7554/eLife.71959

Yano, T., Kassovska-Bratinova, S., Teh, J. S., Winkler, J., Sullivan, K., Isaacs, A., et al. (2011). Reduction of clofazimine by mycobacterial type 2 NADH:Quinone oxidoreductase: A pathway for the generation of bactericidal levels of reactive oxygen species. *J. Biol. Chem.* 286 (12), 10276–10287. doi: 10.1074/jbc.M110.200501

Yano, T., Li, L. S., Weinstein, E., Teh, J. S., and Rubin, H. (2006). Steady-state kinetics and inhibitory action of antitubercular phenothiazines on mycobacterium tuberculosis type-II NADH-menaquinone oxidoreductase (NDH-2). *J. Biol. Chem.* 281 (17), 11456–11463. doi: 10.1074/jbc.M508844200

Yao, R., Wang, B., Fu, L., Li, L., You, K., Li, Y. G., et al. (2022). Sudapyridine (WX-081), a novel compound against mycobacterium tuberculosis. *Microbiol. Spectr.* 10 (1), e0247721. doi: 10.1128/spectrum.02477-21

Yu, W., Chiwala, G., Gao, Y., Liu, Z., Sapkota, S., Lu, Z., et al. (2020). TB47 and clofazimine form a highly synergistic sterilizing block in a second-line regimen for tuberculosis in mice. *BioMed. Pharmacother.* 131, 110782. doi: 10.1016/j.biopha.2020.110782

Yu, W., Yusuf, B., Wang, S., Tian, X., Hameed, H. M. A., Lu, Z., et al. (2021). Sterilizing effects of novel regimens containing TB47, clofazimine, and linezolid in a murine model of tuberculosis. *Antimicrob. Agents Chemother.* 65 (10), e0070621. doi: 10.1128/AAC.00706-21

Zeng, S., Zhang, J., Sun, M., Zhang, X., Cook, G. M., and Zhang, T. (2021). Nitric oxide-dependent electron transport chain inhibition by the cytochrome bc1 inhibitor and pretomanid combination kills mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 65 (9), e0095621. doi: 10.1128/AAC.00956-21

Zhang, Y. J., Ioerger, T. R., Huttenhower, C., Long, J. E., Sassetti, C. M., Sacchettini, J. C., et al. (2012). Global assessment of genomic regions required for growth in mycobacterium tuberculosis. *PloS Pathog.* 8 (9), e1002946. doi: 10.1371/journal.ppat.1002946

Zheng, H., Wang, Y., He, W., Li, F., Xia, H., Zhao, B., et al. (2022). *In vitro* activity of pretomanid against nontuberculous mycobacteria. *Antimicrob. Agents Chemother.* 66 (1), e0181021. doi: 10.1128/AAC.01810-21

Zhou, X., Gao, Y., Wang, W., Yang, X., Yang, X., Liu, F., et al. (2021). Architecture of the mycobacterial succinate dehydrogenase with a membrane-embedded rieske FeS cluster. *Proc. Natl. Acad. Sci. U.S.A.* 118 (15), e2022308118. doi: 10.1073/pnas.2022308118

Zhou, S., Wang, W., Zhou, X., Zhang, Y., Lai, Y., Tang, Y., et al. (2021). Structure of mycobacterium tuberculosis cytochrome bcc in complex with Q203 and TB47, two anti-TB drug candidates. *Elife* 10, e69418. doi: 10.7554/eLife.69418

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Brief Research Report
PUBLISHED 24 August 2022
DOI 10.3389/fcimb.2022.957287

OPEN ACCESS

EDITED BY
Digby Warner,
University of Cape Town, South Africa

REVIEWED BY
Kohta Saito,
NewYork-Presbyterian, United States
Luis Rivas,
Spanish National Research Council (CSIC), Spain
Benfang Lei,
Montana State University, United States

*CORRESPONDENCE
Galina V. Mukamolova
gvm4@leicester.ac.uk

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 30 May 2022
ACCEPTED 29 July 2022
PUBLISHED 24 August 2022

CITATION
Glenn SM, Turapov O, Makarov V, Kell DB and Mukamolova GV (2022) Dimethyl fumarate eliminates differentially culturable *Mycobacterium tuberculosis* in an intranasal murine model of tuberculosis. *Front. Cell. Infect. Microbiol.* 12:957287. doi: 10.3389/fcimb.2022.957287

Dimethyl fumarate eliminates differentially culturable *Mycobacterium tuberculosis* in an intranasal murine model of tuberculosis

Sarah M. Glenn[1], Obolbek Turapov[2], Vadim Makarov[3], Douglas B. Kell[4,5] and Galina V. Mukamolova[2]*

[1]Division of Biomedical Services, University of Leicester, Leicester, United Kingdom, [2]Leicester Tuberculosis Research Group, Department of Respiratory Sciences, University of Leicester, Leicester, United Kingdom, [3]Research Center of Biotechnology, Russian Academy of Sciences, Moscow, Russia, [4]Department of Biochemistry and Systems Biology, Institute of Systems, Molecular and Integrative Biology, Faculty of Health and Life Sciences, University of Liverpool, Liverpool, United Kingdom, [5]The Novo Nordisk Foundation Centre for Biosustainability, Technical University of Denmark, Lyngby, Denmark

Tuberculosis (TB) claims nearly 1.5 million lives annually. Current TB treatment requires a combination of several drugs administered for at least 6 months. *Mycobacterium tuberculosis* (Mtb), the causative agent of TB, can persist in infected humans and animals for decades. Moreover, during infection, Mtb produces differentially culturable bacteria (DCB) that do not grow in standard media but can be resuscitated in liquid media supplemented with sterile Mtb culture filtrates or recombinant resuscitation-promoting factors (Rpfs). Here, we demonstrate that, in an intranasal murine model of TB, Mtb DCB are detectable in the lungs after 4 weeks of infection, and their loads remain largely unchanged during a further 8 weeks. Treatment of the infected mice with dimethyl fumarate (DMF), a known drug with immunomodulatory properties, for 8 weeks eliminates Mtb DCB from the lungs and spleens. Standard TB treatment consisting of rifampicin, isoniazid, and pyrazinamide for 8 weeks reduces Mtb loads by nearly four orders of magnitude but does not eradicate DCB. Nevertheless, no DCB can be detected in the lungs and spleens after 8 weeks of treatment with DMF, rifampicin, isoniazid, and pyrazinamide. Our data suggest that addition of approved anti-inflammatory drugs to standard treatment regimens may improve TB treatment and reduce treatment duration.

KEYWORDS

***Mycobacterium tuberculosis*, tuberculosis, differentially culturable bacteria, treatment, persisters, resuscitation-promoting factor, dimethyl fumarate**

Introduction

Tuberculosis (TB) remains a significant global challenge that affects nearly 10 million people annually (WHO, 2021). TB treatment is long and requires a combination of several drugs, which may result in low compliance and rise of multidrug-resistant TB. The effectiveness of treatment is affected by many factors, namely, the extent of pathological changes and cavitation, status of the immune system, chronic non-infectious diseases, and side effects of drugs (Dartois and Rubin, 2022). Cavitary TB, accompanied by high *Mycobacterium tuberculosis* (Mtb) loads in sputum samples, is more challenging to cure and often requires prolonged treatment (Imperial et al., 2018). It becomes increasingly clear that meeting the WHO STOP TB objectives (WHO, 2008) is not possible without the development of personalised treatment and a deep understanding of host–pathogen interactions, physiological adaptation of Mtb to the immune pressures, and molecular mechanisms of Mtb survival during chemotherapy. The concept of persisters, the enigmatic bacilli that are not killed by bactericidal drugs and persist in infected humans and animals for a long period of time, has highlighted the importance of bacterial physiological state in successful eradication of TB (Gold and Nathan, 2017). While the precise nature of these persisters is obscure, it is generally accepted that heterogenous Mtb populations arise during infection (Manina et al., 2015) and can be recovered from infected tissue or TB clinical samples (Mukamolova et al., 2010; Dhillon et al., 2014; Hu et al., 2015; Chengalroyen et al., 2016; Turapov et al., 2016; Rosser et al., 2018; Dusthackeer et al., 2019). These populations differ by staining patterns (Garton et al., 2008; Manina et al., 2015) or by cultivation requirements (Mukamolova et al., 2010; Turapov et al., 2016; Chengalroyen et al., 2016). Simple growth assays revealed at least three distinct Mtb populations in clinical samples: plateable Mtb, producing colonies on solid agar; non-plateable Mtb, recovering in liquid media; resuscitation-promoting factor (Rpf)–dependent or culture supernatant (CSN)–dependent Mtb, growing in the presence of sterile CSN or recombinant Rpf (Mukamolova et al., 2010; Turapov et al., 2016). Our current understanding of Mtb resuscitation of non-plateable and CSN-dependent Mtb is limited; therefore, the term "differentially culturable bacteria" more accurately defines Mtb that cannot grow in standard media and have special cultivation and resuscitation requirements (Chengalroyen et al., 2016).

Regardless of the nature of resuscitating factor, DCB are apparently more resistant to first-line drugs, enriched in treated patients (Mukamolova et al., 2010; Turapov et al., 2016; McAulay et al., 2018; Beltran et al., 2020) and associated with TB relapse in the Cornell mouse model of TB (Hu et al., 2015; Hu et al., 2019).

We have previously proposed that formation of Rpf-dependent mycobacteria is triggered by the specific *in vivo* environment; the host inflammatory response likely plays a critical role in this process (Turapov et al., 2014). Application of anti-inflammatory compounds reduced the duration of TB treatment, indirectly suggesting that the inflammatory response may trigger persister formation (Gold et al., 2012). However, dynamics of DCB or Rpf-dependent Mtb was not investigated in the study. Here, we show that DCB are generated in the murine lungs after 4 weeks of infection *via* an intranasal route. The treatment of the infected mice with dimethyl fumarate (DMF) for 4 weeks removes DCB, suggesting a strategy for controlling DCB burden. DMF is a well-known drug for management of psoriasis and multiple sclerosis (Rommer et al., 2018; Kourakis et al., 2020). This chemical possesses profound anti-inflammatory properties (Seidel and Roth, 2013). In particular, DMF was shown to reduce NF-κB–mediated pro-inflammatory cytokine release in human peripheral blood mononuclear cells and down-regulate the production of nitric oxide synthase and IL-1β, TNF-α, and IL-6 in cultured microglia (reviewed by Mills et al., 2018). DMF promoted post-ischemic recovery of mice and was well tolerated at a concentration of 45 mg/kg (Yao et al., 2016).

DMF can kill Mtb at a concentration of 12.5 mM (Ruecker et al., 2017); however, its effect on Mtb growth at lower concentrations has not been studied. Our pilot results show that DMF has an inhibitory effect on Mtb growth *in vitro* at a concentration of 25 μM but does not impact on loads of plateable Mtb *in vivo*. Nevertheless, it facilitates the removal of DCB in infected murine organs when used on its own or in combination with standard treatment.

Methods

Organism and media

The Mtb H37Rv strain was grown in 7H9 Middlebrook's broth supplemented with 10% (v/v) oleic acid albumin dextrose (OADC) enrichment, 0.2% (v/v) glycerol, and 0.05% (w/v) Tween 80 (referred to as supplemented 7H9 broth or s7H9). Solid 7H10 agar supplemented with OADC was used for the determination of colony-forming units (CFUs). PANTA supplements were added for the prevention of contamination. CSNs were obtained from logarithmic phase cultures (OD580 ~0.8–1.0) as previously described (Turapov et al., 2016). CSN preparations were passed twice through 0.22-μm filters, and sterility of all batches was confirmed by incubation of CSN aliquots at 37°C for 12 weeks. Media and supplements were purchased from DIFCO™; DMF, rifampicin, isoniazid, and pyrazinamide were purchased from Sigma-Aldrich. For preparation of frozen stocks, bacteria were washed and frozen in phosphate-buffered saline (PBS).

Animal infection experiments

Animal experiments were performed in accordance with the Animals (Scientific Procedures) Act, under project license P7B01C07A. The University of Leicester Ethical Committee and the U.K. Home Office approved the experimental protocols. Six- and eight-week-old female BALB/c mice were bred and maintained in house for the study. Mice were randomised to groups. Mice were lightly anaesthetised with 2.5% (v/v) isoflurane over oxygen (1.8–2 L min^{-1}) and infected intranasally with 5×10^3 mycobacteria resuspended in 50 µl of PBS. For each time point, bacterial loads from five to eight separate mice were determined.

Treatment

After 4 weeks of infection, mice began treatment *via* oral gavage. Formulations were prepared in PEG 400. Four groups of mice included (i) control (PEG 400), (ii) DMF (DMF at 45 mg/kg twice a day as previously described by Yao et al., 2016), (iii) standard treatment RHZ (rifampicin at 10 mg/kg, isoniazid at 25 mg/kg, and pyrazinamide at 150 mg/kg), and (iv) DRHZ (standard treatment RHZ with DMF). Two doses of DMF were given to mice in DMF and DRHZ groups. Mice were treated for up to 8 weeks and Mtb counts determined after 4 and 8 weeks of treatment. Minimum inhibitory concentration of DMF for Mtb growth was determined in s7H9 according to the published protocols (Jorgensen et al., 1999).

Determination of bacterial counts

Mtb loads in organs were assessed by CFU and most probable number (MPN) count assays (Turapov et al., 2014). Briefly, mice were humanely killed by cervical dislocation and immediately dissected. Lungs or spleens were homogenized in 5 ml of 7H9 medium containing sterile MP Biomedicals™ Lysing Matrix S beads using a FastPrep-24 homogenizer (MP Biomedicals). Homogenates were serially diluted in 7H9 medium and used for CFU and MPN count assays. MPN counts were determined in s7H9 (MPN) or in 7H9 containing 50% (v/v) CSN. MPN counts were calculated using the MPN calculator program (Jarvis et al., 2010). Unpaired *t*-test was used for statistical analysis.

Key definitions

DCB_{7H9} Mtb – differentially culturable bacteria that resuscitate in s7H9 liquid media.

DCB_{CSN} Mtb – differentially culturable bacteria that resuscitate in s7H9 liquid media supplemented with sterile CSN.

DCB Mtb – all Mtb bacteria that do not grow on solid media but resuscitate in liquid media.

Plateable Mtb – bacteria that produce colonies on solid 7H10 agar.

Resuscitation Index (RI) – is the ratio of resuscitating Mtb to plateable Mtb. CSN consistently showed the highest resuscitation effect; we therefore determined RI only for CSN. RI = $\log_{10}$ (MPN_CSN ml^{-1}) - $\log_{10}$ (CFU ml^{-1}).

Results

DCB are produced in murine lungs after 4 weeks of infection

We first investigated the dynamics of plateable Mtb and DCB in the control animal group using CFU and MPN count assays. We found that, at 24 h post-infection, CFU, MPN, and MPN_CSN counts were nearly identical (around 3.2 $\log_{10}$ bacteria ml^{-1}), suggesting that, at the onset of experiment, most bacteria were plateable (Figure 1A). As the infection progressed, CFU increased by five orders of magnitude and peaked at 4 weeks of infection; further infection resulted in a substantial decrease in CFU counts, which stabilized at 6 $\log_{10}$ CFU ml^{-1} at the end of the experiment (Figure 1A). The MPN counts obtained in 7H9 showed an opposite pattern, and after the initial lower increase (presumably due to growth inhibition), they progressively increased throughout the study. MPN_CSN counts remained largely unchanged after 4 weeks of infection and were significantly higher than CFU or MPN counts ($p < 0.001$). At 8 weeks of infection, the resuscitation index reflecting the difference between plateable and CSN-resuscitated Mtb was 1.8 (Figure 1B). Our data showed that DCB are produced in the lungs in the intranasal infection model, confirming our previous findings obtained with *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) (Turapov et al., 2014). Thus, generation of DCB *in vivo* is not a species-specific phenomenon and can be observed in Mtb and *M. bovis* (BCG). Importantly, DCB_{CSN} was the dominating Mtb population recovered from the lungs at 4, 8, and 12 weeks of infection.

Treatment with the RHZ combination for 8 weeks significantly reduces bacterial loads but does not eliminate DCB

Exposure of mycobacteria to antimicrobials such as rifampicin or isoniazid leads to the accumulation of Rpf-dependent mycobacteria (Loraine et al., 2016). We next investigated whether standard treatment, consisting of rifampicin (R), isoniazid (H), and pyrazinamide (Z), had any effect on Mtb counts in the lungs after 4

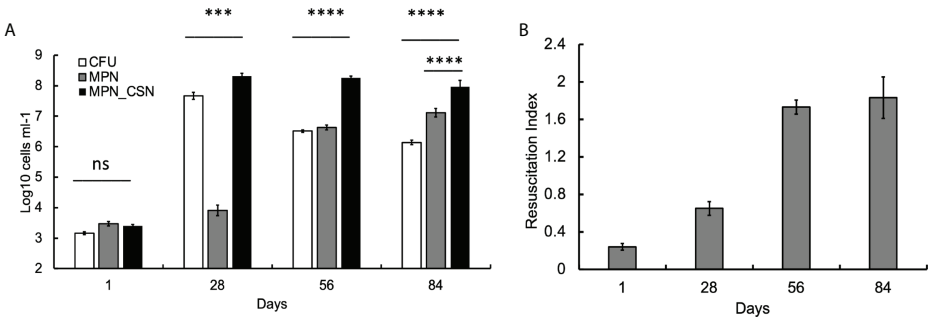


FIGURE 1
Assessment of Mtb loads in the infected murine lungs over a 12-week infection period. Mice were culled after 1, 28, 56 and 84 days of infection. At 56 and 84 days, mice received PEG (no treatment control). **(A)** CFU, MPN and MPN_CSN counts were determined in the lung homogenates. Data shown as means ± STDV (N = 5). **(B)** The resuscitation indices were calculated using the following formula: RI = $\log_{10}$ (MPN_CSN ml^{-1}) – $\log_{10}$ (CFU ml^{-1}). Ns, non-significant; $p > 0.05$; ***$p < 0.001$; ****$p < 0.0001$ unpaired t-test.

and 8 weeks of treatment. Mtb loads of plateable Mtb were reduced by two and three orders of magnitude at 4 and 8 weeks of treatment, respectively (Figures 2A, B). Importantly, this treatment reduced but did not completely eliminate DCB_{CSN} as MPN_CSN counts were significantly higher than CFU counts after 4 and 8 weeks of treatment ($p < 0.05$, 4 weeks and $p < 0.001$, 8 weeks). It was not possible to conclude whether treatment induced differential culturability or failed to remove the DCB produced during infection.

DMF eliminates Mtb DCB from the infected murine lungs

We previously hypothesized that the *in vivo* environment promoted the generation of DCB and Rpf-dependent mycobacteria and immunomodulation might interfere with this process (Turapov et al., 2014). We therefore treated mice with DMF, an approved drug for treatment of multiple sclerosis

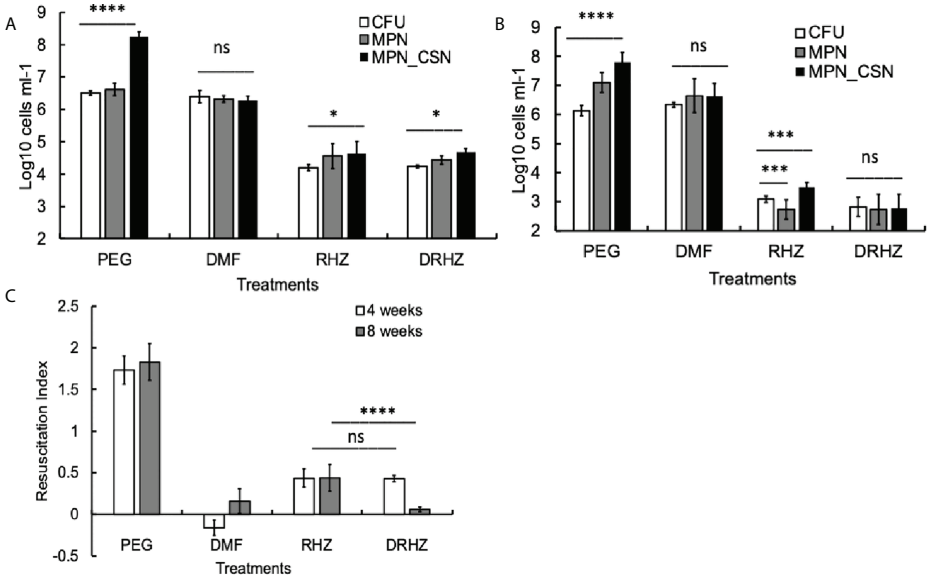


FIGURE 2
Effect of dimethyl fumarate (DMF), standard treatment and standard treatment with DMF on Mtb loads in the infected murine lungs after 4 **(A)** and 8 weeks of treatment **(B)**. Mice were infected intranasally as described in *Methods* and treatment began after 4 weeks of infection. Murine lung homogenates were used for CFU, MPN and MPN_CSN assays. **(C)** Resuscitation indices. Data shown as means ± STDV (N = 7) **(B)**. The resuscitation indices were calculated using the following formula: RI= $\log_{10}$ (MPN_CSN ml^{-1}) – $\log_{10}$ (CFU ml^{-1}). PEG, control PEG 400 group; DMF, dimethyl fumarate group; RHZ, rifampicin, isoniazid and pyrazinamide group; and DRHZ, rifampicin, isoniazid, pyrazinamide and dimethyl fumarate group; Ns, non-significant; $p > 0.05$; *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$ unpaired t-test.

and psoriasis, which possesses anti-inflammatory and immunomodulatory properties (Kourakis et al., 2020). We determined that the minimum inhibitory concentration of DMF for the Mtb strain used in this study and grown in supplemented 7H9 medium is 25 μM; thus, DMF represents a drug with anti-inflammatory and anti-TB effects.

However, DMF treatment for 4 or 8 weeks had no effect on plateable Mtb (Figures 2A, B); the CFU counts from the control and DMF-treated mice did not differ significantly ($p > 0.05$). Importantly, the MPN and MPN_CSN counts were also similar to CFU counts obtained with DMF-treated mice (Figures 2A, B). The RI values for DMF-treated samples were below 0.2, compared with RIs of 1.73 and 1.83 for the control PEG-treated group at 4 and 8 weeks of treatment (Figure 2C). Hence, DMF treatment removed DCB Mtb from the infected lungs.

Addition of DMF to standard regimen removes DCB from the lungs and spleens after 8 weeks of treatment

Finally, we tested whether the addition of DMF to the RHZ regimen would impact on Mtb loads in the infected organs. After 4 weeks of treatment, all Mtb counts in the DRHZ and RHZ groups were very similar (CFU, MPN, and MPN_CSN), with an RI of 0.44, suggesting the presence of DCB (Figures 2A, C). However, 8-week treatment led to a reduction in MPN and MPN_GSN counts in the DHRZ group to 0.06, whereas the RI for RHZ lung samples was 0.43 (Figure 2C). These results suggest that the combined DRHZ treatment eliminated DCB from the lungs after 8 weeks. These findings were further confirmed by assessment of Mtb loads in the infected spleens after 8 weeks of treatment (Figure 3A). DCB_{CSN} were present in the control spleens; however, their proportion was lower as manifested by an RI of 0.5 compared with an RI of 1.83 in the lungs (Figure 3B). Interestingly, the RI value obtained for Mtb from the RHZ-treated spleens was 1.3, even though all Mtb counts were very low (Figure 3B). DMF completely eliminated all Mtb from the spleens because no Mtb were detected when the entire homogenates were used for assessment by CFU and MPN count assays. These findings suggest that while DMF, on its own, has no bactericidal effect on plateable Mtb, it eliminates DCB from infected organs.

Discussion

The prolonged TB treatment creates substantial health, logistical and cost challenges (Dartois and Rubin, 2022). Recent drug trials have identified potential markers of patients who might be treated for shorter or longer periods (Imperial et al., 2018). However, our current understanding of Mtb persisters, the bacteria which are associated with failed treatment, is still limited. The Mtb persisters seemed to be represented by multiple populations, triggered by various factors (Gold and Nathan, 2017; Sarathy and Dartois, 2020). Importantly, some persisters are difficult to detect because they need special resuscitation media for cultivation. These DCB persisters are abundant in clinical samples and are more resistant to treatment. While animal studies have shown that DCB could be removed from the infected organs by increased concentrations of rifampicin (Hu et al., 2015) or bedaquiline (Hu et al., 2019) containing regimens, we still do not have a clear

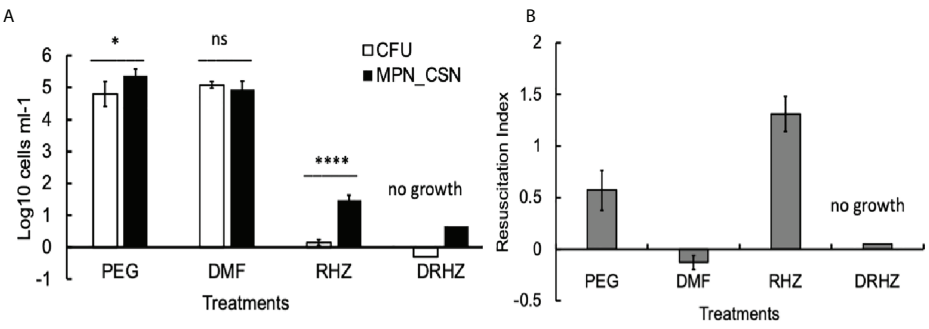


FIGURE 3
Effect of dimethyl fumarate (DMF), standard treatment and standard treatment with DMF on Mtb loads in the infected spleens after 8 weeks of treatment. **(A)** Spleen homogenates were used for CFU and MPN_CSN assays. Data shown as means ± STDV ($N = 5$) **(B)**. The resuscitation indices were calculated using the following formula: RI= $\log_{10}$ (MPN_CSN ml^{-1}) – $\log_{10}$ (CFU ml^{-1}). Ns, non-significant; $p > 0.05$; *$p < 0.05$; ****$p < 0.0001$ unpaired *t*-test. No Mtb were recovered in the DHZR group; the presented bars indicate the limit of detection for the MPN assay 4.6 cell ml^{-1} and 0.25 cell ml^{-1} (CFU). PEG, control PEG 400 group; DMF, dimethyl fumarate group; RHZ, rifampicin, isoniazid and pyrazinamide group; and DRHZ, rifampicin, isoniazid, pyrazinamide and dimethyl fumarate group.

strategy for prevention of DCB formation or DCB removal from TB patients. The intrinsic challenge of DCB persisters is that the immune response directed to destroy Mtb may, in fact, create a specific environment that stimulates the generation of DCB. The excessive immune response results in lung damage and pathology, accompanied by the formation of lesions and cavities, the perfect environment for further persister formation and rise of drug-resistant Mtb (Sarathy and Dartois, 2020). Hence, finding drugs that control Mtb growth and reduce the immune response could provide a plausible solution for the Mtb persister challenge. Here, we present data demonstrating that application of anti-inflammatory drug DMF completely eliminates DCB from the infected lungs and spleens after 8 weeks of treatment. This pilot study was only designed for a relatively short course of infection, and further experiments are necessary to confirm whether the inclusion of DMF may reduce the duration of treatment and prevent TB relapse. It remains to be established whether DCB are particularly vulnerable to DMF or whether DMF breaks an ongoing cycle of DCB formation during the prolonged infection. We are currently testing the effect of DMF on DCB obtained *in vitro*. Future studies will reveal the molecular mechanisms of DMF-mediated elimination of DCB persisters from infected organism by identification of host signalling pathways, careful monitoring of cytokine levels in treated and untreated animals, investigation of metabolomics and application of other immunomodulatory chemicals such as vitamin D, steroid and non-steroid anti-inflammatory drugs (reviewed by Tobin, 2015). Design and synthesis of DMF derivatives with mycobactericidal effect may further improve the observed effects. Identification of individuals with high numbers of DCB using host proteomics (Beltran et al., 2020) or transcriptomics approaches (Singhania et al., 2018) will contribute to the development of patient-tailored TB therapy.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was reviewed and approved by Animal experiments were performed in accordance with the Animals (Scientific Procedures) Act, under project license P7B01C07A.The University of Leicester Ethical Committee and the U.K. Home Office approved the experimental protocols.

Author contributions

GM, VM and DK conceived of and designed the experiments. SG, GM and VM secured funding. SG, OT and GM performed the experiments and analysed the data. All authors contributed to the article and approved the submitted version.

Funding

This research was funded by the Medical Research Council Leicester Confidence in Concept 2019 award (MRC CiC 2019), grant number MC_PC_19043 (SG and GM), and the Russian Science Foundation under grant 21-15-00042 (VM).

Acknowledgments

We thank Professor Andrea Cooper and Dr. John Pearl for the provision of Mtb strain and useful discussions. We acknowledge the Centre for Core Biotechnology Services at the University of Leicester for support with containment level 3 experiments. We are grateful to Jennifer Schofield for technical help with the experiments. We would like to acknowledge the staff at the Division of Biomedical Services, University of Leicester, for their care of the animals and their assistance in experimental procedures. DK thanks the Novo Nordisk Foundation for funding (grant NNF10CC1016517). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Conflict of interest

Author DK was employed by The Novo Nordisk Foundation Centre for Biosustainability.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Beltran, C. G. G., Gallant, J., Venter, R., du Plessis, N., Loxton, A. G., Trost, M., et al. (2020). Investigating non-sterilizing cure in TB patients at the end of successful anti-TB therapy. *Front. Cell. Infect. Microbiol.* 10. doi: 10.3389/fcimb.2020.00443

Chengalroyen, M. D., Beukes, G. M., Gordhan, B. G., Streicher, E. M., Churchyard, G., Hafner, R., et al. (2016). Detection and quantification of differentially culturable tubercle bacteria in sputum from patients with tuberculosis. *Am. J. Respir. Crit. Care Med.* 194, 1532–1540. doi: 10.1164/rccm.201604-0769OC

Dartois, V. A., and Rubin, E. J. (2022). Anti-tuberculosis treatment strategies and drug development: Challenges and priorities. *Nat. Rev. Microbiol.*, 1–17. doi: 10.1038/s41579-022-00731-y

Dhillon, J., Fourie, P. B., and Mitchison, D. A. (2014). Persister populations of mycobacterium tuberculosis in sputum that grow in liquid but not on solid culture media. *J. Antimicrob. Chemother.* 69, 437–440. doi: 10.1093/jac/dkt357

Dusthackeer, A., Balasubramanian, M., Shanmugam, G., Priya, S., Nirmal, C. R., Sam Ebenezer, R., et al. (2019). Differential culturability of mycobacterium tuberculosis in culture-negative sputum of patients with pulmonary tuberculosis and in a simulated model of dormancy. *Front. Microbiol.* 10. doi: 10.3389/fmicb.2019.02381

Garton, N. J., Waddell, S. J., Sherratt, A. L., Lee, S. M., Smith, R. J., Senner, C., et al. (2008). Cytological and transcript analyses reveal fat and lazy persister-like bacilli in tuberculous sputum. *PLoS Med.* 5, e75. doi: 10.1371/journal.pmed.0050075

Gold, B., and Nathan, C. (2017). Targeting phenotypically tolerant mycobacterium tuberculosis. *Microbiol. Spectr.* 5:10.1128/microbiolspec.TBTB2-0031-2016. doi: 10.1128/microbiolspec

Gold, B., Pingle, M., Brickner, S. J., Shah, N., Roberts, J., Rundell, M., et al. (2012). Nonsteroidal anti-inflammatory drug sensitizes mycobacterium tuberculosis to endogenous and exogenous antimicrobials. *Proc. Natl. Acad. Sci. U S A.* 109, 16004–16011. doi: 10.1073/pnas.1214188109

Hu, Y., Liu, A., Ortega-Muro, F., Alameda-Martin, L., Mitchison, D., and Coates, A. (2015). High-dose rifampicin kills persisters, shortens treatment duration, and reduces relapse rate *in vitro* and *in vivo*. front. *Microbiol.* 6. doi: 10.3389/fmicb.2015.00641

Hu, Y., Pertinez, H., Liu, Y., Davies, G., and Coates, A. (2019). Bedaquiline kills persistent mycobacterium tuberculosis with no disease relapse: An *in vivo* model of a potential cure. *J. Antimicrob. Chemother.* 74, 1627–1633. doi: 10.1093/jac/dkz052

Imperial, M. Z., Nahid, P., Phillips, P. P. J., Davies, G. R., Fielding, K., Hanna, D., et al. (2018). A patient-level pooled analysis of treatment-shortening regimens for drug-susceptible pulmonary tuberculosis. *Nat. Med.* 24, 1708–1715. doi: 10.1038/s41591-018-0224-2

Jarvis, B., Wilrich, C., and Wilrich, P. T. (2010). Reconsideration of the derivation of most probable numbers, their standard deviations, confidence bounds and rarity values. *J. Appl. Microbiol.* 109, 1660–1667. doi: 10.1111/j.1365-2672.2010.04792.x

Jorgensen, J. H., Turnidges, J. D., and Washington, J. A. (1999). "Antibacterial susceptibility tests: dilution and disk diffusion methods," in *Manual of clinical microbiology*. Eds. P. R. Murray, E. J. Baron, Pfaller M.A.,, F. C. Tenover and R. H. Yolken (Washington, DC: ASM), 1526–1543.

Kourakis, S., Timpani, C. A., de Haan, J. B., Gueven, N., Fischer, D., and Rybalka, E. (2020). Dimethyl fumarate and its esters: a drug with broad clinical utility? *Pharm. (Basel).* 13, 306. doi: 10.3390/ph13100306

Loraine, J., Pu, F., Turapov, O., and Mukamolova, G. V. (2016). Development of an *in vitro* assay for detection of drug induced resuscitation-promoting factor dependent mycobacteria. *Antimicrob. Agents Chemother.* 60, 6227–6233. doi: 10.1128/AAC.00518-16

Manina, G., Dhar, N., and McKinney, J. D. (2015). Stress and host immunity amplify mycobacterium tuberculosis phenotypic heterogeneity and induce nongrowing metabolically active forms. *Cell Host Microbe* 17, 32–46. doi: 10.1016/j.chom.2014.11.016

McAulay, K., Saito, K., Warrier, T., Walsh, K. F., Mathurin, L. D., Royal-Mardi, G., et al. (2018). Differentially detectable mycobacterium tuberculosis cells in sputum from treatment-naive subjects in Haiti and their proportionate increase after initiation of treatment. *mBio* 9, e02192–e02118. doi: 10.1128/mBio.02192-18

Mills, E. A., Ogrodnik, M. A., Plave, A., and Mao-Draayer, Y. (2018). Emerging understanding of the mechanism of action for dimethyl fumarate in the treatment of multiple sclerosis. *Front. Neurol.* 9. doi: 10.3389/fneur.2018.00005

Mukamolova, G. V., Turapov, O., Malkin, J., Woltmann, G., and Barer, M. R. (2010). Resuscitation-promoting factors reveal an occult population of tubercle bacilli in sputum. *Am. J. Respir. Crit. Care Med.* 181, 174–180. doi: 10.1164/rccm.200905-0661OC

Rommer, P. S., Milo, R., Han, M. H., Satyanarayan, S., Sellner, J., Hauer, L., et al. (2018). Immunological aspects of approved MS therapeutics. *Front. Immunol.* 10. doi: 10.3389/fimmu.2019.01564

Rosser, A., Pareek, M., Turapov, O., Wiselka, M. J., and Mukamolova, G. V. (2018). Differentially culturable tubercule bacilli are generated during non-pulmonary tuberculosis infection. *Am. J. Respir. Crit. Care Med.* 197, 818–821. doi: 10.1164/rccm.201705-1048LE

Ruecker, N., Jansen, R., Trujillo, C., Puckett, S., Jayachandran, P., Piroli., G. G., et al. (2017). Fumarase deficiency causes protein and metabolite succination and intoxicates mycobacterium tuberculosis. *Cell Chem. Biol.* 24, 306–315. doi: 10.1016/j.chembiol.2017.01.005

Sarathy, J. P., and Dartois, V. (2020). Caseum: a niche for mycobacterium tuberculosis drug-tolerant persisters. *Clin. Microbiol. Rev.* 33, e00159–e00119. doi: 10.1128/CMR.00159-19

Seidel, P., and Roth, M. (2013). Anti-inflammatory dimethyl fumarate: a potential new therapy for asthma? *Mediators Inflammation*, 875403. doi: 10.1155/2013/875403

Singhania, A., Wilkinson, R. J., Rodrigue, M., Haldar, P., and O'Garra, A. (2018). The value of transcriptomics in advancing knowledge of the immune response and diagnosis in tuberculosis. *Nat. Immunol.* 19, 1159–1168. doi: 10.1038/s41590-018-0225-9

Tobin, D. M. (2015). Host-directed therapies for tuberculosis. *Cold Spring Harb. Perspect. Med.* 5, a021196. doi: 10.1101/cshperspect.a021196

Turapov, O., Glenn, S., Kana, B., Makarov, V., Andrew, P. W., and Mukamolova, G. V. (2014). The in vivo environment accelerates generation of resuscitation-promoting factor–dependent mycobacteria. *Am. J. Respir. Crit. Care Med.* 190, 1455–1457. doi: 10.1164/rccm.201407-1289LE

Turapov, O., O'Connor, B., Sarybaeva, A. A., Williams, C., Patel, H., Kadyrov, A. S., et al. (2016). Phenotypically adapted mycobacterium tuberculosis populations from sputum are tolerant to first line drugs. *Antimicrob. Agents Chemother.* 60, 2476–2483. doi: 10.1128/AAC.01380-15

WHO (2021) *Global tuberculosis report*. Available at: https://www.who.int/publications/i/item/9789240037021.

World Health Organization (2008). *Global Tuberculosis Programme*. Available at: https://www.who.int/teams/global-tuberculosis-programme/the-end-tb-strategy

Yao, Y., Miao, W., Liu, Z., Han, W., Shi, K., Shen, y., et al. (2016). Dimethyl fumarate and monomethyl fumarate promote post-ischemic recovery in mice. *Transl. Stroke Res.* 7, 535–547. doi: 10.1007/s12975-016-0496-0

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 02 September 2022
DOI 10.3389/fcimb.2022.990312

OPEN ACCESS

EDITED BY
Ben Gold,
Weill Cornell Medicine, United States

REVIEWED BY
Jim Sun,
University of Ottawa, Canada
Md. Aejazur Rahman,
Africa Health Research Institute (AHRI), South Africa
Saurabh Mishra,
Cornell University, United States

*CORRESPONDENCE
Riccardo Manganelli
riccardo.manganelli@unipd.it

†PRESENT ADDRESS
Stefano De Giorgi, Clinical Microbiology, ASST Grande Ospedale Metropolitano Niguarda, Milano, Italy

‡These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 09 July 2022
ACCEPTED 11 August 2022
PUBLISHED 02 September 2022

CITATION
Miotto P, Sorrentino R, De Giorgi S, Provvedi R, Cirillo DM and Manganelli R (2022) Transcriptional regulation and drug resistance in *Mycobacterium tuberculosis*. *Front. Cell. Infect. Microbiol.* 12:990312. doi: 10.3389/fcimb.2022.990312

Transcriptional regulation and drug resistance in *Mycobacterium tuberculosis*

Paolo Miotto[1‡], Rita Sorrentino[1‡], Stefano De Giorgi[2†], Roberta Provvedi[3], Daniela Maria Cirillo[1] and Riccardo Manganelli[2*]

[1]Emerging Bacterial Pathogens Unit, Div. of Immunology, Transplantation and Infectious Diseases IRCCS San Raffaele Scientific Institute, Milano, Italy, [2]Department of Molecular Medicine, University of Padova, Padova, Italy, [3]Department of Biology, University of Padova, Padova, Italy

Bacterial drug resistance is one of the major challenges to present and future human health, as the continuous selection of multidrug resistant bacteria poses at serious risk the possibility to treat infectious diseases in the near future. One of the infection at higher risk to become incurable is tuberculosis, due to the few drugs available in the market against *Mycobacterium tuberculosis*. Drug resistance in this species is usually due to point mutations in the drug target or in proteins required to activate prodrugs. However, another interesting and underexplored aspect of bacterial physiology with important impact on drug susceptibility is represented by the changes in transcriptional regulation following drug exposure. The main regulators involved in this phenomenon in *M. tuberculosis* are the sigma factors, and regulators belonging to the WhiB, GntR, XRE, Mar and TetR families. Better understanding the impact of these regulators in survival to drug treatment might contribute to identify new drug targets and/or to design new strategies of intervention.

KEYWORDS

Mycobacteria, drug resistance, transcriptional regulation, tuberculosis, sigma factors, riboswitch

Introduction

One of the most fascinating aspects of bacteriology is the extremely fast and efficient responsivity of bacteria to external stimuli, which is translated in a fast and precise variation of their transcriptional profile. This capacity is founded on complex regulatory networks based on sigma factors, transcriptional repressors/activators, two component systems, small RNAs or riboswitches able to reshape bacterial physiology allowing the cells to adapt in real time to any external challenge.

Antibacterial drugs can be considered atypical stressors, both causing a direct interference with cellular physiology and inducing secondary stress due to this interference, as in the case of the oxidative stress induced by bactericidal drugs

(Kohanski et al., 2007; Martínez et al., 2020). Consequently, bacteria respond to drug with profound changes in their transcriptional profile that can increase bacterial drug resistance (DR) by target overexpression, drug modification, induction of efflux systems, or simply by helping the cells to respond to the drug-induced stress.

To be noted that whereas DR is usually associated with genetic mutations (usually also referred as genetic resistance), in some cases DR can be developed without chromosomal abnormalities (defined as phenotypic resistance) (Corona and Martinez, 2013). Transcription factors can be involved in both the mechanisms of resistance.

In this paper, we review the impact of transcriptional regulation on drug susceptibility in *M. tuberculosis*.

Sigma factors and drug susceptibility

Sigma factors are small interchangeable cofactors of RNA polymerase able to confer promoter specificity. The *M. tuberculosis* genome encodes 13 sigma factors and it is the obligate pathogen with the higher amount of sigma factor genes per megabase (Rodrigue et al., 2006). Among these sigma factors, only σ^A is essential. The others are dispensable and are activated in response to specific environmental signals. Following their activation, sigma factors switch RNA polymerase holoenzyme promoter-specificity, resulting in a quick change of the bacterial transcriptome leading to the adaptation to the new environment experienced by the bacterium. Mycobacterial sigma factors have been shown to be involved in the response to different conditions endangering the bacterial cells as oxidative stress, alkaline stress, surface stress, low pH, hypoxia, nutrient depletion and heat shock. Drug treatment has been shown to induce a strong stress response in bacteria and the bactericidal activity of some drugs has been primary linked to the stress they induce on the bacteria (Kohanski et al., 2007). Since sigma factors have a primary role in stress response, it is simple to imagine their implication in the establishment of the baseline resistance to drugs.

At least five *M. tuberculosis* sigma factors have been shown to be involved in the basal level of resistance to drugs (Table 1). σ^F (*i*) is induced upon treatment with ethambutol (EMB), rifampin (RIF), streptomycin (STR), and cycloserine (CS) (Michele et al., 1999), while a *sigF* null mutant in CDC1551 is more resistant to RIF (Chen et al., 2000). However, this phenotype was not confirmed in the H37Rv genetic background, suggesting a different role of σ^F in different mycobacterial strains (Hartkoorn et al., 2010).

The extracytoplasmatic function (ECF) sigma factor σ^I (*ii*) directly regulates the expression of the structural gene of KatG, an enzyme required for the activation of isoniazide (INH) (Figure 1A). Consequently, a *sigI* null mutant was more resistant to this drug both in axenic culture and during mice infection, but surprisingly was not attenuated as predictable for a strain expressing lower levels of KatG (Lee et al., 2012).

The ECF sigma factor σ^H (*iii*) is involved in oxidative stress response. One of the genes most induced by σ^H in response to oxidative stress is *rv2466c* (Manganelli et al., 2002), encoding for the mycothiol-dependent reductase DsbA (Rosado et al., 2017). This gene is required for the activation of TP053, a promising thienopyrimidine derivative prodrug, able to kill replicating and non-replicating *M. tuberculosis* (Albesa-Jové et al., 2015). Consistent with these data, a *sigH* null mutant was shown to be resistant to TP053 (Rosado et al., 2017). Since *sigH* is induced upon oxidative stress, it is possible to assume that the activation of TP053 activation increases after intracellular bacteria are exposed to reactive oxygen intermediates.

Both σ^B (*iv*), a member of the primary-like sigma factors, and the ECF sigma factor σ^E (*v*) have been shown to be induced upon exposure to vancomycin (VAN) (Provvedi et al., 2009), and involved in the baseline resistance to several antitubercular drugs. In particular, Pisu and colleagues (Pisu et al., 2017) showed that a *sigE* null mutant was more sensitive to VAN, RIF, STR, gentamicin, INH, and EMB, while a *sigB* null mutant was more sensitive to INH and EMB. Moreover, Yang and colleagues reported that deletion of *sigB* causes increased sensitivity to *para*-aminosalicylic acid and sulfamethoxazole (Yang et al., 2017). Interestingly, Pisu and colleagues showed that σ^B and σ^E are also essential for the development of persistent bacteria able to survive the bactericidal activity of very high concentrations of VAN (*sigE* mutant), STR and INH (both mutants). These data support the hypothesis that σ^E represents a bistable switch involved in persistence development (Balázsi et al., 2008; Sureka et al., 2008; Manganelli and Provvedi, 2010; Tiwari et al., 2010; Zorzan et al., 2021).

Finally, a recent paper demonstrated that σ^E-mediated activation of surface stress response is essential for pyrazinamide (PZA) susceptibility. PZA is only active when bacteria are exposed to low pH, while its derivative pyrazinoic acid is active also at circumneutral pH. However, a mutant overexpressing *sigE* due to the deletion of the gene encoding its anti-sigma factor RseA (Boldrin et al., 2019) was equally sensitive to PZA at both low pH and circumneutral pH, while a *sigE* null mutant was resistant to both PZA and pyrazinoic acid demonstrating that the activation of the σ^E regulon is essential for PZA susceptibility (Thiede et al., 2022). Interestingly, both meropenem and CS, showed synergistic activity with PZA due to their activation of σ^E-mediated surface stress response (Thiede et al., 2022). Since it is well known that σ^E is activated at low pH (Bush, 2018), these data strongly suggest that the role of low pH in PZA susceptibility is the activation of the σ^E response.

WhiB family (WhiB1-7)

M. tuberculosis genome encodes seven proteins belonging to the WhiB superfamily. Proteins belonging to this family are

TABLE 1 Principal transcription factors (TF) in *M. tuberculosis* with established mechanisms of action involved in drug resistance (DR) (transcription factors known for their homology with other mycobacteria are described in the text but not reported in this table).

TF	EXPERIMENTAL CONDITION TESTED	TARGET	EFFECT DURING DRUG-RELATED STRESS	REFERENCES
SigB	Deletion		Increased sensitivity to INH and EMB	(Provvedi et al., 2009)
SigE	Deletion		Increased sensitivity to VAN, RIF, STR, gentamicin, INH, PZA and EMB	(Pisu et al., 2017)
SigE	Constitutive expression		Sensitive to PZA	(Thiede et al., 2022)
SigF	Deletion		Increased resistance to RIF*	(Hartkoorn et al., 2010)
SigH	Deletion	*Rv2466c/dsbA*	Increased resistance to TP053	(Manganelli et al., 2002; Rosado et al., 2017)
SigI	Deletion	*katG*	Increased resistance to INH	(Lee et al., 2012)
WhiB3	(Observed) overexpression		Increased tolerance to RIF, MFX, MTX and AMK	(Rodriguez et al., 2014)
WhiB3	(Observed) overexpression	*egt* operon (*Rv3700c-Rv3704c*)	Increased sensitivity to RIF, INH, BDQ and CFZ	(Saini et al., 2016; Mavi et al., 2020)
WhiB4		β-lactamase	Tolerance to ampicillin	(Mishra et al., 2017)
WhiB7	(Mutated) over-expression	*eis, erm37* and *tap*	Increased resistance to STR, LZD, KAN, AMK	(Reeve et al., 2013; Köser et al., 2013; Vargas et al., 2021)
WhiB7	Inactivation	*eis, erm37* and *tap*	Hypersusceptibility to macrolides and clarithromycin**	(Li et al., 2022)
Rv0023	(Observed) overexpression	*ndh*	Tolerance to INH and ETO	(Gupta et al., 2020)
Rv0273c		*inhA*	Increased sensitivity to INH	(Zhu et al., 2018)
Rv0324	Deletion		Hypersensitivity to BDQ	(Peterson et al., 2016)
Rv0678	(Mutated) Inactivation	*mmpS5-mmpL5* operon	Resistance to BDQ and CFZ	(Kadura et al., 2020)
Rv0880	Deletion		Hypersensitivity to BDQ	(Peterson et al., 2016)
Rv1152	(Observed) overexpression		Resistance to VAN	(Zeng et al., 2016; Deng et al., 2022)
Rv3082c	(Mutated) overexpression	*mymA* operon (*Rv3083 to Rv3089*)	Resistance to ETO and thiooxadiazole 3	(Grant et al., 2016)

*Only in CDC1551 genetic background; **lineage-specific mutation (L1).

small transcriptional regulators and are exclusively present in Actinobacteria. They are characterized by four cysteine residues that bind a [4Fe–4S] cluster and by a five residues (G[V/I]WGG) motif, while their putative role is that to sense O_2 and nitric oxide (Bansal et al., 2017). Two of them (WhiB1 and WhiB2) are essential for growth, while others were shown to be important for several aspects of mycobacterial physiology as redox homeostasis, DR, dormancy and reactivation (Bansal et al., 2017).

WhiB transcriptional factors are involved in stress response and their genes are often upregulated in multidrug resistant (MDR) clinical isolates. Many *whiB* genes showed altered expression during drug treatment: *whiB6* is downregulated by CS, *whiB2* is upregulated by CS, EMB and INH, *whiB7* is upregulated by macrolides, fluoroquinolones and aminoglycosides (Morris et al., 2005; Geiman et al., 2006; Burian et al., 2012; Chatterjee et al., 2013).

Within the WhiB family, a key player in DR is WhiB7, a transcription factor inducing stress response and promoting low levels of resistance to several antibacterial drugs including macrolides, tetracyclines, and some aminoglycosides (Morris et al., 2005; Burian et al., 2013; Reeves et al., 2013). Recently, an elegant experiment based on CRISPR interference identified *whiB7* as the unique gene responsible for increased susceptibility to ribosome-targeting drugs STR and linezolid (LZD) (Li et al., 2022). Among the genes relevant for DR to antibiotics inhibiting translation (i.e. macrolides), *rv2416c* (*eis*) encodes an aminoglycoside acetyltransferase which inactivate the drugs, whereas 23S rRNA methylation by Rv1988 (Erm37) confers resistance to macrolides. Another gene found to be associated with DR to different drugs including STR is *rv1258c* (*tap*), which encodes for an efflux pump. All these DR genes are part of the WhiB7 regulon (Figure 1B). Of particular relevance is the link between mutations affecting the expression of *whiB7* observed by Reeves and colleagues leading to enhanced expression of *rv2416c* (*eis*) (low-level KAN and amikacin resistance) and of *rv1258c* (*tap*) (low-level STR resistance) (Reeves et al., 2013). Interestingly, loss-of-function mutations in the arginine biosynthesis pathway were found to up-regulate the expression of *whiB7*, and *eis* genes, thus conferring tolerance to KAN (Schrader et al., 2021). These mutations mapping in the *rv2747* (*argA*) and *rv1655* (*argD*) genes were associated with increased survival during RIF exposure, as well as with minimum inhibitory concentration (MIC) increase to clarithromycin, again related to the up-regulation of *whiB7*. Modern Beijing isolates and one phylogenetically intermediate Beijing isolate harbored a loss-of-function mutation in *tap*; thus

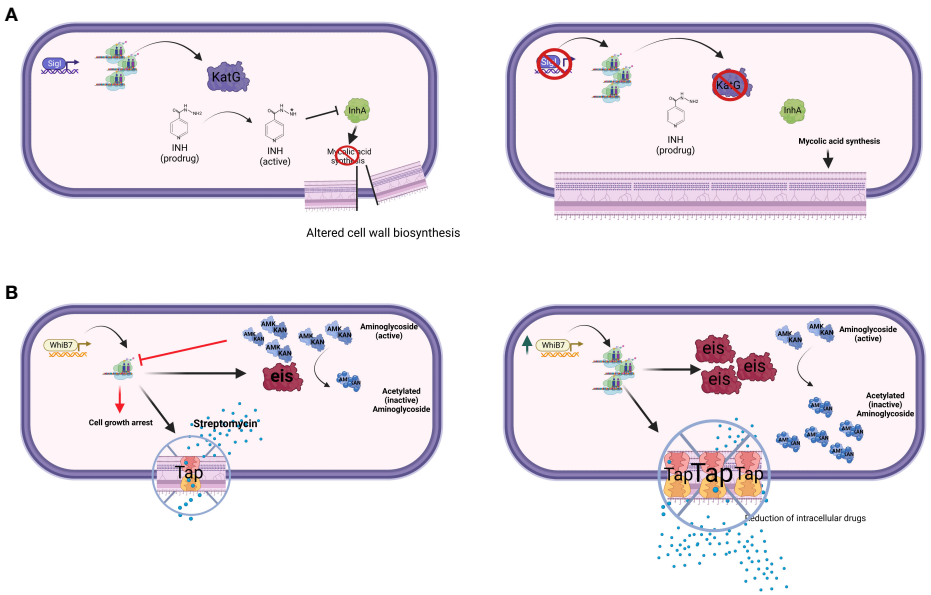


FIGURE 1
The figure schematizes two major drug-resistance mechanisms mediated by transcriptional factors. **(A)** Transcriptional factor down-regulation. The ECF sigma factor SigI controls the transcription of *katG;* mutant strains lacking SigI are more resistant to INH (Lee et al., 2012). **(B)** Transcriptional factor over-expression. WhiB7 regulon contains key genes involved in DR, such as *rv2416c* (*eis*) linked to low-level KAN and AMK resistance, and *rv1258c* (*tap*) whose overexpression is linked to low-level STR resistance (Reeves et al., 2013). Point mutations in *tap,* V219A and S292L, have been associated with resistance to PZA, INH, and STR (Liu et al., 2019). Created with BioRender.com.

whiB7 mutations would not translate into low-level STR resistance, but would lead to low-level KAN and AMK resistance only, despite this epistatic interaction has not been proven in clinical isolates yet (Köser et al., 2013; Vargas et al., 2021). Interestingly, a lineage-specific mutation present in the L1 Indo-Oceanic clade inactivates WhiB7, thus making strains belonging to this phylogenetic branch hypersusceptible to macrolides and clarithromycin (Li et al., 2022).

WhiB7 is involved also in physiological stress responses and virulence (Buriánková et al., 2004; Geiman et al., 2006; Zaunbrecher et al., 2009; Homolka et al., 2010; Adams et al., 2011; Kim et al., 2012; Larsson et al., 2012; Ramón-García et al., 2012). Interestingly, the role of WhiB7 in providing intrinsic DR is dependent on its interactions with the principal sigma factor σ^A, and mutations in either *whiB7* or *sigA* preventing their interaction, have been found to cause multidrug susceptibility (Burian et al., 2013; Lilic et al., 2021). In *M. smegmatis*, WhiB7 was found to positively regulate *ms3140*, a gene homolog of *rv1473* and encoding for an ABC efflux pump involved in macrolide transport (Duan et al., 2019). A similar central role for WhiB7 in DR is found in Actinobacteria, including other mycobacterial opportunistic pathogens such as *Mycobacterium abscessus* (Ramón-García et al., 2013; Hurst-Hess et al., 2017; Pryjma et al., 2017).

The role of other WhiB family members is more nuanced. WhiB4 regulates β-lactamase expression, thus inducing antibiotic tolerance in *M. tuberculosis* (Mishra et al., 2017). WhiB2 is part of a regulatory loop involving *rv1830* (*mcdR*) ultimately fine-tuning mycobacterial cell division and adaptation to stress response, including increased mutation rates during antibiotic challenge (Zhou et al., 2022). Recent genome-wide association analysis (GWAS) approaches have also identified novel associations between mutations in the *whiB6* region and aminoglycosides resistance (Farhat et al., 2019). Mutations in *whiB2* and *whiB6* have been observed within patient microevolution during antimycobacterial treatment, however their role in DR was not fully elucidated (Liu et al., 2015; Xu et al., 2018). WhiB3 was the only transcriptional regulator whose structural gene was induced in a model of adaptation to growth with lipids as the sole carbon source (Rodríguez et al., 2014). In this lipid environment, WhiB3 drove increased drug tolerance to RIF, moxifloxacin (MFX), metronidazole (MTZ), and AMK. Interestingly, similar to WhiB7, WhiB3 was found to interact with σ^A (Burian et al., 2013). Several antimycobacterial compounds can produce an oxidative burst as part of their antimicrobial mechanism (Gurumurthy et al., 2013; Piccaro et al., 2014; Shetty and Dick, 2018). Therefore, transcriptional factors involved in redox homeostasis are relevant in maintaining a reducing microenvironment to avoid DNA damage and macromolecules (i.e. protein and small RNA) misfolding. WhiB3 has been described to negatively regulate the *egt* operon (*rv3700c-rv3704c*) encoding for ergothioneine,

which together with mycothiol, plays an important role in maintaining the oxidoreduction balance within the bacterial cell (Saini et al., 2016). Both ergothioneine and mycothiol have been proved to be triggered from a wide range of stimuli, from starvation or hypoxia to microenvironmental acidification (i.e. phagolysosome acidification post macrophage infection) (Mavi et al., 2020). Saini and colleagues tested RIF, INH, bedaquiline (BDQ) and clofazimine (CFZ) in *rv3704c* and *rv3701c* deficient strains and observed a MIC reduction for all the tested drugs. Accordingly, resistance and tolerance to antimycobacterial drugs impairing redox homeostasis have been directly linked to the intracellular accumulation of ergothioneine (Saini et al., 2016). On the other side, Xu and colleagues produced several strains with mutations in mycothiol-related enzymes demonstrating that such mutants are resistant to both INH and ethionamide (ETO) (Xu et al., 2011).

GntR family transcriptional regulators

Transcription factors of the GntR family are widely shared among bacteria, and the firsts members of this family have been described as a gluconate operon repressor in *B. subtilis* (Vindal et al., 2007; Suvorova et al., 2015). GntR members contain a DNA-binding domain with a structural motif helix-turn-helix (HTH) at their N-terminal, conserved among all the family members, and a more variable C-terminal domain that has been used to divide the GntR factors into six subfamilies (Suvorova et al., 2015). Most of the characterized GntR family members are transcriptional repressors, although some exceptions exist. The *M. tuberculosis* genome encodes for a large number of GntR family transcription factors, although their role and regulation are still poorly described (Cole et al., 1998).

Among the techniques used to identify transcription factors potentially relevant for DR, over-expressing libraries for transcriptional regulators under selection on high drug concentrations have been shown to be successful tools. For example, Hu and colleagues (Hu et al., 2015) identified a hypothetical transcription factor encoded by the *ms0535* gene as a potential contributor to INH resistance in *M. smegmatis*. Sequence analysis showed that Ms0535 belongs to the GntR family (FadR sub-family). Ms0535 acts as a transcriptional activator for the expression of its own structural gene and a major facilitator superfamily permease gene *ms0534* in the same operon, thus triggering INH resistance. Interestingly, the two genes are not responsive to INH, although their over-expression increases INH resistance. Both *ms0535* and *ms0534* are absent in *M. tuberculosis*, thus they can contribute to explain the differences in INH resistance between the two species.

In *M. tuberculosis* there are at least eight putative GntR-like proteins: Rv0043c, Rv0165c (Mce1R), Rv0494, Rv0586 (Mce2R), Rv0792c (MoyR), Rv1152, Rv3060c, and Rv3575c (Vindal et al., 2007; Chauhan et al., 2021). Among them, Rv0494 has an ortholog in *M. smegmatis* (Ms2173). *M. smegmatis* mutants for Ms2173 showed altered INH and RIF susceptibility (its over-expression led to increased INH and RIF susceptibility), likely due to the regulatory activity of this transcription factor on membrane-associated transporter genes (Rao et al., 2012).

Rv1152 is involved in the regulation of cell wall permeability (Zeng et al., 2016; Deng et al., 2022). This transcriptional regulator is involved in acid and cell surface stress response and plays an important role in determining VAN resistance by negatively regulating genes responsive to this glycopeptide antibiotic. Indeed, *M. smegmatis* overexpressing *M. tuberculosis* Rv1152 showed an increased resistance to VAN, whereas deleting its homologous gene (*ms5174*) established increased sensitivity that could be restored by complementation with *rv1152* (Zeng et al., 2016).

Deletion of *rv0792c* impaired the ability of *M. tuberculosis* to infect guinea pigs, however no difference was observed in survival during exposure to INH, RIF, or LEV (Chauhan et al., 2021). A role in DR for the remaining GntR family members has yet to be identified, although some of them have been found upregulated in drug resistant isolates. Interestingly, protein levels of Rv0043c were found less abundant in lineage 7 (L7), however the phenotypic outcomes of these findings remain unknown (Yimer et al., 2020).

Xenobiotic response element (XRE) family transcriptional regulators

XRE response element are among the most widespread regulatory elements in bacteria. They are characterized by a conserved HTH DNA binding domain at their N-terminus and a highly variable C-terminal region. The *M. tuberculosis* genome encodes for seven members of this family: Rv0023, Rv0465c (RamB), Rv0474, Rv1129 (PrpR), Rv2017, Rv2021, and Rv3849 (EspR).

Rv0023 is a transcription factor modulating nearly 900 genes, and its regulon is enriched for NAD reductases (Rustad et al., 2014). Given the link with NADH/NAD^+ regulation, Gupta and coll. explored the role of Rv0023 in INH and ETO tolerance. The overexpression of Rv0023 conferred increased INH and ETO tolerance in *M. smegmatis* by downregulating the expression of the *ndh* gene, which encodes for a NADH dehydrogenase. This leads to increased NADH cellular concentration and subsequent inhibition of drug-NAD^+ adducts formation, which are essential for INH activity (Gupta et al., 2020). Furthermore, the study found that Rv0023 is also a negative regulator of *whiB5*.

Rv0465c (RamB), and Rv1129 (PrpR) are involved in the regulation of propionate and acetate metabolisms, respectively. A link between the two metabolic pathways and conditional

drug tolerance has been established; however, its biological meaning remains unclear and needs further elucidation (Hicks et al., 2018; Tang et al., 2019; Hicks et al., 2020).

rv2017 was found to be deleted or disrupted by IS*6110* in several drug resistant isolates; however, the link between this gene and specific drug resistant phenotype has yet to be defined (Klopper et al., 2020; Perdigão et al., 2020; Antoine et al., 2021). Rv3849 (RspR) was predicted to regulate *ponA1*, a gene involved in cell wall synthesis relevant for altered fitness in *M. tuberculosis* during RIF treatment (Farhat et al., 2013; Kieser et al., 2015; Kieser et al., 2015). The remaining XRE transcription regulators in *M. tuberculosis* are uncharacterized for their role in DR.

MarR family transcriptional regulators

There are at least nine genes in the genome of *M. tuberculosis* annotated as MarR-like proteins. One of the most studied MarR-like family transcriptional regulator is Rv0678. This is a transcriptional repressor of the *mmpS5-mmpL5* operon, which encodes an efflux pump able to transport BDQ and CFZ (Milano et al., 2009; Andries et al., 2014). Mutations in *rv0678* affecting its binding activity to the promoter region of the *mmpS5-mmpL5* operon are relevant markers of BDQ and CFZ resistance in *M. tuberculosis* (Kadura et al., 2020). Peterson and coll. described BDQ tolerance mediated by Rv0880 and Rv0324, regulators belonging to the MarR and ArsR family, respectively (Peterson et al., 2016). Knockout mutant strains for *rv0324* and *rv0880* showed hypersensitivity to BDQ, without affecting the susceptibility to other antimycobacterial drugs with unrelated mechanisms of action. Drug tolerance is mediated by the transcriptional cascades modulated by the two transcription factors rather than being caused by genetic mutations. Interestingly, the Rv0324 regulon correlates with nutrient-limited stress condition, which has important implications since BDQ killing depends upon glycolytic pathways (Mackenzie et al., 2020).

Resistance to a novel pyrido-benzimidazole with potent mycobactericidal activity was found to be mediated by mutations in the gene encoding the MarR-like Rv2887 transcription factor (Warrier et al., 2016; Gao et al., 2017). Mutations in this gene negatively affects the ability of the transcription factor to bind its target DNA sequences, ultimately leading to the upregulation of downstream genes. Among them, *rv0560c* was found to encode for a benzoquinone methyltransferase able to N-methylate and thus inactivate the pyrido-benzimidazole compound (Warrier et al., 2016). Mutations in Rv2887 were also found to abrogate susceptibility to a new imidazopyridine-based drug candidate (Winglee et al., 2015). In this case, DR is mediated by the upregulation of efflux pumps yet to be further identified.

Rv2327 has been hypothesized to participate in INH antibiotic response given its role in the regulation of *fbpA* and *fbpC* (encoding antigen 85 complex A and C, respectively), which are involved in cell wall biosynthesis and over-expressed in response to INH treatment (Nguyen et al., 2005; Romero et al., 2010). However, the putative mechanism (e.g. direct interaction Rv2327-INH or other) has not been further explored. Other members of the family have not been directly linked with DR in *M. tuberculosis* so far.

TetR family transcriptional regulators

Regulators of the TetR family usually repress transcription binding their target DNA sequence through a conserved HTH motif present at their N-terminus. Target DNA is released following a structural change of the protein caused by the binding of a specific effector molecule to a ligand-binding pocket situated at the C-terminus of the protein (Balhana et al., 2015). Among the pathways under the control of these regulators, drug efflux is probably the most studied. The TetR family regulator Rv3066 was described to repress the transcription of *rv3065 (mmr)*, a small multidrug resistance (SMR) efflux pump (Bolla et al., 2012). Similarly, Rv1219c was reported to regulate the transcription of the ATP-binding cassette (ABC) transporter encoded by *rv1217c-rv1218c* (Kumar et al., 2014). Both the SMR and ABC transporters have been found overexpressed in MDR clinical isolates, however experiments with knockout strains, recently challenged their role in direct transport of relevant anti-TB drugs (Wang et al., 2013; Shahi et al., 2021; Remm et al., 2022). A recent study matching genomic mutations and increased MIC levels to several drugs identified mutations mapping in the low-affinity binding domain of Rv1219c associated with increased MIC for INH, thus proposing that the repression of the *rv1217c-rv1218c* is somehow linked with increased levels of resistance to this first-line drug (Consortium et al., 2021).

Three members of these transcriptional regulators have been directly linked with DR: (*i*) Rv0275 (InbR) is able to directly interact with INH, and its overexpression is associated with increased resistance to this drug, whereas knockout mutants showed increased susceptibility to several anti-TB drugs (Yang et al., 2018). Genes involved in the INH pathway such as *iniABC* were shown to belong to its regulon; (*ii*) *Rv3855* (EthR) is well-known for its regulatory role on *ethA* (*rv3854c*), which encodes a Baeyer-Villager monooxygenase involved in the activation of ETO (Engohang-Ndong et al., 2004). Despite the role of mutations affecting *ethA* in ETO resistance is evident (Ushtanit et al., 2022), mutations in its transcriptional regulator seems relatively rare in clinical isolates (da Silva et al., 2018; Mugumbate et al., 2021); (*iii*) Rv0273c (EtbR) is a transcriptional repressor of the *inhA* gene, which encodes the target of INH. EMB can bind EtbR, increasing its repressing activity on *inhA* transcription, thus increasing susceptibility to INH (Zhu et al., 2018).

Further relationships between TetR family transcriptional regulators and DR levels in *M. tuberculosis* have to be discovered yet. Of note, Rv0302, Rv1816, and Rv3249c are predicted to regulate *mmpL3* and *mmpL11*, two relevant drug target candidates (Domenech et al., 2005; Chou et al., 2015; Delmar et al., 2015).

Other transcriptional regulators and post-translational modifications affecting transcriptional factors

Among additional transcriptional regulators reported to affect DR in mycobacteria we can mention the members of the AraC/XylS and the SmtB/ArsR families. The AraC/XylS family of transcription factors includes hundreds of positive regulators (Gallegos et al., 1997; Egan, 2002). In *M. tuberculosis* at least nine members of the AraC/XylS family have been described: Rv0023, Rv0465c (RamB), Rv0474, Rv1129 (PrpR), Rv2017, Rv2021, Rv3082c (VirS), and Rv3849 (EspR). Mutations in *rv3082c* (*virS*) were found to mediate resistance to a new putative antimicrobial compound (defined as thiooxadiazole 3) and ETO by affecting the expression of the *mymA* operon, which is responsible for the activation of these molecules (Grant et al., 2016). Other members of the family are involved in several regulatory functions related to carbon metabolism, stress response, and pathogenesis.

At least 12 ArsR family homologs, including Rv0324, Rv2034 and the metal sensors Rv0827c (KmtR), Rv1994c (CmtR), Rv2358 (SmtB), and Rv3744 (NmtR), have been described in *M. tuberculosis*. Beside the already mentioned role of Rv0324 in BDQ tolerance (Peterson et al., 2016), no further links with DR have been found for this family of transcriptional regulators. A study showed the Rv2034, a regulator of the ArsR family, regulates *whiB7* expression, but its role on the WhiB7 regulon in terms of drug tolerance/resistance has not been further explored (Gao et al., 2012).

Additional transcriptional regulators have been reported to affect DR in mycobacteria. For example, the histone-like Lsr2 protein (encoded by the *rv3597c* gene) is involved in several regulatory functions involving cell wall biosynthesis, transport, and responses to antibiotic treatment. Lsr2 represses INH-mediated induction of *iniBAC* and *efpA* (Colangeli et al., 2007). Interestingly, *iniBAC* is also under the control of another transcriptional regulator: IniR (Rv0339c) (Boot et al., 2017). Similarly, another histone-like protein, HupB (Rv2986c, also known as MDP-1), negatively regulates *katG* expression, thus affecting phenotypic tolerance to INH in *M. tuberculosis* (Niki et al., 2012). Another example is Rv1267c (EmbR), which regulates *embABC* transcription, well-known to be relevant for EMB resistance (Sharma et al., 2006). An additional case of interest relates to CFZ. Li and coll. described a link between the transcriptional regulator Rv1453 and CFZ resistance, where the overexpression of the gene was associated with increased MIC to the drug (Li et al., 2021).

Several transcription factors are modified post-translationally, thus adding an additional layer of control that can modify the interaction between bacteria and drugs. Among those previously cited, EmbR is positively regulated by phosphorylation, whereas HupB acetylation and methylation alter INH susceptibility in mycobacteria (Arora et al., 2021). Similarly, PknB phosphorylates the histon-like protein Lsr2, thus reducing its DNA binding affinity (Alqaseer et al., 2019). Rv3701c (part of the *egt* operon), is under the strict regulation of the serine/threonine-protein kinase PknD, and its phosphorylated form fails to catalyzes ergothioneine biosynthesis (Richard-Greenblatt et al., 2015). Therefore, it is plausible that these additional regulatory layers have a role in fine-tuning redox homeostasis, and thus drug tolerance. Moreover, transcription factors are not universally conserved in the *M. tuberculosis complex*, thus this genetic diversity has to be taken into account systematically when studying genotypic features in relationship with phenotypic drug susceptibility (Köser et al., 2012; Chiner-Oms et al., 2018; Chiner-Oms et al., 2019).

Targeting transcriptional regulation: A new frontier for drug discovery

As DR has become a crucial worldwide problem, new strategies to design innovative generations of antibacterial drugs has been implemented. Targeting transcriptional regulation is one of them. An interesting example is that of EthR. The mycobacterial monoxygenase EthA is the activator of several thiocarbamide-containing drugs, including ETO. Its expression is regulated by EthR, a transcriptional repressor. Synthetic compounds selected to inhibit EthR-DNA interaction have been shown to increase *ethA* expression, boosting bacterial sensitivity to ETO (Willand et al., 2009; Flipo et al., 2012; Nikiforov et al., 2017). Another emerging and promising strategy is targeting riboswitches (Deigan and Ferré-D'Amaré, 2011; Lünse et al., 2014; Dar et al., 2016; Panchal and Brenk, 2021). Metabolite-binding riboswitches are non-coding RNAs that bind specifically to metabolites and regulate downstream gene expression depending on the metabolite concentration. These sequences are composed of two domains: an aptamer that binds the metabolites/ligands, and a response platform that allows the expression or repression of downstream genes. The binding of ligand leads either to transcriptional and/or translational termination of downstream gene expression (switch-OFF) or to the expression of downstream genes (switch-ON) (Bastet et al., 2018; Yadav et al., 2020). Riboswitches occur almost exclusively in prokaryotes and are involved in the metabolism of essential amino acids and metabolites. Although not yet thoroughly studied, some riboswitches are present in pathogenic bacteria, including *M. tuberculosis*, and play an important role in

controlling essential genes. Indeed, many of the classes of riboswitches are fundamental in controlling the expression of genes involved in virulence (Blount and Breaker, 2006; Lünse et al., 2014). Being involved in the expression of essential genes and absent in eukaryotic genomes, riboswitches have become interesting targets for the design of innovative antibacterial strategies. The general idea is to target the pocket recognizing the metabolite to induce a transition of the riboswitch from the ON to the OFF configuration turning off the regulated gene. Riboswitches-based antibacterial drugs could be broad-spectrum, in the case of riboswitches present in an extended range of pathogenic species, or narrow spectrum in the case of species-specific riboswitches (Blount and Breaker, 2006; Panchal and Brenk, 2021). Using this rational some antibacterial compounds targeting riboswitches have already been identified (recently reviewed in [Panchal and Brenk, 2021)]. *M. tuberculosis* is predicted to have at least 16 riboswitches from the Rfam database belonging to different classes (Kalvari et al., 2018). However, only few riboswitches have been validated in this species (Schwenk and Arnvig, 2018) as the cyclic-di-AMP sensing riboswitch regulating *rpfA* (Arnvig and Young, 2012; Nelson et al., 2013), the riboswitch regulating *rpfB* (Schwenk et al., 2018), and the cobalamin-dependent riboswitch responsible for the downregulation of *metE* in the presence of cobalamin (Warner et al., 2007). The latter case is particularly interesting to show the potential importance of riboswitches as drug targets. *M. tuberculosis* has two methionine synthases: MetE, whose expression is repressed by cobalamin and MetH, which requires cobalamin for its functionality. In the clinical isolate CDC1551 MetH is not functional due to a mutation in its structural gene, so MetE is the only methionine synthase in this strain. Warner and coll. (Warner et al., 2007) showed that indeed this strain is unable to grow in the presence of cobalamin due to the repression of *metE* expression. Mutants resistant to cobalamin showed mutations at the level of the riboswitch confirming the role of this regulatory element in *metE* regulation.

Conclusions

Drug exposure represents a stress for the bacterium, which reacts by changing its transcriptional profile thus activating different stress regulons responsible of setting its own basal level of susceptibility to a specific drug. Sometimes, mutations in transcriptional regulators can structurally modify the bacterial transcriptional profile resulting in a constitutive change in the susceptibility to a given drug. Moreover, as outlined above, post-transcriptional regulation can further modify transcriptional regulation.

Understanding the role of transcription factors in DR pathways is critical not only to improve our knowledge in resistance mechanisms or to detect new genetic markers of resistance. Unravelling the transcriptomic networks would indeed open to new therapeutic opportunities. As an example, it has been recently reported how DR in *M. tuberculosis* could give rise to collateral sensitivity to β-lactam drugs, where basically anti-TB drugs induces the expression of the gene encoding the transcriptional repressor BlaI and its downstream genes *atpH*, and *sigC*, which ultimately inhibits intrinsic β-lactam resistance (Trigos et al., 2021). Finally, small non-coding RNAs and riboswitches have been described to modulate antibiotic tolerance, and resistance in several bacteria (Dar et al., 2016; Dersch et al., 2017; Zhang et al., 2020). The role (if any) in DR of transcriptional and/or post-transcriptional regulation mediated by these molecules in *M. tuberculosis* has not been elucidated yet and requires further investigation.

Author contributions

PM: Writing - original draft, conceptualization, investigation. RS: Writing - original draft, visualization, investigation. SG; Writing - review & editing, investigation. RP: Writing - review & editing. DMC: Writing - review & editing. RM: Writing - original draft, conceptualization, investigation, supervision. All authors contributed to the article and approved the submitted version.

Funding

RM laboratory is founded from the Innovative Medicines Initiative 2 Joint Undertaking (JU) under grant agreement no 853989. RS was supported by the Italian Ministry of Health "Ricerca Finalizzata 2016" under grant agreement GR-2016-02364014 to PM.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Adams, K. N., Takaki, K., Connolly, L. E., Wiedenhoft, H., Winglee, K., Humbert, O., et al. (2011). Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell* 145, 39–53. doi: 10.1016/j.cell.2011.02.022

Albesa-Jové, D., Comino, N., Tersa, M., Mohorko, E., Urresti, S., Dainese, E., et al. (2015). The redox state regulates the conformation of Rv2466c to activate the antitubercular prodrug TP053. *J. Biol. Chem.* 290, 31077–31089. doi: 10.1074/jbc.M115.677039

Alqaseer, K., Turapov, O., Barthe, P., Jagatia, H., De Visch, A., Roumestand, C., et al. (2019). Protein kinase b controls *Mycobacterium tuberculosis* growth *via* phosphorylation of the transcriptional regulator Lsr2 at threonine 112. *Mol. Microbiol.* 112, 1847–1862. doi: 10.1111/mmi.14398

Andries, K., Villellas, C., Coeck, N., Thys, K., Gevers, T., Vranckx, L., et al. (2014). Acquired resistance of *Mycobacterium tuberculosis* to bedaquiline. *PloS One* 9, e102135. doi: 10.1371/journal.pone.0102135

Antoine, R., Gaudin, C., and Hartkoorn, R. C. (2021). Intragenic distribution of IS6110 in clinical *Mycobacterium tuberculosis* strains: Bioinformatic evidence for gene disruption leading to underdiagnosed antibiotic resistance. *Microbiol. Spectr.* 9, e0001921. doi: 10.1128/Spectrum.00019-21

Arnvig, K., and Young, D. (2012). Non-coding RNA and its potential role in *Mycobacterium tuberculosis* pathogenesis. *RNA Biol.* 9, 427–436. doi: 10.4161/rna.20105

Arora, G., Bothra, A., Prosser, G., Arora, K., and Sajid, A. (2021). Role of post-translational modifications in the acquisition of drug resistance in *Mycobacterium tuberculosis*. *FEBS J.* 288, 3375–3393. doi: 10.1111/febs.15582

Balázsi, G., Heath, A. P., Shi, L., and Gennaro, M. L. (2008). The temporal response of the *Mycobacterium tuberculosis* gene regulatory network during growth arrest. *Mol. Syst. Biol.* 4, 225. doi: 10.1038/msb.2008.63

Balhana, R. J., Singla, A., Sikder, M. H., Withers, M., and Kendall, S. L. (2015). Global analyses of TetR family transcriptional regulators in mycobacteria indicates conservation across species and diversity in regulated functions. *BMC Genomics* 16, 479. doi: 10.1186/s12864-015-1696-9

Bansal, R., Anil Kumar, V., Sevalkar, R. R., Singh, P. R., and Sarkar, D. (2017). *Mycobacterium tuberculosis* virulence-regulator PhoP interacts with alternative sigma factor SigE during acid-stress response. *Mol. Microbiol.* 104, 400–411. doi: 10.1111/mmi.13635

Bastet, L., Turcotte, P., Wade, J. T., and Lafontaine, D. A. (2018). Maestro of regulation: Riboswitches orchestrate gene expression at the levels of translation, transcription and mRNA decay. *RNA Biol.* 15, 679–682. doi: 10.1080/15476286.2018.1451721

Blount, K. F., and Breaker, R. R. (2006). Riboswitches as antibacterial drug targets. *Nat. Biotechnol.* 24, 1558–1564. doi: 10.1038/nbt1268

Boldrin, F., Cioetto Mazzabò, L., Anoosheh, S., Palù, G., Gaudreau, L., Manganelli, R., et al. (2019). Assessing the role of Rv1222 (RseA) as an anti-sigma factor of the *Mycobacterium tuberculosis* extracytoplasmic sigma factor SigE. *Sci. Rep.* 9, 4513. doi: 10.1038/s41598-019-41183-4

Bolla, J. R., Do, S. V., Long, F., Dai, L., Su, C. C., Lei, H. T., et al. (2012). Structural and functional analysis of the transcriptional regulator Rv3066 of *Mycobacterium tuberculosis*. *Nucleic Acids Res.* 40, 9340–9355. doi: 10.1093/nar/gks677

Boot, M., van Winden, V. J. C., Sparrius, M., van de Weerd, R., Speer, A., Ummels, R., et al. (2017). Cell envelope stress in mycobacteria is regulated by the novel signal transduction ATPase IniR in response to trehalose. *PLoS Genet.* 13, e1007131. doi: 10.1371/journal.pgen.1007131

Buriánková, K., Doucet-Populaire, F., Dorson, O., Gondran, A., Ghnassia, J. C., Weiser, J., et al. (2004). Molecular basis of intrinsic macrolide resistance in the *Mycobacterium tuberculosis* complex. *Antimicrob. Agents Chemother.* 48, 143–150. doi: 10.1128/AAC.48.1.143-150.2004

Burian, J., Ramón-García, S., Howes, C. G., and Thompson, C. J. (2012). WhiB7, a transcriptional activator that coordinates physiology with intrinsic drug resistance in *Mycobacterium tuberculosis*. *Expert Rev. Anti Infect. Ther.* 10, 1037–1047. doi: 10.1586/eri.12.90

Burian, J., Yim, G., Hsing, M., Axerio-Cilies, P., Cherkasov, A., Spiegelman, G. B., et al. (2013). The mycobacterial antibiotic resistance determinant WhiB7 acts as a transcriptional activator by binding the primary sigma factor SigA (RpoV). *Nucleic Acids Res.* 41, 10062–10076. doi: 10.1093/nar/gkt751

Bush, M. J. (2018). The actinobacterial WhiB-like (Wbl) family of transcription factors. *Mol. Microbiol.* 110, 663–676. doi: 10.1111/mmi.14117

Chatterjee, A., Saranath, D., Bhatter, P., and Mistry, N. (2013). Global transcriptional profiling of longitudinal clinical isolates of *Mycobacterium tuberculosis* exhibiting rapid accumulation of drug resistance. *PloS One* 8, e54717. doi: 10.1371/journal.pone.0054717

Chauhan, N. K., Anand, A., Sharma, A., Dhiman, K., Gosain, T. P., Singh, P., et al. (2021). Structural and functional characterization of Rv0792c from *Mycobacterium tuberculosis*: identifying small molecule inhibitors against GntR protein. *bioRxiv*. doi: 10.1101/2021.09.17.460839

Chen, P., Ruiz, R. E., Li, Q., Silver, R. F., and Bishai, W. R. (2000). Construction and characterization of a *Mycobacterium tuberculosis* mutant lacking the alternate sigma factor gene, sigF. *Infect. Immun.* 68, 5575–5580. doi: 10.1128/IAI.68.10.5575-5580.2000

Chiner-Oms, Á, Berney, M., Boinett, C., González-Candelas, F., Young, D. B., Gagneux, S., et al. (2019). Genome-wide mutational biases fuel transcriptional diversity in the *Mycobacterium tuberculosis* complex. *Nat. Commun.* 10, 3994. doi: 10.1038/s41467-019-11948-6

Chiner-Oms, Á, González-Candelas, F., and Comas, I. (2018). Gene expression models based on a reference laboratory strain are poor predictors of *Mycobacterium tuberculosis* complex transcriptional diversity. *Sci. Rep.* 8, 3813. doi: 10.1038/s41598-018-22237-5

Chou, T. H., Delmar, J. A., Wright, C. C., Kumar, N., Radhakrishnan, A., Doh, J. K., et al. (2015). Crystal structure of the *Mycobacterium tuberculosis* transcriptional regulator Rv0302. *Protein Sci.* 24, 1942–1955. doi: 10.1002/pro.2802

Colangeli, R., Helb, D., Vilchèze, C., Hazbón, M. H., Lee, C. G., Safi, H., et al. (2007). Transcriptional regulation of multi-drug tolerance and antibiotic-induced responses by the histone-like protein Lsr2 in *M. tuberculosis*. *PLoS Pathog.* 3, e87. doi: 10.1371/journal.ppat.0030087

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., et al. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393, 537–544. doi: 10.1038/31159

The CRyPTIC Consortium. Earle, S. G., Wilson, D. J. (2022). Genome-wide association studies of global *Mycobacterium tuberculosis* resistance to 13 antimicrobials in 10,228 genomes identify new resistance mechanisms. *PLoS Biol.* 20 (8), e3001755. doi: 10.1371/journal.pbio.3001755

Corona, F., and Martinez, J. L. (2013). Phenotypic resistance to antibiotics. *Antibiotics (Basel)* 2, 237–255. doi: 10.3390/antibiotics2020237

Dar, D., Shamir, M., Mellin, J. R., Koutero, M., Stern-Ginossar, N., Cossart, P., et al. (2016). Term-seq reveals abundant ribo-regulation of antibiotics resistance in bacteria. *Science* 352, aad9822. doi: 10.1126/science.aad9822

da Silva, D. A., Ferreira, N. V., Rego, A. M., Barbosa, P. C. P., Machado, R. F., Pimentel, A., et al. (2018). Integrated analysis of ethionamide resistance loci in *Mycobacterium tuberculosis* clinical isolates. *Tuberculosis* 113, 163–174. doi: 10.1016/j.tube.2018.08.010

Deigan, K. E., and Ferré-D'Amaré, A. R. (2011). Riboswitches: discovery of drugs that target bacterial gene-regulatory RNAs. *Acc Chem. Res.* 44, 1329–1338. doi: 10.1021/ar200039b

Delmar, J. A., Chou, T. H., Wright, C. C., Licon, M. H., Doh, J. K., Radhakrishnan, A., et al. (2015). Structural basis for the regulation of the MmpL transporters of *Mycobacterium tuberculosis*. *J. Biol. Chem.* 290, 28559–28574. doi: 10.1074/jbc.M115.683797

Deng, W., Zheng, Z., Chen, Y., Yang, M., Yan, J., Li, W., et al. (2022). Deficiency of GntR family regulator MSMEG_5174 promotes Mycobacterium smegmatis resistance to aminoglycosides via manupulating purine metabolism. *Front. Microbiol.* 13, 919538. doi: 10.3389/fmicb.2022.919538

Dersch, P., Khan, M. A., Mühlen, S., and Görke, B. (2017). Roles of regulatory RNAs for antibiotic resistance in bacteria and their potential value as novel drug targets. *Front. Microbiol.* 8, 803. doi: 10.3389/fmicb.2017.00803

Domenech, P., Reed, M. B., and Barry, C. E. (2005). Contribution of the *Mycobacterium tuberculosis* MmpL protein family to virulence and drug resistance. *Infect. Immun.* 73, 3492–3501. doi: 10.1128/IAI.73.6.3492-3501.2005

Duan, W., Li, X., Ge, Y., Yu, Z., Li, P., Li, J., et al. (2019). *Mycobacterium tuberculosis* Rv1473 is a novel macrolides ABC efflux pump regulated by WhiB7. *Future Microbiol.* 14, 47–59. doi: 10.2217/fmb-2018-0207

Egan, S. M. (2002). Growing repertoire of AraC/XylS activators. *J. Bacteriol.* 184, 5529–5532. doi: 10.1128/JB.184.20.5529-5532.2002

Engohang-Ndong, J., Baillat, D., Aumercier, M., Bellefontaine, F., Besra, G. S., Locht, C., et al. (2004). EthR, a repressor of the TetR/CamR family implicated in ethionamide resistance in mycobacteria, octamerizes cooperatively on its operator. *Mol. Microbiol.* 51, 175–188. doi: 10.1046/j.1365-2958.2003.03809.x

Farhat, M. R., Freschi, L., Calderon, R., Ioerger, T., Snyder, M., Meehan, C. J., et al. (2019). GWAS for quantitative resistance phenotypes in *Mycobacterium tuberculosis* reveals resistance genes and regulatory regions. *Nat. Commun.* 10, 2128. doi: 10.1038/s41467-019-10110-6

Farhat, M. R., Shapiro, B. J., Kieser, K. J., Sultana, R., Jacobson, K. R., Victor, T. C., et al. (2013). Genomic analysis identifies targets of convergent positive selection

in drug-resistant *Mycobacterium tuberculosis*. *Nat. Genet.* 45, 1183–1189. doi: 10.1038/ng.2747

Flipo, M., Willand, N., Lecat-Guillet, N., Hounsou, C., Desroses, M., Leroux, F., et al. (2012). Discovery of novel n-phenylphenoxyacetamide derivatives as EthR inhibitors and ethionamide boosters by combining high-throughput screening and synthesis. *J. Med. Chem.* 55, 6391–6402. doi: 10.1021/jm300377g

Gallegos, M. T., Schleif, R., Bairoch, A., Hofmann, K., and Ramos, J. L. (1997). Arac/XylS family of transcriptional regulators. *Microbiol. Mol. Biol. Rev.* 61, 393–410. doi: 10.1128/mmbr.61.4.393-410.1997

Gao, Y. R., Li, D. F., Fleming, J., Zhou, Y. F., Liu, Y., Deng, J. Y., et al. (2017). Structural analysis of the regulatory mechanism of MarR protein Rv2887 in *M. tuberculosis*. *Sci. Rep.* 7, 6471. doi: 10.1038/s41598-017-01705-4

Gao, C. H., Yang, M., and He, Z. G. (2012). Characterization of a novel ArsR-like regulator encoded by Rv2034 in *Mycobacterium tuberculosis*. *PLoS One* 7, e36255. doi: 10.1371/journal.pone.0036255

Geiman, D. E., Raghunand, T. R., Agarwal, N., and Bishai, W. R. (2006). Differential gene expression in response to exposure to antimycobacterial agents and other stress conditions among seven *Mycobacterium tuberculosis* whiB-like genes. *Antimicrob. Agents Chemother.* 50, 2836–2841. doi: 10.1128/AAC.00295-06

Grant, S. S., Wellington, S., Kawate, T., Desjardins, C. A., Silvis, M. R., Wivagg, C., et al. (2016). Baeyer-villiger monooxygenases EthA and MymA are required for activation of replicating and non-replicating *Mycobacterium tuberculosis* inhibitors. *Cell Chem. Biol.* 23, 666–677. doi: 10.1016/j.chembiol.2016.05.011

Gupta, S. K., Angara, R. K., Yousuf, S., Reddy, C. G., and Ranjan, A. (2020). Ectopic Expression of Rv0023 Mediates Isoniazid/Ethionamide Tolerance *via* Altering NADH/NAD+ in *Mycobacterium smegmatis*. *Front. Microbiol.* 11, 3. doi: 10.3389/fmicb.2020.00003

Gurumurthy, M., Rao, M., Mukherjee, T., Rao, S. P., Boshoff, H. I., Dick, T., et al. (2013). A novel F(420) -dependent anti-oxidant mechanism protects *Mycobacterium tuberculosis* against oxidative stress and bactericidal agents. *Mol. Microbiol.* 87, 744–755. doi: 10.1111/mmi.12127

Hartkoorn, R. C., Sala, C., Magnet, S. J., Chen, J. M., Pojer, F., and Cole, S. T. (2010). Sigma factor f does not prevent rifampin inhibition of RNA polymerase or cause rifampin tolerance in *Mycobacterium tuberculosis*. *J. Bacteriol* 192, 5472–5479. doi: 10.1128/JB.00687-10

Hicks, N. D., Giffen, S. R., Culviner, P. H., Chao, M. C., Dulberger, C. L., Liu, Q., et al. (2020). Mutations in dnaA and a cryptic interaction site increase drug resistance in *Mycobacterium tuberculosis*. *PloS Pathog.* 16, e1009063. doi: 10.1371/journal.ppat.1009063

Hicks, N. D., Yang, J., Zhang, X., Zhao, B., Grad, Y. H., Liu, L., et al. (2018). Clinically prevalent mutations in *Mycobacterium tuberculosis* alter propionate metabolism and mediate multidrug tolerance. *Nat. Microbiol.* 3, 1032–1042. doi: 10.1038/s41564-018-0218-3

Homolka, S., Niemann, S., Russell, D. G., and Rohde, K. H. (2010). Functional genetic diversity among *Mycobacterium tuberculosis* complex clinical isolates: delineation of conserved core and lineage-specific transcriptomes during intracellular survival. *PloS Pathog.* 6, e1000988. doi: 10.1371/journal.ppat.1000988

Hurst-Hess, K., Rudra, P., and Ghosh, P. (2017). *Mycobacterium abscessus* WhiB7 regulates a species-specific repertoire of genes to confer extreme antibiotic resistance. *Antimicrob. Agents Chemother.* 11, 61. doi: 10.1128/AAC.01347-17

Hu, J., Zhao, L., and Yang, M. (2015). A GntR family transcription factor positively regulates mycobacterial isoniazid resistance by controlling the expression of a putative permease. *BMC Microbiol.* 15, 214. doi: 10.1186/s12866-015-0556-8

Kadura, S., King, N., Nakhoul, M., Zhu, H., Theron, G., Köser, C. U., et al. (2020). Systematic review of mutations associated with resistance to the new and repurposed *Mycobacterium tuberculosis* drugs bedaquiline, clofazimine, linezolid, delamanid and pretomanid. *J. Antimicrob. Chemother.* 75, 2031–2043. doi: 10.1093/jac/dkaa136

Kalvari, I., Nawrocki, E. P., Argasinska, J., Quinones-Olvera, N., Finn, R. D., Bateman, A., et al. (2018). Non-coding RNA analysis using the rfam database. *Curr. Protoc. Bioinf.* 62, e51. doi: 10.1002/cpbi.51

Kieser, K. J., Baranowski, C., Chao, M. C., Long, J. E., Sassetti, C. M., Waldor, M. K., et al. (2015). Peptidoglycan synthesis in *Mycobacterium tuberculosis* is organized into networks with varying drug susceptibility. *Proc. Natl. Acad. Sci. U.S.A.* 112, 13087–13092. doi: 10.1073/pnas.1514135112

Kieser, K. J., Boutte, C. C., Kester, J. C., Baer, C. E., Barczak, A. K., Meniche, X., et al. (2015). Phosphorylation of the peptidoglycan synthase PonA1 governs the rate of polar elongation in mycobacteria. *PloS Pathog.* 11, e1005010. doi: 10.1371/journal.ppat.1005010

Kim, K. H., An, D. R., Song, J., Yoon, J. Y., Kim, H. S., Yoon, H. J., et al. (2012). *Mycobacterium tuberculosis* eis protein initiates suppression of host immune responses by acetylation of DUSP16/MKP-7. *Proc. Natl. Acad. Sci. U.S.A.* 109, 7729–7734. doi: 10.1073/pnas.1120251109

Klopper, M., Heupink, T. H., Hill-Cawthorne, G., Streicher, E. M., Dippenaar, A., de Vos, M., et al. (2020). A landscape of genomic alterations at the root of a near-untreatable tuberculosis epidemic. *BMC Med.* 18, 24. doi: 10.1186/s12916-019-1487-2

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., and Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 130, 797–810. doi: 10.1016/j.cell.2007.06.049

Köser, C. U., Bryant, J. M., Parkhill, J., and Peacock, S. J. (2013). Consequences of whiB7 (Rv3197A) mutations in Beijing genotype isolates of the *Mycobacterium tuberculosis* complex. *Antimicrob. Agents Chemother.* 57, 3461. doi: 10.1128/AAC.00626-13

Köser, C. U., Feuerriegel, S., Summers, D. K., Archer, J. A., and Niemann, S. (2012). Importance of the genetic diversity within the *Mycobacterium tuberculosis* complex for the development of novel antibiotics and diagnostic tests of drug resistance. *Antimicrob. Agents Chemother.* 56, 6080–6087. doi: 10.1128/AAC.01641-12

Kumar, N., Radhakrishnan, A., Wright, C. C., Chou, T. H., Lei, H. T., Bolla, J. R., et al. (2014). Crystal structure of the transcriptional regulator Rv1219c of *Mycobacterium tuberculosis*. *Protein Sci.* 23, 423–432. doi: 10.1002/pro.2424

Larsson, C., Luna, B., Ammerman, N. C., Maiga, M., Agarwal, N., and Bishai, W. R. (2012). Gene expression of *Mycobacterium tuberculosis* putative transcription factors whiB1-7 in redox environments. *PloS One* 7, e37516. doi: 10.1371/journal.pone.0037516

Lee, J. H., Ammerman, N. C., Nolan, S., Geiman, D. E., Lun, S., Guo, H., et al. (2012). Isoniazid resistance without a loss of fitness in *Mycobacterium tuberculosis*. *Nat. Commun.* 3, 753. doi: 10.1038/ncomms1724

Li, Y., Fu, L., Zhang, W., Chen, X., and Lu, Y. (2021). The transcription factor Rv1453 regulates the expression of qor and confers resistant to clofazimine in *Mycobacterium tuberculosis*. *Infect. Drug Resist.* 14, 3937–3948. doi: 10.2147/IDR.S324043

Lilic, M., Darst, S. A., and Campbell, E. A. (2021). Structural basis of transcriptional activation by the *Mycobacterium tuberculosis* intrinsic antibiotic-resistance transcription factor WhiB7. *Mol. Cell* 81, 2875–2886, e2875. doi: 10.1016/j.molcel.2021.05.017

Li, S., Poulton, N. C., Chang, J. S., Azadian, Z. A., DeJesus, M. A., Ruecker, N., et al. (2022). CRISPRi chemical genetics and comparative genomics identify genes mediating drug potency in *Mycobacterium tuberculosis*. *Nat. Microbiol.* 7, 766–779. doi: 10.1038/s41564-022-01130-y

Liu, J., Shi, W., Zhang, S., Hao, X., Maslov, D. A., Shur, K. V., et al. (2019). Mutations in efflux pump Rv1258c (Tap) cause resistance to pyrazinamide, isoniazid, and streptomycin in M. tuberculosis. *Front. Microbiol.* 10, 216. doi: 10.3389/fmicb.2019.00216

Liu, Q., Via, L. E., Luo, T., Liang, L., Liu, X., Wu, S., et al. (2015). Within patient microevolution of *Mycobacterium tuberculosis* correlates with heterogeneous responses to treatment. *Sci. Rep.* 5, 17507. doi: 10.1038/srep17507

Lünse, C. E., Schüller, A., and Mayer, G. (2014). The promise of riboswitches as potential antibacterial drug targets. *Int. J. Med. Microbiol.* 304, 79–92. doi: 10.1016/j.ijmm.2013.09.002

Mackenzie, J. S., Lamprecht, D. A., Asmal, R., Adamson, J. H., Borah, K., Beste, D. J. V., et al. (2020). Bedaquiline reprograms central metabolism to reveal glycolytic vulnerability in *Mycobacterium tuberculosis*. *Nat. Commun.* 11, 6092. doi: 10.1038/s41467-020-19959-4

Manganelli, R., and Provvedi, R. (2010). An integrated regulatory network including two positive feedback loops to modulate the activity of sigma(E) in mycobacteria. *Mol. Microbiol.* 75, 538–542. doi: 10.1111/j.1365-2958.2009.07009.x

Manganelli, R., Voskuil, M. I., Schoolnik, G. K., Dubnau, E., Gomez, M., and Smith, I. (2002). Role of the extracytoplasmic-function sigma factor sigma(H) in *Mycobacterium tuberculosis* global gene expression. *Mol. Microbiol.* 45, 365–374. doi: 10.1046/j.1365-2958.2002.03005.x

Martínez, S. R., Durantini, A. M., Becerra, M. C., and Cosa, G. (2020). Real-time single-cell imaging reveals accelerating lipid peroxyl radical formation in *Escherichia coli* Triggered by a Fluoroquinolone Antibiotic. *ACS Infect. Dis.* 6, 2468–2477. doi: 10.1021/acsinfecdis.0c00317

Mavi, P. S., Singh, S., and Kumar, A. (2020). Reductive stress: New insights in physiology and drug tolerance of *Mycobacterium*. *Antioxid Redox Signal* 32, 1348–1366. doi: 10.1089/ars.2019.7867

Michele, T. M., Ko, C., and Bishai, W. R. (1999). Exposure to antibiotics induces expression of the *Mycobacterium tuberculosis* sigF gene: implications for chemotherapy against mycobacterial persistors. *Antimicrob. Agents Chemother.* 43, 218–225. doi: 10.1128/AAC.43.2.218

Milano, A., Pasca, M. R., Provvedi, R., Lucarelli, A. P., Manina, G., Ribeiro, A. L., et al. (2009). Azole resistance in *Mycobacterium tuberculosis* is mediated by the MmpS5-MmpL5 efflux system. *Tuberculosis (Edinb)* 89, 84–90. doi: 10.1016/j.tube.2008.08.003

Mishra, S., Shukla, P., Bhaskar, A., Anand, K., Baloni, P., Jha, R. K., et al. (2017). Efficacy of β-lactam/β-lactamase inhibitor combination is linked to WhiB4-mediated changes in redox physiology of *Mycobacterium tuberculosis*. *Elife* 26, 6. doi: 10.7554/eLife.25624.037

Morris, R. P., Nguyen, L., Gatfield, J., Visconti, K., Nguyen, K., Schnappinger, D., et al. (2005). Ancestral antibiotic resistance in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12200–12205. doi: 10.1073/pnas.0505446102

Mugumbate, G., Nyathi, B., Zindoga, A., and Munyuki, G. (2021). Application of computational methods in understanding mutations in *Mycobacterium tuberculosis drug resistance*. *Front. Mol. Biosci.* 8, 643849. doi: 10.3389/fmolb.2021.643849

Nelson, J. W., Sudarsan, N., Furukawa, K., Weinberg, Z., Wang, J. X., and Breaker, R. R. (2013). Riboswitches in eubacteria sense the second messenger c-di-AMP. *Nat. Chem. Biol.* 9, 834–839. doi: 10.1038/nchembio.1363

Nguyen, L., Chinnapapagari, S., and Thompson, C. J. (2005). FbpA-dependent biosynthesis of trehalose dimycolate is required for the intrinsic multidrug resistance, cell wall structure, and colonial morphology of *Mycobacterium smegmatis*. *J. Bacteriol.* 187, 6603–6611. doi: 10.1128/JB.187.19.6603-6611.2005

Nikiforov, P. O., Blaszczyk, M., Surade, S., Boshoff, H. I., Sajid, A., Delorme, V., et al. (2017). Fragment-sized EthR inhibitors exhibit exceptionally strong ethionamide boosting effect in whole-cell *Mycobacterium tuberculosis* assays. *ACS Chem. Biol.* 12, 1390–1396. doi: 10.1021/acschembio.7b00091

Niki, M., Tateishi, Y., Ozeki, Y., Kirikae, T., Lewin, A., Inoue, Y., et al. (2012). A novel mechanism of growth phase-dependent tolerance to isoniazid in mycobacteria. *J. Biol. Chem.* 287, 27743–27752. doi: 10.1074/jbc.M111.333385

Panchal, V., and Brenk, R. (2021). Riboswitches as Drug Targets for Antibiotics. *Antibiotics*. 10, 45. doi: 10.3390/antibiotics10010045

Perdigão, J., Gomes, P., Miranda, A., Maltez, F., Machado, D., Silva, C., et al. (2020). Using genomics to understand the origin and dispersion of multidrug and extensively drug resistant tuberculosis in Portugal. *Sci. Rep.* 10, 2600. doi: 10.1038/s41598-020-59558-3

Peterson, E. J. R., Ma, S., Sherman, D. R., and Baliga, N. S. (2016). Network analysis identifies Rv0324 and Rv0880 as regulators of bedaquiline tolerance in *Mycobacterium tuberculosis*. *Nat. Microbiol.* 1, 16078. doi: 10.1038/nmicrobiol.2016.78

Piccaro, G., Pietraforte, D., Giannoni, F., Mustazzolu, A., and Fattorini, L. (2014). Rifampin induces hydroxyl radical formation in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 58, 7527–7533. doi: 10.1128/AAC.03169-14

Pisu, D., Provvedi, R., Espinosa, D. M., Payan, J. B., Boldrin, F., Palù, G., et al. (2017). The alternative sigma factors SigE and SigB are involved in tolerance and persistence to antitubercular drugs. *Antimicrob. Agents Chemother.* 12, 61. doi: 10.1128/AAC.01596-17

Provvedi, R., Boldrin, F., Falciani, F., Palù, G., and Manganelli, R. (2009). Global transcriptional response to vancomycin in *Mycobacterium tuberculosis*. *Microbiology* 155, 1093–1102. doi: 10.1099/mic.0.024802-0

Pryjma, M., Burian, J., Kuchinski, K., and Thompson, C. J. (2017). Antagonism between front-line antibiotics clarithromycin and amikacin in the treatment of *Mycobacterium abscessus* infections is mediated by the whiB7 gene. *Antimicrob. Agents Chemother.* 11, 61. doi: 10.1128/AAC.01353-17

Ramón-García, S., Mick, V., Dainese, E., Martín, C., Thompson, C. J., De Rossi, E., et al. (2012). Functional and genetic characterization of the tap efflux pump in *Mycobacterium bovis* BCG. *Antimicrob. Agents Chemother.* 56, 2074–2083. doi: 10.1128/AAC.05946-11

Ramón-García, S., Ng, C., Jensen, P. R., Dosanjh, M., Burian, J., Morris, R. P., et al. (2013). WhiB7, an Fe-S-dependent transcription factor that activates species-specific repertoires of drug resistance determinants in actinobacteria. *J. Biol. Chem.* 288, 34514–34528. doi: 10.1074/jbc.M113.516385

Rao, M., Liu, H., Yang, M., Zhao, C., and He, Z. G. (2012). A copper-responsive global repressor regulates expression of diverse membrane-associated transporters and bacterial drug resistance in mycobacteria. *J. Biol. Chem.* 287, 39721–39731. doi: 10.1074/jbc.M112.383604

Reeves, A. Z., Campbell, P. J., Sultana, R., Malik, S., Murray, M., Plikaytis, B. B., et al. (2013). Aminoglycoside cross-resistance in *Mycobacterium tuberculosis* due to mutations in the 5' untranslated region of whiB7. *Antimicrob. Agents Chemother.* 57, 1857–1865. doi: 10.1128/AAC.02191-12

Remm, S., Earp, J. C., Dick, T., Dartois, V., and Seeger, M. A. (2022). Critical discussion on drug efflux in *Mycobacterium tuberculosis*. *FEMS Microbiol. Rev.* 09, 46. doi: 10.1093/femsre/fuab050

Richard-Greenblatt, M., Bach, H., Adamson, J., Peña-Diaz, S., Li, W., Steyn, A. J., et al. (2015). Regulation of ergothioneine biosynthesis and its effect on *Mycobacterium tuberculosis* growth and infectivity. *J. Biol. Chem.* 290, 23064–23076. doi: 10.1074/jbc.M115.648642

Rodrigue, S., Provvedi, R., Jacques, P. E., Gaudreau, L., and Manganelli, R. (2006). The sigma factors of *Mycobacterium tuberculosis*. *FEMS Microbiol. Rev.* 30, 926–941. doi: 10.1111/j.1574-6976.2006.00040.x

Rodríguez, J. G., Hernández, A. C., Helguera-Repetto, C., Aguilar Ayala, D., Guadarrama-Medina, R., Anzóla, J. M., et al. (2014). Global adaptation to a lipid environment triggers the dormancy-related phenotype of *Mycobacterium tuberculosis*. *mBio* 5, e01125–e01114. doi: 10.1128/mBio.01125-14

Romero, I. C., Mehaffy, C., Burchmore, R. J., Dobos-Elder, K., Brennan, P., and Walker, J. (2010). Identification of promoter-binding proteins of the fbp a and c genes in *Mycobacterium tuberculosis*. *Tuberculosis* 90, 25–30. doi: 10.1016/j.tube.2009.10.001

Rosado, L. A., Wahni, K., Degiacomi, G., Pedre, B., Young, D., de la Rubia, A. G., et al. (2017). The antibacterial prodrug activator Rv2466c is a mycothiol-dependent reductase in the oxidative stress response of *Mycobacterium tuberculosis*. *J. Biol. Chem.* 292, 13097–13110. doi: 10.1074/jbc.M117.797837

Rustad, T. R., Minch, K. J., Ma, S., Winkler, J. K., Hobbs, S., Hickey, M., et al. (2014). Mapping and manipulating the *Mycobacterium tuberculosis* transcriptome using a transcription factor overexpression-derived regulatory network. *Genome Biol.* 15, 502. doi: 10.1186/s13059-014-0502-3

Saini, V., Cumming, B. M., Guidry, L., Lamprecht, D. A., Adamson, J. H., Reddy, V. P., et al. (2016). Ergothioneine maintains redox and bioenergetic homeostasis essential for drug susceptibility and virulence of *Mycobacterium tuberculosis*. *Cell Rep.* 14, 572–585. doi: 10.1016/j.celrep.2015.12.056

Schrader, S. M., Botella, H., Jansen, R., Ehrt, S., Rhee, K., Nathan, C., et al. (2021). Multiform antimicrobial resistance from a metabolic mutation. *Sci. Adv.* 08, 7. doi: 10.1126/sciadv.abh2037

Schwenk, S., and Arnvig, K. B. (2018). Regulatory RNA in *Mycobacterium tuberculosis*, back to basics. *Pathog. Dis.* 06 01, 76. doi: 10.1093/femspd/fty035

Schwenk, S., Moores, A., Nobeli, I., McHugh, T. D., and Arnvig, K. B. (2018). Cell-wall synthesis and ribosome maturation are co-regulated by an RNA switch in *Mycobacterium tuberculosis*. *Nucleic Acids Res.* 46, 5837–5849. doi: 10.1093/nar/gky226

Shahi, F., Khosravi, A. D., Tabandeh, M. R., and Salmanzadeh, S. (2021). Investigation of the Rv3065, Rv2942, Rv1258c, Rv1410c, and Rv2459 efflux pump genes expression among multidrug-resistant *Mycobacterium tuberculosis* clinical isolates. *Heliyon* 7, e07566. doi: 10.1016/j.heliyon.2021.e07566

Sharma, K., Gupta, M., Pathak, M., Gupta, N., Koul, A., Sarangi, S., et al. (2006). Transcriptional control of the mycobacterial embCAB operon by PknH through a regulatory protein, EmbR, *in vivo*. *J. Bacteriol* 188, 2936–2944. doi: 10.1128/JB.188.8.2936-2944.2006

Shetty, A., and Dick, T. (2018). Mycobacterial cell wall synthesis inhibitors cause lethal ATP burst. *Front. Microbiol.* 9, 1898. doi: 10.3389/fmicb.2018.01898

Sureka, K., Ghosh, B., Dasgupta, A., Basu, J., Kundu, M., and Bose, I. (2008). Positive feedback and noise activate the stringent response regulator rel in mycobacteria. *PloS One* 3, e1771. doi: 10.1371/journal.pone.0001771

Suvorova, I. A., Korostelev, Y. D., and Gelfand, M. S. (2015). GntR family of bacterial transcription factors and their DNA binding motifs: Structure, positioning and Co-evolution. *PloS One* 10, e0132618. doi: 10.1371/journal.pone.0132618

Tang, S., Hicks, N. D., Cheng, Y. S., Silva, A., Fortune, S. M., and Sacchettini, J. C. (2019). Structural and functional insight into the *Mycobacterium tuberculosis* protein PrpR reveals a novel type of transcription factor. *Nucleic Acids Res.* 47, 9934–9949. doi: 10.1093/nar/gkz724

Thiede, J. M., Dillon, N. A., Howe, M. D., Aflakpui, R., Modlin, S. J., Hoffner, S. E., et al. (2022). Pyrazinamide susceptibility is driven by activation of the SigE-dependent cell envelope stress response in *Mycobacterium tuberculosis*. *mBio* 01, e0043921. doi: 10.1128/mbio.00439-21

Tiwari, A., Balázsi, G., Gennaro, M. L., and Igoshin, O. A. (2010). The interplay of multiple feedback loops with post-translational kinetics results in bistability of mycobacterial stress response. *Phys. Biol.* 7, 036005. doi: 10.1088/1478-3975/7/3/036005

Trigos, A. S., Goudey, B. W., Bedő, J., Conway, T. C., Faux, N. G., and Wyres, K. L. (2021). Collateral sensitivity to β-lactam drugs in drug-resistant tuberculosis is driven by the transcriptional wiring of BlaI operon genes. *mSphere* 6, e0024521. doi: 10.1128/mSphere.00245-21

Ushtanit, A., Kulagina, E., Mikhailova, Y., Makarova, M., Safonova, S., and Zimenkov, D. (2022). Molecular Determinants of Ethionamide Resistance in Clinical Isolates of *Mycobacterium tuberculosis*. *Antibiotics (Basel)*. 20, 11. doi: 10.3390/antibiotics11020133

Vargas, R., Freschi, L., Spitaleri, A., Tahseen, S., Barilar, I., Niemann, S., et al. (2021). Role of epistasis in amikacin, kanamycin, bedaquiline, and clofazimine resistance in *Mycobacterium tuberculosis* complex. *Antimicrob. Agents Chemother.* 65, e0116421. doi: 10.1128/AAC.01164-21

Vindal, V., Ranjan, S., and Ranjan, A. (2007). In silico analysis and characterization of GntR family of regulators from *Mycobacterium tuberculosis*. *Tuberculosis* 87, 242–247. doi: 10.1016/j.tube.2006.11.002

Wang, K., Pei, H., Huang, B., Zhu, X., Zhang, J., Zhou, B., et al. (2013). The expression of ABC efflux pump, Rv1217c-Rv1218c, and its association with multidrug resistance of *Mycobacterium tuberculosis* in China. *Curr. Microbiol.* 66, 222–226. doi: 10.1007/s00284-012-0215-3

Warner, D. F., Savvi, S., Mizrahi, V., and Dawes, S. S. (2007). A riboswitch regulates expression of the coenzyme B12-independent methionine synthase in *Mycobacterium tuberculosis*: implications for differential methionine synthase function in strains H37Rv and CDC1551. *J. Bacteriol* 189, 3655–3659. doi: 10.1128/JB.00040-07

Warrier, T., Kapilashrami, K., Argyrou, A., Ioerger, T. R., Little, D., Murphy, K. C., et al. (2016). N-methylation of a bactericidal compound as a resistance mechanism in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 113, E4523–E4530. doi: 10.1073/pnas.1606590113

Willand, N., Dirié, B., Carette, X., Bifani, P., Singhal, A., Desroses, M., et al. (2009). Synthetic EthR inhibitors boost antituberculous activity of ethionamide. *Nat. Med.* 15, 537–544. doi: 10.1038/nm.1950

Winglee, K., Lun, S., Pieroni, M., Kozikowski, A., and Bishai, W. (2015). Mutation of Rv2887, a marR-like gene, confers *Mycobacterium tuberculosis* resistance to an imidazopyridine-based agent. *Antimicrob. Agents Chemother.* 59, 6873–6881. doi: 10.1128/AAC.01341-15

Xu, Y., Liu, F., Chen, S., Wu, J., Hu, Y., Zhu, B., et al. (2018). *In vivo* evolution of drug-resistant *Mycobacterium tuberculosis* in patients during long-term treatment. *BMC Genomics* 19, 640. doi: 10.1186/s12864-018-5010-5

Xu, X., Vilchèze, C., Av-Gay, Y., Gómez-Velasco, A., and Jacobs, W. R. (2011). Precise null deletion mutations of the mycothiol synthesis genes reveal their role in isoniazid and ethionamide resistance in *Mycobacterium smegmatis*. *Antimicrob. Agents Chemother.* 55, 3133–3139. doi: 10.1128/AAC.00020-11

Yadav, B., Sellamuthu, B., and Tyagi, R. D. (2020). "12 - riboswitches and aptamers: potential future targets to control drug-resistant bacteria," in *Current developments in biotechnology and bioengineering* (Amsterdam, The Netherlands: Elsevier), 421–462.

Yang, S. S., Hu, Y. B., Wang, X. D., Gao, Y. R., Li, K., Zhang, X. E., et al. (2017). Deletion of sigB causes increased sensitivity to para-aminosalicylic acid and sulfamethoxazole in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 10, 61. doi: 10.1128/AAC.00551-17

Yang, J. S., Kim, K. J., Choi, H., and Lee, S. H. (2018). Delamanid, bedaquiline, and linezolid minimum inhibitory concentration distributions and resistance-related gene mutations in multidrug-resistant and extensively drug-resistant tuberculosis in Korea. *Ann. Lab. Med.* 38, 563–568. doi: 10.3343/alm.2018.38.6.563

Yimer, S. A., Kalayou, S., Homberset, H., Birhanu, A. G., Riaz, T., Zegeye, E. D., et al. (2020). Lineage-specific proteomic signatures in the *Mycobacterium tuberculosis* complex reveal differential abundance of proteins involved in virulence, DNA repair, CRISPR-cas, bioenergetics and lipid metabolism. *Front. Microbiol.* 11, 550760. doi: 10.3389/fmicb.2020.550760

Zaunbrecher, M. A., Sikes, R. D., Metchock, B., Shinnick, T. M., and Posey, J. E. (2009). Overexpression of the chromosomally encoded aminoglycoside acetyltransferase eis confers kanamycin resistance in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20004–20009. doi: 10.1073/pnas.0907925106

Zeng, J., Deng, W., Yang, W., Luo, H., Duan, X., Xie, L., et al. (2016). *Mycobacterium tuberculosis* Rv1152 is a novel GntR family transcriptional regulator involved in intrinsic vancomycin resistance and is a potential vancomycin adjuvant target. *Sci. Rep.* 6, 28002. doi: 10.1038/srep28002

Zhang, J., Liu, G., Zhang, X., Chang, Y., Wang, S., He, W., et al. (2020). Aminoglycoside riboswitch control of the expression of integron associated aminoglycoside resistance adenyltransferases. *Virulence* 11, 1432–1442. doi: 10.1080/21505594.2020.1836910

Zhou, W., Huang, S., Cumming, B. M., Zhang, Y., Tang, W., Steyn, A. J. C., et al. (2022). A feedback regulatory loop containing McdR and WhiB2 controls cell division and DNA repair in mycobacteria. *mBio. Mar.* 31, e0334321. doi: 10.1128/mbio.03343-21

Zhu, C., Liu, Y., Hu, L., Yang, M., and He, Z. G. (2018). Molecular mechanism of the synergistic activity of ethambutol and isoniazid against. *J. Biol. Chem.* 293, 16741–16750. doi: 10.1074/jbc.RA118.002693

Zorzan, I., Del Favero, S., Giaretta, A., Manganelli, R., Di Camillo, B., and Schenato, L. (2021). Mathematical modelling of SigE regulatory network reveals new insights into bistability of mycobacterial stress response. *BMC Bioinf.* 22, 558. doi: 10.1186/s12859-021-04372-5

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 09 September 2022
DOI 10.3389/fcimb.2022.974101

OPEN ACCESS

EDITED BY
Ben Gold,
Weill Cornell Medicine, United States

REVIEWED BY
Michael Berney,
Albert Einstein College of Medicine, United States
Gregory T. Robertson,
Colorado State University, United States

*CORRESPONDENCE
Tanya Parish
tanya.parish@seattlechildrens.org

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 20 June 2022
ACCEPTED 18 August 2022
PUBLISHED 09 September 2022

CITATION
Bhagwat A, Deshpande A and Parish T (2022) How *Mycobacterium tuberculosis* drug resistance has shaped anti-tubercular drug discovery. *Front. Cell. Infect. Microbiol.* 12:974101. doi: 10.3389/fcimb.2022.974101

How *Mycobacterium tuberculosis* drug resistance has shaped anti-tubercular drug discovery

Amala Bhagwat†, Aditi Deshpande† and Tanya Parish*

Center for Global Infectious Disease Research, Seattle Children's Research Institute, Seattle, WA, United States

Drug resistance is an increasing problem for the treatment of tuberculosis. The prevalence of clinical isolates with pre-existing resistance needs to be considered in any drug discovery program. Non-specific mechanisms of resistance such as increased efflux or decreased permeability need to be considered both in developing individual drug candidates and when designing novel regimens. We review a number of different approaches to develop new analogs and drug combinations or improve efficacy of existing drugs that may overcome or delay the appearance of clinical resistance. We also discuss the need to fully characterize mechanisms of resistance and cross-resistance to existing drugs to ensure that novel drugs will be clinically effective.

KEYWORDS

Mycobacterium tuberculosis, antibiotic resistance, drug discovery, antibiotic tolerance, antibacterial

Introduction

Tuberculosis (TB) remains a major global health problem causing ~1.5 million deaths in 2020 (World Health Organization, 2021). Treatment of TB is complicated owing to the unique capacity of the causative bacterium (Mycobacterium tuberculosis), to survive within the human body. Although the bacilli are unable to replicate in acidic or hypoxic environments, such as found in the granuloma, M. tuberculosis can persist in these environments for lengthy periods. Latent TB infection (LTBI), in which the bacilli remain in the body without clinical symptoms poses a unique problem for the diagnosis and treatment of TB. In addition, drug resistance is a major problem which can result from both inherent and acquired resistance mechanisms. Thus TB control programs require both new drugs to overcome existing resistance. and rapid detection tests for drug resistance.

Although treatment of TB became possible with the discovery of streptomycin, there are few antibiotics available for modern use. The major frontline drugs for drug-susceptible TB,

isoniazid and rifampicin have been supplemented with the recent addition of the new agents active against drug resistant TB, such as bedaquiline (a diarylquinolone), delaminid and pretomanid (nitroimidazoles) and repurposing of linezolid (an oxazolidinone), but the pipeline is still inadequate (Libardo et al., 2018; Oh et al., 2021). As for other antibiotics, resistance to streptomycin was observed very soon after its clinical use. Early clinical trials demonstrated the utility of combination regimens (Fox, 1979; Mitchison, 1985; Grosset, 1989) with the additional advantage that resistance to multiple agents is harder to acquire. However, given the length of time the frontline agents have been in use, a rise in drug resistance has been seen for rifampicin and isoniazid both singly and in combination (multi-drug resistance or MDR). Extensively drug resistant (XDR) strains are resistant to rifampicin, isoniazid and a fluoroquinolone.

Drug resistance in TB is largely mediated by chromosomal mutations, as there are no reports of horizontal gene transfer. However, there are multiple routes by which the bacilli can become resistant, not all of which involve mutation of the drug target. Mutations in the target which reduce or alleviate drug-binding do occur, as does mutation in the promoter leading to increased target expression. Drug inactivation, such as with the beta lactamases that degrade the beta lactams or modifying enzymes such as acetyl/methyl transferases are another resistance mechanism reported in M. tuberculosis (Zaunbrecher et al., 2009; Kurz and Bonomo, 2012). Other mechanisms which do not involve chromosomal mutation have been noted *in vitro* such as mistranslation of proteins leading to phenotypic resistance to rifampicin (Javid et al., 2014). In addition, changes in drug uptake or efflux are non-specific processes which can affect sensitivity to multiple drugs from the same or different chemical classes. M. tuberculosis has a lipid-rich outer cell wall which imparts intrinsic resistance by acting as a permeability barrier, and changes in cell wall composition can affect drug uptake (Jarlier and Nikaido, 1994). M. tuberculosis also has a variety of efflux systems which, if up-regulated, can lead to resistance (Rodrigues et al., 2017). For pro-drugs, such as isoniazid, occurrence of mutation in the activating enzymes can lead to drug resistance (Zhang et al., 1992; Zhang and Yew, 2009; Seifert et al., 2015). Given the variety of resistance mechanisms and the possibility of resistance to multiple drugs, an evaluation of the resistance mechanisms for new drugs is an important component of the drug discovery process, alongside the standard considerations of pre-existing resistance and resistance frequency.

The need for lengthy treatments (>6 months for drug sensitive TB) with multiple agents poses issues with adherence that can lead to the selection of resistant subpopulations during treatment. There is an urgent need to develop agents with new mechanisms that are not affected by pre-existing resistance, but also to shorten the duration of TB treatment to restrict the emergence of resistance. Thus, drug discovery for TB has been strongly shaped by the prevalence of existing resistance mechanisms, as well as the rate of resistance appearing in the clinic.

Drug discovery for TB has adopted several approaches which attempt to develop new agents to address the issue of pre-existing resistance and to the appearance of new resistance mechanisms. Several approaches have been used including: (i) Generating analogs which overcome resistance by binding to the target in a different fashion; (ii) Restoring sensitivity to antibiotics using booster or adjunct molecules; (iii) Using combinations to generate new regimens to minimize the appearance of resistance. We will review recent successes in these approaches and address some of the additional factors that should be considered when developing new agents (Table 1).

Development of analogs of existing drugs

The standard drug regimen for TB has a high success rate for cure when used with drug sensitive strains. Thus, there has been a lot of emphasis on developing new analogs of these successful antibiotics, but which can overcome pre-existing resistance.

New RNA polymerase inhibitors

The DNA-dependent RNA polymerase is the target of the frontline drug rifampicin. The majority of clinical resistance results from mutation in a hotspot of 81bp in the coding region of the target RpoB (Telenti et al., 1993; Mboowa et al., 2014; Zaw, 2018). Mutations in clinical isolates which result in changes in the hydrogen bonding and van der Waals interaction between RpoB and rifampicin are associated with clinical resistance (Li et al., 2021). Knowledge of the binding mechanism can be used to design novel derivatives which retain binding or to find molecules that bind to different sites on the RNA polymerase. Other members of the rifamycin class such as rifampin, rifapentine and rifabutin have the same pharmacophore which can result in cross-resistance (Alfarisi et al., 2017; Tiberi et al., 2017; Farhat et al., 2019). For example, H526C mutations lead to resistance to both rifampicin and rifabutin (Cavusoglu et al., 2004). Molecules with alternative binding sites/modes are of interest, for example fidaxomicin has *in vitro* activity against M. tuberculosis and a class of N-aroyl-N-aryl-phenyl-alaninamides were identified that bind to RNA polymerase and inhibit M. tuberculosis without cross-resistance (Lin et al., 2017; Kirsch et al., 2022). Development of these alternative RNA polymerase inhibitors could supplant rifampicin in a regimen and overcome clinical resistance.

New InhA inhibitors

Isoniazid is one of the earliest anti-tubercular drugs and works *via* inhibition of InhA, a component of FAS-II (fatty acid

synthase) involved in synthesis of mycolic acids, key cell wall components. Isoniazid is a prodrug which is activated intracellularly by the KatG catalase-peroxidase (Zhang et al., 1992). The activated molecule forms an adduct with NAD(H) at the active site of the enzyme (Banerjee et al., 1994; Rawat et al., 2003). There are multiple routes to isoniazid resistance: (i) mutations in KatG (most commonly S315T) which reduce its enzymatic activity leading to lack of activation of isoniazid; (ii) mutations in the target InhA which lead to lack of binding (Tseng et al., 2015); and (iii) mutations in the promoter region which lead to increased expression of InhA (Seifert et al., 2015). A combination of mutations in the promoter and InhA are often seen clinically with highly resistant strains (Seifert et al., 2015).

In order to generate analogs which overcome resistance, the development of direct InhA inhibitors which do not require activation shows promise. Early work on triclosan and its derivatives confirmed that it was possible to develop alternative inhibitors for InhA (Armstrong et al., 2020; Rodriguez et al., 2020; Chetty et al., 2021) and multiple scaffolds, as well as a natural product, have been identified which can inhibit InhA (Pan and Tonge, 2012). These newer analogs generally do not require activation and bind directly to InhA, thus they can overcome resistance due to KatG and InhA mutation. A series of hydroxy-pyridones which do not require activation are active against common isoniazid resistant clinical strains (Manjunatha and Smith, 2015), as are several classes of thiadiazoles which inhibit InhA directly (Šink et al., 2015; Martínez-Hoyos et al., 2016). In addition, diazaborines which do not require activation or binding to NADH have been developed (Xia et al., 2018) which are active against isoniazid resistant clinical isolates. These also demonstrate good activity against both replicating and non-replicating bacteria (Flint et al., 2020) suggesting they might be able both to overcome pre-existing resistance and shorten therapy by eliminating persistent organisms. The natural product pyridomycin also targets InhA, as a competitive binder for NADH and is active against most clinically-resistant isolates (Hartkoorn et al., 2012). In addition to overcoming existing resistance new analogs which do not require activation would have a lower frequency of resistance, so drug resistance in the clinic would likely appear more slowly. This has been demonstrated in animal models, where the diazborine AN12855 had a lower frequency of resistance in mice as compared to INH (Robertson 2019).

New gyrase inhibitors

Fluoroquinolones are broad-spectrum antibiotics with bactericidal activity which target DNA gyrase and DNA topoisomerase. In M. tuberculosis, DNA gyrase is the sole target, since it lacks the topoisomerase (Nagaraja et al., 2017; Aubry, 2004) Fluoroquinolones are attractive since they have activity against replicating, non-replicating and intracellular M. tuberculosis. Resistance to fluoroquinolones in M. tuberculosis is due to mutations in DNA gyrase (Avalos et al., 2015); high level resistance is generally conferred by mutation in the GyrA subunit in the quinolone resistance determining region covering codons 74-113 (Soudani et al., 2010; Singh et al., 2015; Singh et al., 2021; Chaoui et al., 2018). A single mutation can lead to resistance to the entire class of fluoroquinolones, therefore novel agents with different binding modes would be useful.

One approach to overcome resistance encoded by gyrA mutations, has been to identify novel scaffolds that target gyrase *in vitro*. Examples include the naphthyridone/aminopiperidines (Gibson et al., 2019) and alkoxytriazoloquinolones (Carta et al., 2019). The spiropyrimidinetrione series has activity against M. tuberculosis strains with mutations in gyrase suggesting a potential to overcome fluoroquinolone resistance (Basarab et al., 2022). In addition, the possibility of targeting GyrB has been addressed (Stokes et al., 2020); for example, the aminopyrazinamides and 2-amino-5-phenylthiophene-3-

TABLE 1 Examples of resistance mechanisms to current TB drugs and approaches to overcome resistance (references in text).

Drug	Resistance mechanisms in TB	Strategies
Rifampicin	Mutation in *rpoB* hotspot region	New rifamycins New inhibitor classes
Isoniazid	Mutations in *katG* (lack of pro-drug activation) Mutations in *inhA* and promoter region (loss of binding to target)	Analogs which do not require activation Direct *inhA* inhibitors
Fluoro-quinolones	Mutations in *gyrA/B* (loss of binding to target)	Novel scaffolds Gyrase ATPase inhibitors GyrB inhibitors
Ethionamide	Mutations in *ethA* (lack of pro-drug activation)	Increase activation of pro-drug (disruption of EthR-DNA binding) Alternative mechanisms of prodrug activation (increased expression of MymA)
Beta-lactams	Beta lactamase inactivation	Beta- lactamase inhibitors Beta- lactamase resistant analogs
Aminoglycosides	Mutation in ribosomal RNA and protein (*rrs*, *rspL*) Inactivation by *eis* acetyl transferase	Eis inhibitors
All	Non-specific or intrinsic resistance e.g. increased efflux Antibiotic tolerance	Targeting efflux pumps e.g. EfpA

carboxamide (Shirude et al., 2013; Saxena et al., 2015) which target GyrB have good potency *in vitro*.

Restoring/improving the activity of existing agents

M. tuberculosis is intrinsically resistant (or can become resistant) to several classes of antibiotics *via* expression of drug-metabolizing enzymes. The bacilli also have efflux systems which can minimize intracellular accumulation and target engagement. Examples of efforts to overcome these intrinsic resistance mechanisms are described below and may lead to new strategies for prolonging the useful life of an antibiotic and/or reducing the required dose.

Ethionamide (ETH) is a prodrug which is activated by M. tuberculosis EthA to form an NAD-adduct which binds to InhA and inhibits mycolic acid synthesis (similar to the mode of action of isoniazid) (Vannelli et al., 2002). EthA, a flavin mono-oxygenase, is negatively regulated by the transcriptional regulator EthR. Inhibition of EthR leads to up-regulation of EthA which increases the activity of ETH. Small molecule inhibitors which disrupt EthR-DNA binding are able to "boost" the activity of ETH significantly, leading to activity *in vivo* at reduced doses (Willand et al., 2009) and could improve the clinical utility of ETH.

Ethionamide efficacy can also be "boosted" by the N-acylated 4-phenylpiperidine series (Flipo et al., 2022). These molecules interact with the VirS transcriptional regulator leading to the increased expression of MymA, a monooxygenase which activates ethionamide. This approach was successful in overcoming ethionamide resistance due to EthA mutations *in vitro* and in an animal model of infection.

M. tuberculosis is intrinsically resistant to beta lactams due to the expression of beta lactamase, but this can be reversed by the addition of beta lactamase inhibitors. For example, meropenem is highly effective *in vitro* when combined with clavulanate, as are the cephalosporins (Hugonnet et al., 2009; Ramón-García et al., 2016). The clinical effectiveness of meropenem is less clear, due to tolerability issues (De Jager et al., 2022), but this has led to an increased effort to find new beta lactams (Gold et al., 2022).

The M. tuberculosis acetyltransferase Eis can modify aminoglycosides thereby inactivating them (Willby et al., 2016). Increased expression of the enzyme leads to kanamycin resistance (Zaunbrecher et al., 2009) whereas inactivation of Eis restores kanamycin sensitivity. Several series of Eis inhibitors have been identified Willby et al., 2016; Punetha et al., 2020; (Punetha et al., 2021). Although kanamycin is unlikely to be used clinically since it is not orally available, this approach does lend proof of concept to the idea that targeting antibiotic modifying enzymes can overcome intrinsic resistance.

Drug efflux is a common mechanism of intrinsic resistance in many bacterial species, and M. tuberculosis encodes many efflux systems (Louw et al., 2009; Rodrigues et al., 2017). Differences in the expression or activity of efflux pumps in clinical isolates has been linked to resistance and over-expression of several systems (mmr, mmpL7, Rv1285c, p55 and efpA) was noted in response to drug treatment (Machado et al., 2017). Increased efflux is linked to antibiotic tolerance and the development of drug resistance (Pasipanodya and Gumbo, 2011). Thus targeting efflux and/or specific efflux pumps has been proposed as a way to improve efficacy of drugs and reduce resistance, although inhibiting efflux non-specifically can have issues with selectivity and/or toxicity (Rodrigues et al., 2020). Inhibitors of the EfpA efflux pump were recently identified (Johnson et al., 2019). EfpA plays a role in antibiotic tolerance in mycobacteria since its over-expression led to decreased uptake of several antibiotics including moxifloxacin (Rai and Mehra, 2021). Thus inhibitors of this system might have a dual function, since inhibition of EfpA inhibits growth, but could also prevent induction of tolerance.

Using combinations to reduce resistance

Combination regimens

The general consensus in anti-bacterial drug discovery is that the appearance of resistance occurs within a decade of widespread use for any new drug. If resistance can be delayed, this prolongs the useful life of a new drug. Standard TB therapy consists of a four drug regimen, partly because the drugs are insufficient on their own, but also because the combination of drugs can be very effective in delaying the appearance of resistance. Since the majority of target-based resistance is due to chromosomal mutations in M. tuberculosis, combining drugs is an effective way to reduce the frequency of resistance (since bacteria would need to be resistant to more than one agent simultaneously at the outset). Thus the development of new regimens, rather than individual drugs, is standard practice for TB. However, there are still additional considerations for generating the best regimens. In particular, the resistance mechanism(s) for each drug in the regimen needs to be different. Combining drugs which hit different targets is not sufficient to prevent cross-resistance, due to the possibility of non-specific resistance mechanisms. Recent experience using monotherapy with bedaquiline has demonstrated that low level clinical resistance can appear quickly and that it can involve non-specific mechanisms, such as increased drug efflux (see below). Therefore considering the susceptibility of novel agents to common resistance mechanisms is important.

Dual targeting molecules

An alternative approach to developing individual agents for a combination regimen is to develop agents that simultaneously inhibit more than one target. This has been proposed both for targets from the same family as well as for targets with different active sites. For example, uridine derivatives that target multiple Mur enzymes (involved in the same pathway of peptidoglycan synthesis) have been identified (Kumari et al., 2022), as well as "ionized non-classical antifolates" that target both dihydrofolate reductase and thymidylate synthase (Hajian et al., 2019); the thiophene carboxamide IMB-T130 which targets both tyrosyl-tRNA synthetase and dehydroquinate synthase (Zhu et al., 2015; Zhu et al., 2018); and SQ109 which targets both MmpL3 and respiration (Kai et al., 2014; Li et al., 2014, 3). Although this approach could be useful to reduce the frequency of resistance to a single agent it may pose difficulties with respect to optimization for multiple targets, dosing and pharmacokinetics due to variation in the expression level, essentiality and vulnerability of the targets.

Overcoming drug tolerance and eradicating persistent organisms

Antibiotic tolerance is assumed to be one of the major reasons that TB therapy takes many months; the persistence of genotypically sensitive, but phenotypically resistant bacilli may be a consequence of the physiological state(s) induced by host-induced stresses such as acidic pH, hypoxia or nutrient starvation (Mandal et al., 2019). Antibiotic tolerance is a precursor to the appearance of drug resistant bacilli since it allows for extended periods of survival in fluctuating concentrations of antimicrobial agents. Therefore, developing novel drugs that can shorten therapy would be a major advance in preventing or delaying the appearance of resistant isolates in the clinic. A number of groups have conducted high throughput screens to identify agents which target non-replicating organisms induced by different *in vitro* stresses including hypoxia, low pH, nitric oxide, cholesterol and nutrient starvation, as well as multi-stress models combining these (reviewed in (Parish, 2020). Such screens have identified numerous scaffolds for investigation. The most advanced compound GSK286, which was identified in a macrophage screen, targets cholesterol metabolism and is currently in a Phase I clinical trial (GlaxoSmithKline, 2022; Nuermberger et al., 2022).

The impact of broad resistance mechanisms on early drug discovery

Bedaquiline, a member of class of diarylquinolines, inhibits ATP generation by binding to the C subunit of F0-F1 of the ATP synthase. High level resistance results from mutations in AtpE which reduce binding affinity. However, other mechanisms of resistance are found including mutations in the transcriptional repressor Rv0678 (efflux pump regulator) (Andries et al., 2014) and pepQ (Hartkoorn et al., 2014; Almeida et al., 2016). Mutations in Rv0678 lead to upregulation of the MmpL5/MmpS5 efflux system and increased efflux of the drug. Since this system also effluxes other drug classes, including azoles, clofazimine and macozinone (Hartkoorn et al., 2014; Chen et al., 2022; Guo et al., 2022), the appearance of these mutations in clinical isolates will lead to cross-resistance to multiple antimycobacterial classes. Similarly mutations in pepQ result in resistance to other agents such as macozinone (Chen et al., 2022; Guo et al., 2022), This underscores the need to determine mechanisms of resistance for new agents for both low-level and high-level resistance. In addition, since Rv0678 mutations occur in clinical isolates (Andries et al., 2014), mutant strains with these SNPs should form part of any clinical isolate panel used for routine testing during drug discovery.

Determining mechanisms of resistance during the discovery phase

Phenotypic screening has been very successful in identifying new scaffolds for development. The disadvantage of whole cell screens is that the target is not known from the outset, so much effort has been put into developing target identification and validation methods. One of the most commonly-used methods is to isolate resistant mutants and characterize the chromosomal mutations. This can provide valuable information about potential target(s) and insight into the mechanism(s) of resistance. In these studies, the major focus has been on determining the frequency of resistance and of identifying mutations that lead to high level resistance.

Identification of the target and mutations that affect inhibitor binding can be invaluable in designing new analogs. However, there can be a disconnect between the mutations found *in vitro* and those that arise *in vivo* during treatment. For example, complete loss of KatG activity results in attenuation of M. tuberculosis but is the most common mechanism of isoniazid resistance isolated in vitro. In contrast, mutations which reduce the activity of KatG are more often seen *in vivo* (Vilchèze and Jacobs, 2014). Similarly the spectrum of mutations seem for linezolid are different *in vitro* from *in vivo* (Lee et al., 2012; McNeil et al., 2017). In clinical isolates of M. tuberculosis, resistance-conferring mutations are often accompanied by compensatory mutations that increase the overall fitness of the pathogen by restoring the activity of the drug target (Alame Emane et al., 2021). High level resistance can result from multiple mutations in the drug target which may

affect binding and/or activity. As noted above, non-specific resistance mechanisms can also lead to low level resistance. Thus identifying mechanisms of resistance that arise using both *in vitro* and *in vivo* using relevant infection models are important to include in drug discovery efforts, as well as testing against a large panel of isogenic strains and clinical isolates.

Conclusion

Drug discovery for tuberculosis is notoriously difficult due to the nature of the bacterium and the pathology of the disease. The existence of resistance in clinical isolates and the probability of resistance developing to new agents in the clinic poses further restraints on drug development. Several approaches to deal with the prevalence of clinically-resistant isolates have been tried including the development of analogs of existing frontline drugs and potentiation of the efficacy of existing drugs. The development of novel combination regimens aims to reduce the appearance of resistance. In practical terms, during the development of novel anti-microbials, a wide range of clinical isolates carrying known resistance-associated mutations should form part of a screening panel. Ideally, such a panel would also include strains with decreased permeability and increased efflux. In addition, a full characterization of mutations that lead to low level and high-level resistance *in vitro* and *in vivo* should form part of the characterization of any drug candidate.

Author contributions

All authors wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was funded by NIAID of the National Institutes of Health under award number R01AI129360 and the Department of Defense Office of the Congressionally Directed Medical Research Programs under award number PR191269.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Alame Emane, A. K., Guo, X., Takiff, H. E., and Liu, S. (2021). Drug resistance, fitness and compensatory mutations in mycobacterium tuberculosis. *Tuberculosis* 129, 102091. doi: 10.1016/j.tube.2021.102091

Alfarisi, O., Alghamdi, W. A., Al-Shaer, M. H., Dooley, K. E., and Peloquin, C. A. (2017). Rifampin vs. rifapentine: what is the preferred rifamycin for tuberculosis? *Expert Rev. Clin. Pharmacol.* 10, 1027–1036. doi: 10.1080/17512433.2017.1366311

Almeida, D., Ioerger, T., Tyagi, S., Li, S.-Y., Mdluli, K., Andries, K., et al. (2016). Mutations in *pepQ* confer low-level resistance to bedaquiline and clofazimine in mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 60, 4590–4599. doi: 10.1128/AAC.00753-16

Andries, K., Villellas, C., Coeck, N., Thys, K., Gevers, T., Vranckx, L., et al. (2014). Acquired resistance of mycobacterium tuberculosis to bedaquiline. *PloS One* 9, e102135. doi: 10.1371/journal.pone.0102135

Armstrong, T., Lamont, M., Lanne, A., Alderwick, L. J., and Thomas, N. R. (2020). Inhibition of mycobacterium tuberculosis InhA: Design, synthesis and evaluation of new di-triclosan derivatives. *Bioorg Med. Chem.* 28, 115744. doi: 10.1016/j.bmc.2020.115744

Aubry, A., Pan, X.-S., Fisher, L. M., Jarlier, V., and Cambau, E. (2004). Mycobacterium tuberculosis DNA gyrase: interaction with quinolones and correlation with antimycobacterial drug activity. *Antimicrob. Agents Chemother.* 48, 1281–88. doi: 10.1128/AAC.48.4.1281-1288.2004

Avalos, E., Catanzaro, D., Catanzaro, A., Ganiats, T., Brodine, S., Alcaraz, J., et al. (2015). Frequency and geographic distribution of gyrA and gyrB mutations associated with fluoroquinolone resistance in clinical mycobacterium tuberculosis isolates: a systematic review. *PloS One* 10, e0120470. doi: 10.1371/journal.pone.0120470

Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., et al. (1994). inhA, a gene encoding a target for isoniazid and ethionamide in mycobacterium tuberculosis. *Science* 263, 227–230. doi: 10.1126/science.8284673

Basarab, G. S., Ghorpade, S., Gibhard, L., Mueller, R., Njoroge, M., Peton, N., et al. (2022). Spiropyrimidinetriones: a class of DNA gyrase inhibitors with activity against mycobacterium tuberculosis and without cross-resistance to fluoroquinolones. *Antimicrobial Agents Chemotherapy* 66, e02192–e02121. doi: 10.1128/aac.02192-21

Carta, A., Bua, A., Corona, P., Piras, S., Briguglio, I., Molicotti, P., et al. (2019). Design, synthesis and antitubercular activity of 4-alkoxy-triazoloquinolones able to inhibit the m. tuberculosis DNA gyrase. *Eur. J. Medicinal Chem.* 161, 399–415. doi: 10.1016/j.ejmech.2018.10.031

Cavusoglu, C., Karaca-Derici, Y., and Bilgic, A. (2004). *In-vitro* activity of rifabutin against rifampicin-resistant mycobacterium tuberculosis isolates with known rpoB mutations. *Clin. Microbiol. Infection* 10, 662–665. doi: 10.1111/j.1469-0691.2004.00917.x

Chaoui, I., Oudghiri, A., and El Mzibri, M. (2018). Characterization of gyrA and gyrB mutations associated with fluoroquinolone resistance in mycobacterium tuberculosis isolates from Morocco. *J. Global Antimicrobial Resistance* 12, 171–174. doi: 10.1016/j.jgar.2017.10.003

Chen, X., Li, Y., Wang, B., and Lu, Y. (2022). Identification of mutations associated with macozinone-resistant in mycobacterium tuberculosis. *Curr. Microbiol.* 79, 205. doi: 10.1007/s00284-022-02881-x

Chetty, S., Armstrong, T., Sharma Kharkwal, S., Drewe, W. C., De Matteis, C. I., Evangelopoulos, D., et al. (2021). New InhA inhibitors based on expanded triclosan and di-triclosan analogues to develop a new treatment for tuberculosis. *Pharm. (Basel)* 14, 361. doi: 10.3390/ph14040361

De Jager, V., Gupte, N., Nunes, S., Barnes, G. L., van Wijk, R. C., Mostert, J., et al. (2022). Early bactericidal activity of meropenem plus clavulanate (with or without rifampin) for tuberculosis: The COMRADE randomized, phase 2A clinical trial. *Am. J. Respir. Crit. Care Med.* 205, 1228–1235. doi: 10.1164/rccm.202108-1976OC

Farhat, M. R., Sixsmith, J., Calderon, R., Hicks, N. D., Fortune, S. M., and Murray, M. (2019). Rifampicin and rifabutin resistance in 1003 mycobacterium tuberculosis clinical isolates. *J. Antimicrob. Chemother.* 74, 1477–1483. doi: 10.1093/jac/dkz048

Flint, L., Korkegian, A., and Parish, T. (2020). InhA inhibitors have activity against non-replicating mycobacterium tuberculosis. *PloS One* 15, e0239354. doi: 10.1371/journal.pone.0239354

Flipo, M., Frita, R., Bourotte, M., Martínez-Martínez, M. S., Boesche, M., Boyle, G. W., et al. (2022). The small-molecule SMARt751 reverses mycobacterium tuberculosis resistance to ethionamide in acute and chronic mouse models of tuberculosis. *Sci. Trans. Med.* 14, eaaz6280. doi: 10.1126/scitranslmed.aaz6280

Fox, W. (1979). The chemotherapy of pulmonary tuberculosis: A review. *Chest* 76, 785–796. doi: 10.1378/chest.76.6.785

Gibson, E. G., Oviatt, A. A., Cacho, M., Neuman, K. C., Chan, P. F., and Osheroff, N. (2019). Bimodal actions of a Naphthyridone/Aminopiperidine-based antibacterial that targets gyrase and topoisomerase IV. *Biochemistry* 58, 4447–4455. doi: 10.1021/acs.biochem.9b00805

GlaxoSmithKline (2022) *A randomised, double blind (Sponsor unblinded), placebo-controlled, first time in human study to evaluate the safety, tolerability and pharmacokinetics of single and repeat oral doses and the food effect of GSK2556286 in healthy adult participants* (clinicaltrials.gov). Available at: https://clinicaltrials.gov/ct2/show/NCT04472897 (Accessed June 15, 2022).

Gold, B., Zhang, J., Quezada, L. L., Roberts, J., Ling, Y., Wood, M., et al. (2022). Identification of β-lactams active against *Mycobacterium tuberculosis* by a consortium of pharmaceutical companies and academic institutions. *ACS Infect. Dis.* 8, 557–573. doi: 10.1021/acsinfecdis.1c00570

Grosset, J. H. (1989). Present status of chemotherapy for tuberculosis. *Rev. Infect. Dis.* 11 Suppl 2, S347–S352. doi: 10.1093/clinids/11.supplement_2.s347

Guo, Q., Bi, J., Lin, Q., Ye, T., Wang, Z., Wang, Z., et al. (2022). Whole genome sequencing identifies novel mutations associated with bedaquiline resistance in mycobacterium tuberculosis. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.807095

Hajian, B., Scocchera, E., Shoen, C., Krucinska, J., Viswanathan, K., G-Dayanandan, N., et al. (2019). Drugging the folate pathway in mycobacterium tuberculosis: The role of multi-targeting agents. *Cell Chem. Biol.* 26, 781–791.e6. doi: 10.1016/j.chembiol.2019.02.013

Hartkoorn, R. C., Sala, C., Neres, J., Pojer, F., Magnet, S., Mukherjee, R., et al. (2012). Towards a new tuberculosis drug: pyridomycin – nature's isoniazid. *EMBO Mol. Med.* 4, 1032–1042. doi: 10.1002/emmm.201201689

Hartkoorn, R. C., Uplekar, S., and Cole, S. T. (2014). Cross-resistance between clofazimine and bedaquiline through upregulation of MmpL5 in mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 58, 2979–2981. doi: 10.1128/AAC.00037-14

Hugonnet, J.-E., Tremblay, L. W., Boshoff, H. I., Barry, C. E., and Blanchard, J. S. (2009). Meropenem-clavulanate is effective against extensively drug-resistant mycobacterium tuberculosis. *Science* 323, 1215–1218. doi: 10.1126/science.1167498

Jarlier, V., and Nikaido, H. (1994). Mycobacterial cell wall: structure and role in natural resistance to antibiotics. *FEMS Microbiol. Lett.* 123, 11–18. doi: 10.1111/j.1574-6968.1994.tb07194.x

Javid, B., Sorrentino, F., Toosky, M., Zheng, W., Pinkham, J. T., Jain, N., et al. (2014). Mycobacterial mistranslation is necessary and sufficient for rifampicin phenotypic resistance *Proc Natl Acad Sci USA*. 111(3), 1132–1137. doi: 10.1073/pnas.1317580111.

Johnson, E. O., LaVerriere, E., Office, E., Stanley, M., Meyer, E., Kawate, T., et al. (2019). Large-Scale chemical-genetics yields new m. tuberculosis inhibitor classes. *Nature* 571, 72–78. doi: 10.1038/s41586-019-1315-z

Kai, L., Lici, A. S-B., Xinxin, F., Ashutosh, U., Venugopal, P, Benoit, L., et al. (2014). Multitarget drug discovery for tuberculosis and other infectious diseases. *J. medicinal Chem.* 57(7), 3126–39. doi: 10.1021/jm500131s.

Kirsch, S. H., Haeckl, F. P. J., and Müller, R. (2022). Beyond the approved: target sites and inhibitors of bacterial RNA polymerase from bacteria and fungi. *Nat. Prod. Rep.* 39(6), 1226–1263. doi: 10.1039/D1NP00067E

Kumari, M., Waseem, M., and Subbarao, N. (2022). Discovery of multi-target mur enzymes inhibitors with anti-mycobacterial activity through a scaffold approach. *J. Biomol Struct. Dyn* 1–22. doi: 10.1080/07391102.2022.2040593

Kurz, S. G., and Bonomo, R. A. (2012). Reappraising the use of β-lactams to treat tuberculosis. *Expert Rev. Anti Infect. Ther.* 10, 999–1006. doi: 10.1586/eri.12.96

Lee, M., Lee, J., Carroll, M. W., Choi, H., Min, S., Song, T., et al. (2012). Linezolid for treatment of chronic extensively drug-resistant tuberculosis. *N Engl. J. Med.* 367, 1508–1518. doi: 10.1056/NEJMoa1201964

Libardo, M. D. J., Boshoff, H. I. M., and Barry, C. E. (2018). The present state of the tuberculosis drug development pipeline. *Curr. Opin. Pharmacol.* 42, 81–94. doi: 10.1016/j.coph.2018.08.001

Li, M.-C., Lu, J., Lu, Y., Xiao, T.-Y., Liu, H.-C., Lin, S.-Q., et al. (2021). rpoB mutations and effects on rifampin resistance in mycobacterium tuberculosis. *Infect. Drug Resist.* 14, 4119–4128. doi: 10.2147/IDR.S333433

Lin, W., Mandal, S., Degen, D., Liu, Y., Ebright, Y. W., Li, S., et al. (2017). Structural basis of mycobacterium tuberculosis transcription and transcription inhibition. *Mol. Cell* 66, 169–179.e8. doi: 10.1016/j.molcel.2017.03.001

Li, W., Upadhyay, A., Fontes, F. L., North, E. J., Wang, Y., Crans, D. C., et al. (2014). Novel insights into the mechanism of inhibition of MmpL3, a target of multiple pharmacophores in mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 58, 6413–6423. doi: 10.1128/AAC.03229-14

Louw, G. E., Warren, R. M., Gey van Pittius, N. C., McEvoy, C. R. E., Van Helden, P. D., and Victor, T. C. (2009). A balancing act: efflux/influx in mycobacterial drug resistance. *Antimicrob. Agents Chemother.* 53, 3181–3189. doi: 10.1128/AAC.01577-08

Machado, D., Coelho, T. S., Perdigão, J., Pereira, C., Couto, I., Portugal, I., et al. (2017). Interplay between mutations and efflux in drug resistant clinical isolates of mycobacterium tuberculosis. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.00711

Mandal, S., Njikan, S., Kumar, A., Early, J. V., and Parish, T. (2019). The relevance of persisters in tuberculosis drug discovery. *Microbiol. (Reading)* 165, 492–499. doi: 10.1099/mic.0.000760

Manjunatha, U. H., and Smith, P. W. (2015). Perspective: Challenges and opportunities in TB drug discovery from phenotypic screening. *Bioorg Med. Chem.* 23, 5087–5097. doi: 10.1016/j.bmc.2014.12.031

Martínez-Hoyos, M., Perez-Herran, E., Gulten, G., Encinas, L., Álvarez-Gómez, D., Alvarez, E., et al. (2016). Antitubercular drugs for an old target: GSK693 as a promising InhA direct inhibitor. *EBioMedicine* 8, 291–301. doi: 10.1016/j.ebiom.2016.05.006

Mboowa, G., Namaganda, C., and Ssengooba, W. (2014). Rifampicin resistance mutations in the 81 bp RRDR of rpoB gene in mycobacterium tuberculosis clinical isolates using Xpert®MTB/RIF in Kampala, Uganda: a retrospective study. *BMC Infect. Dis.* 14, 481. doi: 10.1186/1471-2334-14-481

McNeil, M. B., Dennison, D. D., Shelton, C. D., and Parish, T. (2017). *In vitro* isolation and characterization of oxazolidinone-resistant mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 61, e01296–e01217. doi: 10.1128/AAC.01296-17

Mitchison, D. A. (1985). The action of antituberculosis drugs in short-course chemotherapy. *Tubercle* 66, 219–225. doi: 10.1016/0041-3879(85)90040-6

Nagaraja, V., Godbole, A. A., Henderson, S. R., and Maxwell, A. (2017). DNA Topoisomerase I and DNA gyrase as targets for TB therapy. *Drug Discovery Today* 22, 510–518. doi: 10.1016/j.drudis.2016.11.006

Nuermberger, E. L., Martinez-Martinez, M. S., Sanz, O., Urones, B., Esquivias, J., Soni, H., et al. (2022). GSK2556286 is a novel antitubercular drug candidate effective *in vivo* with the potential to shorten tuberculosis treatment. *Antimicrob Agents Chemother.* 66 (6), e00132-22 doi: 10.1128/aac.00132-22

Oh, S., Trifonov, L., Yadav, V. D., Barry, C. E., and Boshoff, H. I. (2021). Tuberculosis drug discovery: A decade of hit assessment for defined targets. *Front. Cell. Infect. Microbiol.* 11. doi: 10.3389/fcimb.2021.611304

Pan, P., and Tonge, P. J. (2012). Targeting InhA, the FASII enoyl-ACP reductase: SAR studies on novel inhibitor scaffolds. *Curr. Top. Med. Chem.* 12, 672–693. doi: 10.2174/156802612799984535

Parish, T. (2020). *In vitro* drug discovery models for mycobacterium tuberculosis relevant for host infection. *Expert Opin. Drug Discovery* 15, 349–358. doi: 10.1080/17460441.2020.1707801

Pasipanodya, J. G., and Gumbo, T. (2011). A new evolutionary and pharmacokinetic-pharmacodynamic scenario for rapid emergence of resistance to single and multiple anti-tuberculosis drugs. *Curr. Opin. Pharmacol.* 11, 457–463. doi: 10.1016/j.coph.2011.07.001

Punetha, A., Green, K. D., Garzan, A., Thamban Chandrika, N., Willby, M. J., Pang, A. H., et al. (2021). Structure-based design of haloperidol analogues as inhibitors of acetyltransferase eis from mycobacterium tuberculosis to overcome kanamycin resistance. *RSC Med. Chem.* 12, 1894–1909. doi: 10.1039/d1md00239b

Punetha, A., Ngo, H. X., Holbrook, S. Y. L., Green, K. D., Willby, M. J., Bonnett, S. A., et al. (2020). Structure-guided optimization of inhibitors of acetyltransferase eis from mycobacterium tuberculosis. *ACS Chem. Biol.* 15, 1581–1594. doi: 10.1021/acschembio.0c00184

Rai, D., and Mehra, S. (2021). The mycobacterial efflux pump EfpA can induce high drug tolerance to many antituberculosis drugs, including moxifloxacin, in mycobacterium smegmatis. *Antimicrob. Agents Chemother.* 65, e0026221. doi: 10.1128/AAC.00262-21

Ramón-García, S., González del Río, R., Villarejo, A. S., Sweet, G. D., Cunningham, F., Barros, D., et al. (2016). Repurposing clinically approved cephalosporins for tuberculosis therapy. *Sci. Rep.* 6, 34293. doi: 10.1038/srep34293

Rawat, R., Whitty, A., and Tonge, P. J. (2003). The isoniazid-NAD adduct is a slow, tight-binding inhibitor of InhA, the mycobacterium tuberculosis enoyl reductase: adduct affinity and drug resistance. *Proc. Natl. Acad. Sci. U.S.A.* 100, 13881–13886. doi: 10.1073/pnas.2235848100

Robertson, G. T., Ektnitphong, V. A., Scherman, M. S., McNeil, M. B., Dennison, D., Korkegian, A., et al (2019). Efficacy and Improved Resistance Potential of a Cofactor-Independent InhA Inhibitor of Mycobacterium tuberculosis in the C3HeB/FeJ Mouse Model. *Antimicrob. Agents Chemother.* 63, e02071–18. doi: 10.1128/AAC.02071-18

Rodrigues, L., Cravo, P., and Viveiros, M. (2020). Efflux pump inhibitors as a promising adjunct therapy against drug resistant tuberculosis: a new strategy to revisit mycobacterial targets and repurpose old drugs. *Expert Rev. Anti Infect. Ther.* 18, 741–757. doi: 10.1080/14787210.2020.1760845

Rodrigues, L., Parish, T., Balganesh, M., and Ainsa, J. A. (2017). Antituberculosis drugs: reducing efflux=increasing activity. *Drug Discovery Today* 22, 592–599. doi: 10.1016/j.drudis.2017.01.002

Rodriguez, F., Saffon, N., Sammartino, J. C., Degiacomi, G., Pasca, M. R., and Lherbet, C. (2020). First triclosan-based macrocyclic inhibitors of InhA enzyme. *Bioorg Chem.* 95, 103498. doi: 10.1016/j.bioorg.2019.103498

Saxena, S., Samala, G., Renuka, J., Sridevi, J. P., Yogeeswari, P., and Sriram, D. (2015). Development of 2-amino-5-phenylthiophene-3-carboxamide derivatives as novel inhibitors of mycobacterium tuberculosis DNA GyrB domain. *Bioorganic Medicinal Chem.* 23, 1402–1412. doi: 10.1016/j.bmc.2015.02.032

Seifert, M., Catanzaro, D., Catanzaro, A., and Rodwell, T. C. (2015). Genetic mutations associated with isoniazid resistance in mycobacterium tuberculosis: A systematic review. *PloS One* 10, e0119628. doi: 10.1371/journal.pone.0119628

Shirude, P. S., Madhavapeddi, P., Tucker, J. A., Murugan, K., Patil, V., Basavarajappa, H., et al. (2013). Aminopyrazinamides: Novel and specific GyrB inhibitors that kill replicating and nonreplicating mycobacterium tuberculosis. *ACS Chem. Biol.* 8, 519–523. doi: 10.1021/cb300510w

Singh, P., Jain, A., Dixit, P., Prakash, S., Jaiswal, I., Venkatesh, V., et al. (2015). Prevalence of gyrA and b gene mutations in fluoroquinolone-resistant and -sensitive clinical isolates of mycobacterium tuberculosis and their relationship with MIC of ofloxacin. *J. Antibiotics* 68, 63–66. doi: 10.1038/ja.2014.95

Singh, P. K., Singh, U., and Jain, A. (2021). Emergence of specific gyrA mutations associated high-level fluoroquinolone-resistant mycobacterium tuberculosis among multidrug-resistant tuberculosis cases in north India. *Microbial Drug Resistance* 27, 647–651. doi: 10.1089/mdr.2020.0240

Šink, R., Sosič, I., Živec, M., Fernandez-Menendez, R., Turk, S., Pajk, S., et al. (2015). Design, synthesis, and evaluation of new thiadiazole-based direct inhibitors of enoyl acyl carrier protein reductase (InhA) for the treatment of tuberculosis. *J. Med. Chem.* 58, 613–624. doi: 10.1021/jm501029r

Soudani, A., Hadjfredj, S., Zribi, M., Messaoud, T., Masmoudi, A., Majed, B., et al. (2010). First report of molecular characterization of fluoroquinolone-resistant mycobacterium tuberculosis isolates from a Tunisian hospital. *Clin. Microbiol. Infection* 16, 1454–1457. doi: 10.1111/j.1469-0691.2010.03087.x

Stokes, S. S., Vemula, R., and Pucci, M. J. (2020). Advancement of GyrB inhibitors for treatment of infections caused by mycobacterium tuberculosis and non-tuberculous mycobacteria. *ACS Infect. Dis.* 6, 1323–1331. doi: 10.1021/acsinfecdis.0c00025

Telenti, A., Imboden, P., Marchesi, F., Lowrie, D., Cole, S., Colston, M. J., et al. (1993). Detection of rifampicin-resistance mutations in mycobacterium tuberculosis. *Lancet* 341, 647–650. doi: 10.1016/0140-6736(93)90417-f

Tiberi, S., Buchanan, R., Caminero, J. A., Centis, R., Arbex, M. A., Salazar, M., et al. (2017). The challenge of the new tuberculosis drugs. *La Presse Médicale* 46, e41–e51. doi: 10.1016/j.lpm.2017.01.016

Tseng, S.-T., Tai, C.-H., Li, C.-R., Lin, C.-F., and Shi, Z.-Y. (2015). The mutations of katG and inhA genes of isoniazid-resistant mycobacterium tuberculosis isolates in Taiwan. *J. Microbiology Immunol. Infection* 48, 249–255. doi: 10.1016/j.jmii.2013.08.018

Vannelli, T. A., Dykman, A., and Ortiz de Montellano, P. R. (2002). The antituberculosis drug ethionamide is activated by a flavoprotein monooxygenase*. *J. Biol. Chem.* 277, 12824–12829. doi: 10.1074/jbc.M110751200

Vilchèze, C., and Jacobs, W. R. (2014). Resistance to isoniazid and ethionamide in mycobacterium tuberculosis: Genes, mutations, and causalities. *Microbiol. Spectr.* 2, MGM2-0014–2013. doi: 10.1128/microbiolspec.MGM2-0014-2013

Willand, N., Dirié, B., Carette, X., Bifani, P., Singhal, A., Desroses, M., et al. (2009). Synthetic EthR inhibitors boost antituberculous activity of ethionamide. *Nat. Med.* 15, 537–544. doi: 10.1038/nm.1950

Willby, M. J., Green, K. D., Gajadeera, C. S., Hou, C., Tsodikov, O. V., Posey, J. E., et al. (2016). Potent inhibitors of acetyltransferase eis overcome kanamycin resistance in mycobacterium tuberculosis. *ACS Chem. Biol.* 11, 1639–1646. doi: 10.1021/acschembio.6b00110

World Health Organization (2021) *Global tuberculosis report 2021*. Available at: https://www.who.int/publications-detail-redirect/9789240037021 (Accessed January 17, 2022).

Xia, Y., Zhou, Y., Carter, D. S., McNeil, M. B., Choi, W., Halladay, J., et al. (2018). Discovery of a cofactor-independent inhibitor of mycobacterium tuberculosis InhA. *Life Sci. Alliance* 1, e201800025. doi: 10.26508/lsa.201800025

Zaunbrecher, M. A., Sikes, R. D., Metchock, B., Shinnick, T. M., and Posey, J. (2009). Overexpression of the chromosomally encoded aminoglycoside acetyltransferase eis confers kanamycin resistance in mycobacterium tuberculosis. *E. Proc. Natl. Acad. Sci. U.S.A.* 106, 20004–20009. doi: 10.1073/pnas.0907925106

Zaw, M. T. (2018). Mutations inside rifampicin-resistance determining region of rpoB gene associated with rifampicin-resistance in mycobacterium tuberculosis. *J. Infection Public Health* 11, 605–610. doi: 10.1016/j.jiph.2018.04.005.

Zhang, Y., Heym, B., Allen, B., Young, D., and Cole, S. (1992). The catalase-peroxidase gene and isoniazid resistance of mycobacterium tuberculosis. *Nature* 358, 591–593. doi: 10.1038/358591a0

Zhang, Y., and Yew, W. W. (2009). Mechanisms of drug resistance in mycobacterium tuberculosis. *Int. J. Tuberculosis Lung Dis.* 13, 1320–1330.

Zhu, N., Lin, Y., Li, D., Gao, N., Liu, C., You, X., et al. (2015). Identification of an anti-TB compound targeting the tyrosyl-tRNA synthetase. *J. Antimicrobial Chemotherapy* 70, 2287–2294. doi: 10.1093/jac/dkv110

Zhu, N., Wang, X., Li, D., Lin, Y., You, X., Jiang, J., et al. (2018). IMB-T130 targets 3-dehydroquinate synthase and inhibits mycobacterium tuberculosis. *Sci. Rep.* 8, 17439. doi: 10.1038/s41598-018-35701-z

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 09 September 2022
DOI 10.3389/fcimb.2022.949370

OPEN ACCESS

EDITED BY
Ben Gold,
Weill Cornell Medicine, United States

REVIEWED BY
Daniel Fitzgerald,
NewYork-Presbyterian, United States
Kohta Saito,
NewYork-Presbyterian, United States

*CORRESPONDENCE
Bavesh D. Kana
bavesh.kana@wits.ac.za

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 20 May 2022
ACCEPTED 15 August 2022
PUBLISHED 09 September 2022

CITATION
Gordhan BG, Sewcharran A, Letsoalo M, Chinappa T, Yende-Zuma N, Padayatchi N, Naidoo K and Kana BD (2022) Detection of differentially culturable tubercle bacteria in sputum from drug-resistant tuberculosis patients. *Front. Cell. Infect. Microbiol.* 12:949370. doi: 10.3389/fcimb.2022.949370

Detection of differentially culturable tubercle bacteria in sputum from drug-resistant tuberculosis patients

Bhavna G. Gordhan[1], Astika Sewcharran[1], Marothi Letsoalo[2], Thilgavathy Chinappa[2], Nonhlanhla Yende-Zuma[2,3], Nesri Padayatchi[2,3], Kogieleum Naidoo[2,3] and Bavesh D. Kana[1]*

[1]Department of Science and Technology/National Research Foundation Centre of Excellence for Biomedical Tuberculosis (TB) Research, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand and the National Health Laboratory Service, Johannesburg, South Africa, [2]Centre for the AIDS Programme of Research in South Africa (CAPRISA), University of KwaZulu-Natal, Durban, South Africa, [3]South African Medical Research Council (SAMRC)-Centre for the AIDS Programme of Research in South Africa (CAPRISA) Human Immunodeficiency Virus- Tuberculosis (HIV-TB) Pathogenesis and Treatment Research Unit, University of KwaZulu-Natal, Durban, South Africa

Several studies described the presence of non-replicating, drug-tolerant differentially culturable tubercle bacteria (DCTB) in sputum from patients with active tuberculosis (TB). These organisms are unable to form colonies on agar but can be recovered in liquid media supplemented with culture filtrate as a source of growth factors. Herein, we undertook to investigate the response of DCTB during the treatment of individuals with drug-resistant TB. A cohort of 100 participants diagnosed with rifampicin-resistant TB were enrolled and prospectively followed to monitor response to therapy using routine culture and limiting dilution assays, supplemented with culture filtrate (CF) to quantify DCTB. Fifteen participants were excluded due to contamination, and of the remaining 85 participants, 29, 49, and 7 were infected with rifampicin mono-resistant (RMR), multidrug-resistant (MDR), or extremely drug-resistant (XDR) TB, respectively. Analysis of baseline sputum demonstrated that CF supplementation of limiting dilution assays detected notable amounts of DCTB. Prevalence of DCTB was not influenced by smear status or mycobacterial growth indicator tube time to positivity. CF devoid of resuscitation promoting factors (Rpfs) yielded a greater amount of DCTB in sputum from participants with MDR-TB compared with those with RMR-TB. A similar effect was noted in DCTB assays without CF supplementation, suggesting that CF is dispensable for the detection of DCTB from drug-resistant strains. The HIV status of participants, and CD4 count, did not affect the amount of DCTB recovered. During treatment with second-line drug regimens, the probability of detecting DCTB from sputum specimens in liquid media with or without CF was higher compared with colony forming units, with DCTB detected up to 16 weeks post treatment. Collectively, these data point to differences in the ability of drug-resistant strains to respond to CF and Rpfs. Our findings demonstrate the possible utility of DCTB assays to

diagnose and monitor treatment response for drug-resistant TB, particularly in immune compromised individuals with low CD4 counts.

KEYWORDS

tuberculosis, drug resistance, differentially culturable tubercle bacteria (DCTB), culture filtrate (CF), resuscitation promoting factors (Rpfs)

Introduction

Drug-resistant tuberculosis (DR-TB) is a global health crises, with 206,030 people notified with multidrug-resistant or rifampicin mono-resistant TB (MDR/RMR-TB) in 2019, a 10% increase from 186,883 in 2018 (World Health Organization, 2020). MDR *Mycobacterium tuberculosis* strains display resistance to the first-line drugs rifampicin and isoniazid, while pre-extremely drug-resistant (pre-XDR) strains display additional resistance to fluoroquinolones or injectables. XDR strains are MDR with additional resistance to fluoroquinolones and injectables. Further resistance to drugs used to treat XDR-TB results in totally drug-resistant (TDR) strains that are often programmatically untreatable (Dheda et al., 2017). The high mortality associated with drug-resistant TB, often driven by HIV coinfection in certain endemic countries, represents a growing public health threat. Although new drugs such as bedaquiline and delamanid, as well as repurposed drugs such as linezolid and clofazimine, are available for treatment of DR-TB, limited access to these agents and/or the inability to generate an effective combinatorial regimen drives poor outcomes. The high cost, long treatment duration (18–24 months), and debilitating or life-threatening toxicity of second-line drugs pose further challenges in the management of DR-TB (Dookie et al., 2018).

Despite widespread rollout of molecular diagnostics for TB, early detection of drug resistance is limited to rifampicin resistance only (Boehme et al., 2010). The current gold standard for testing *M. tuberculosis* drug susceptibility (DST) is culture-based, which is protracted and delays appropriate linkage to care. This can lead to inappropriate prescription of drugs with devastating side effects or underdosing leading to further progression of the disease and resistance (Schaberg et al., 1996; Melchionda et al., 2013). Hence, improving the efficiency of current culture-based diagnostic approaches is expected to have a beneficial effect on the diagnostics value chain. In this regard, detection of non-replicating, non-platable bacterial populations becomes paramount. Mathematical modeling of sequential bacteriological load data from clinical trials showed a negative correlation between mycobacterial growth indicator tube (MGIT) and colony forming units (CFU) on agar plates, suggesting the presence of a greater mycobacterial load in liquid than on solid medium (Bowness et al., 2015). Several studies demonstrated the presence of differentially culturable/detectable tubercle bacteria (DCTB/DDTB) in sputum that are unable to grow on agar plates but can be resuscitated in liquid media supplemented with culture filtrate (CF) as a source of growth stimulatory factors (Mukamolova et al., 2010; Chengalroyen et al., 2016; Dartois et al., 2016; Dusthackeer et al., 2019; Gordhan et al., 2021). The growth stimulatory effect of CF has been ascribed to the five resuscitating promoting factors (Rpfs) from *M. tuberculosis*, which display the ability to resuscitate non-culturable bacteria (Mukamolova et al., 2002; Kana et al., 2008; Mukamolova et al., 2010). However, sputum samples also harbor a population of Rpf-independent DCTB, which do not require Rpfs for growth, and CF-independent DCTB, which are able to spontaneously resuscitate in liquid media without CF (Chengalroyen et al., 2016). These data underscore the complexity of phenotypically diverse populations in sputum (Dartois et al., 2016).

Detection of DCTB facilitates diagnostic pickup of viable *M. tuberculosis* in patients with negative results by routine testing, particularly those with HIV coinfection (Dusthackeer et al., 2019; McIvor et al., 2021). In addition to Rpfs, cAMP and fatty acids were effective in the detection of non-culturable bacterial populations in a laboratory model; however, these compounds offered no benefit in the recovery of such organisms from sputum samples (Shleeva et al., 2013; Gordhan et al., 2021). DCTB also display drug tolerance, compared with replicating bacteria, suggesting that early TB treatment eliminates only the conventionally culturable sub-population and not the total *M. tuberculosis* bacillary load (Turapov et al., 2016; Saito et al., 2017). Supporting this hypothesis, DCTB were shown to increase significantly after 2 weeks of treatment with standard first-line drugs in HIV-negative Haitian subjects with drug-sensitive TB, suggesting that drug treatment reduces CFU and concurrently increases the proportion of DCTB (McAulay et al., 2018).

A recent comparative analysis for the presence of DCTB prior to and after treatment from participants with DR-TB revealed that sputum from 29% of subjects displayed DCTB prior to treatment initiation, which remained steady after 2 weeks of treatment with second-line drugs. However, after 2 months of treatment, the proportion of DCTB reduced to almost half in subjects with drug-susceptible TB, while no DCTB was

detected in sputum from subjects with DR-TB (Zainabadi et al., 2021). These observations suggest that DCTB from DR *M. tuberculosis* strains may be more susceptible to clearance with second-line drugs. Herein, we undertook to further investigate this effect in DR strains as this has important implications for treatment duration. We also probed Rpf dependency of DCTB in DR *M. tuberculosis*. Our study was nested in a large randomized observational cohort of HIV-positive and -negative individuals from South Africa. The resulting data correlated with previous findings showing the presence of DCTB in the pretreatment sputum, the proportion of which was significantly influenced by the drug resistance status. Unlike our previous findings with drug-susceptible TB, DCTB levels did not correlate with the HIV status or CD4 counts of the participant. The longitudinal analysis assessed the effect of CF on the probability of detecting DCTB over treatment time compared with other standard readouts of bacterial load.

Results

Quantification of DCTB in sputum specimens from patients with DR-TB using CF from *M. tuberculosis*

The treatment timeline and participant disposition flow chart for the cohort used is shown in Figure 1A. This cohort was part of a primary study aimed at investigating the use of whole genome sequencing to direct regimen building. Eligible participants were recruited based on rifampicin resistance on GeneXpert at screening, and a sputum sample was taken from individuals who consented for inclusion. Participants initiated a standard DR-TB treatment regimen upon detection of rifampicin resistance, with regimen modification after drug susceptibility testing 4 weeks later (randomization). Intervention arm patients received an individualized DR-TB regimen based on whole genome sequence (WGS) results, but the primary outcome of this was not available at the time of this submission as the study is ongoing. Participants were followed longitudinally for 25 months (Figure 1A). We screened 138 individuals for DR-TB and enrolled 100. The large number of screen failures was due to negative culture results (n=21) and six participants were sensitive to rifampicin. Exclusion of seven individuals was based on the clinician's decision while one individual declined enrolment. Exclusion of a further three individuals was based on the following criteria: one did not return timeously, the second was an investigator decision, and for the third, no reason for exclusion was recorded. A summary of baseline participant demographics and laboratory data is provided in Table 1. All included participants were sputum GeneXpert-positive with medium or high bacterial loads and a MGIT time to positivity of 7, 6, and 9 days for RMR, MDR, and XDR participants, respectively. A total of 29 participants displayed RMR, 49 were infected with MDR-TB, while 5 were infected with pre-XDR and 2 with XDR-TB. For demographics, we combined the pre-XDR and XDR categories in Table 1. In all three resistance groups, there were more men, and the median age of participants was 32 (RMR), 36 (MDR), and 31 (XDR) years. After exclusion of 15 participants, sputum at screening from the remaining 85 individuals was analyzed using the most probable number (MPN—to detect DCTB) assay and colony forming unit (CFU—to detect conventionally culturable bacteria) assessments as outlined in Figure 1B and described further in Supplementary Information. Of these, 7% (6/85) of the participants received anti-TB drugs prior to the baseline measure (the numbers of days of treatment were 1, 1, 1, 1, 11, and 16, with a median of 0 days of TB treatment for all 85 participants). The participants were stratified into three groups based on their drug resistance status; the percentage harboring DCTB in the RMR and MDR groups was equivalent (approximately, 70%–75%, Supplementary Figure 1A), while in the XDR group, the amount of DCTB was reduced to half and a greater proportion of participants displayed no DCTB. As previously reported (Chengalroyen et al., 2016), we were able to demonstrate that the CF from *M. tuberculosis* with or without Rpfs was necessary to recover the maximum amount of DCTB in sputum from patients infected with DR *M. tuberculosis* compared with specimens tested in media without CF (Supplementary Figure 1B). This was not seen in pre-XDR/XDR isolates, which was most likely due to the small sample size; hence, this group was excluded from subsequent analyses. Our analysis to assess the effect of CF on the recovery of DCTB included specimens wherein no DCTB were detected (MPN/CFU<1, Supplementary Figure 1B). This approach allowed for a comparative assessment of CF supplementation in the detection of DCTB. We next sought to determine if drug resistance status (RMR or MDR) affected the amount of DCTB recovered, and for this, we excluded data for all specimens with no DCTB. No differences in the recovery of DCTB between specimens containing RMR or MDR isolates were noted when complete CF (detecting CF-dependent DCTB) was used in MPN assays (Figure 1C). In contrast, the use of CF without Rpfs (detecting Rpf-independent DCTB) or complete exclusion of CF (detecting CF-independent DCTB) from MPN assays yielded more DCTB in specimens with MDR isolates (Figure 1C).

Next, we assessed if the smear status of participants influenced the detection of DCTB. Smear-positive participants demonstrated the presence of DCTB in a significantly higher proportion (76%) of samples compared with smear-negative samples (56%) (Figure 2A). In contrast, there was no significant difference in MGIT time to positivity between samples with detectable levels of DCTB and those that had no DCTB (Figure 2B). In our previous work, we demonstrated that participants with HIV-TB coinfection harbored lower amounts of DCTB when compared with their HIV-uninfected counterparts (Chengalroyen et al., 2016). In contrast, the HIV

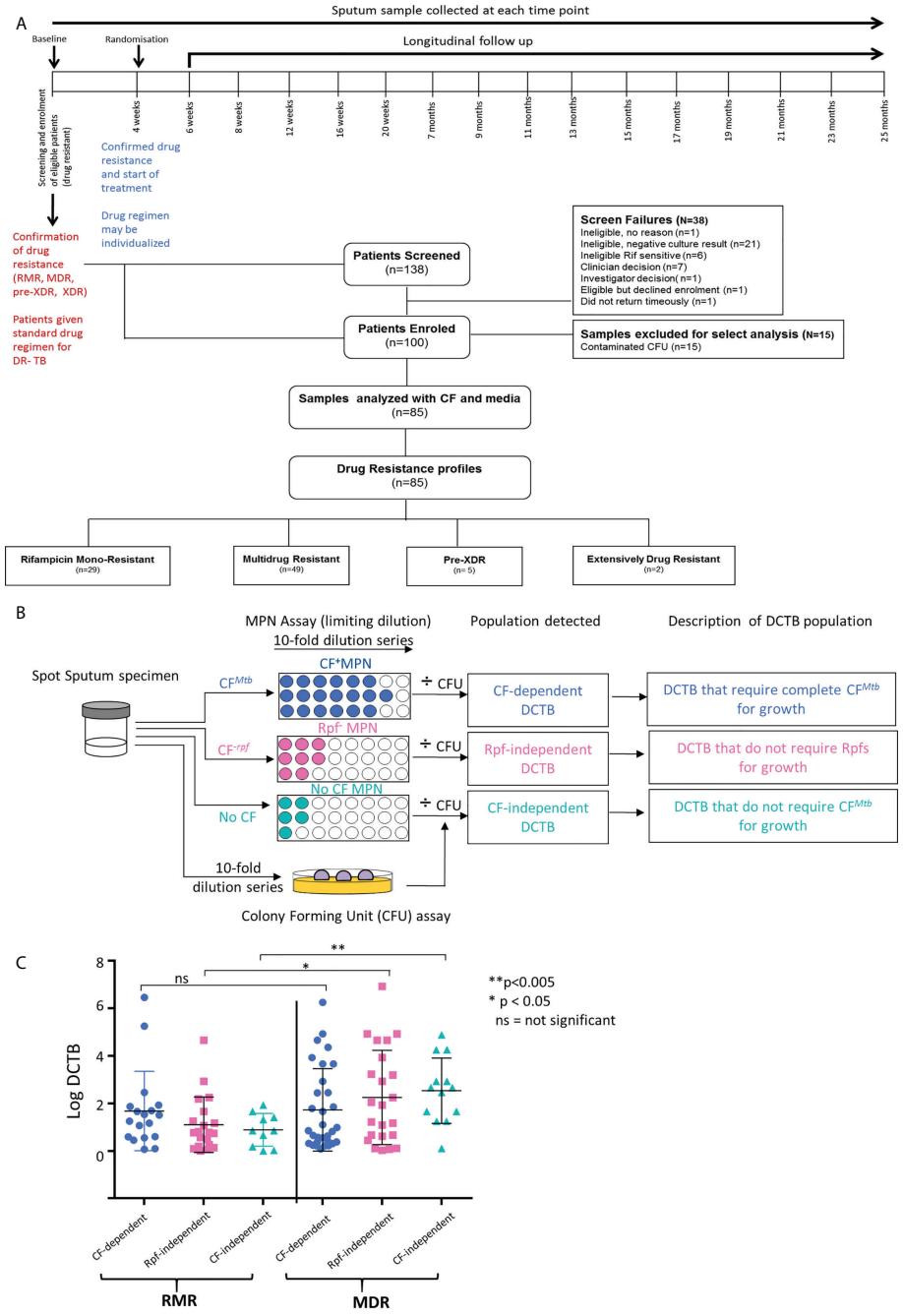


FIGURE 1
Participant disposition flow chart and assessment of DCTB in baseline sputum samples. **(A)** Study timeline and participant disposition flow chart for individuals recruited in this study. **(B)** Flow chart for DCTB assessment of sputum specimens in real time obtained at baseline (screening). Sputum samples were decontaminated and the resulting bacteria assessed by CFU and Most Probable Number (MPN) limiting dilution assays containing CF with and without Rpfs to detect DCTB. To control for the effect of CF in growth stimulation, fresh Middlebrook media was used (No CF MPN). DCTB count was obtained by dividing the MPN values (with or without CF) by CFU counts, the latter depicting conventionally culturable bacteria. **(C)** Median DCTB counts from different MPN assays and resistance categories (RMR, rifampicin mono-resistant; MDR, multidrug resistant). Values from specimens that had detectable levels of DCTB were used. Error bars depict the interquartile range. An unpaired t-test was used, where *p-value <0.05, **p-value <0.005 and ns, not significant.

TABLE 1 Demographics and laboratory diagnostic data for study participants.

	Measurement		
	Rif-mono-resistant participants (n=29)	**Multidrug-resistant participants (n=49)**	**Pre and extremely drug-resistant participants (n=7[$])**
Variables (n=85)			
Demographics			
Sex:			
Women (%)	14 (48)	17 (35)	1(14)
Men (%)	15 (52)	32 (65)	6 (86)
Age, yr, Median (IQR)	32 (28–39.5)	36 (29–43)	31 (24–34)
Weight, kg, Median (IQR)	59.5 (47.3–67.5)	57.5 (52.5–64.25)	55 (45.5–58.0)
Height, mm, Median (IQR)	167 (162.3–173.2)	167 (162.5–175)	168 (163.5–170)
BMI, Median (IQR)	19 (17.1–23.6)	20.10 (19.1–21.5)	19 (18.4–19.3)
HIV Status, n (%)			
Positive	22 (76)	32 (65)	4 (57)
Negative	7 (24)	17 (35)	3 (43)
CD4 Count*, median (IQR)	235.0 (146.5–334.0)	285.0 (149.3–376.8)	63.0 (19.0–130.0)
Conventional TB diagnosis, n (%)			
Smear grade negative	8 (28)	14 (29)	3 (43)
Smear grade positive	21(72)	35 (71)	4 (57)
MGIT Time to Positivity, d, median (IQR)	7.0 (6.0–13.5)	8.0 (5.0–11.75)	9.0 (8.0–13.0)
Log Bacterial load by MPN assay#			
CF-dependent MPN, log median (IQR)	4.29 (2.4–5.6)	3.93 (2.3–5.7)	3.66 (0.0–4.2)
Rpf-independent MPN, log median (IQR)	4.26 (2.3–5.6)	3.60 (1.7–5.3)	3.66 (0.0–4.1)
CF-independent MPN, log median (IQR)	1.66 (0.4–4.6)	1.66 (0.0–2.9)	1.66 (0.0–2.7)
CFU, log median (IQR)	3.85 (0.0–5.2)	3.21 (0.0–4.9)	2.11 (0.0–3.8)
Amount of DCTB (MPN/CFU)#			
CF-dependent DCTB, log median (IQR)	0.55 (0.0–1.6)	0.32 (0.0–1.7)	0.0 (0.0–1.9)
Rpf-independent DCTB, log median (IQR) CF-independent DCTB, log median (IQR)	0.27 (0.0–1.1) 0.0 (0.0–0.4)	0.0 (0.0–1.6) 0.0 (0.0–0.7)	0.0 (0.0–2.2) 0.0 (0.0–0.0)

Definition of abbreviations: yr, years; d, days; kg, kilograms; mm, millimeters; BMI, body mass index; IQR, interquartile range; MGIT, mycobacterial growth indicator tube.
*Only in people who are HIV infected.
#On screening specimen.
$These represent a combination of pre-XDR (n=5) and XDR (n=2).

status of participants with DR-TB did not affect the prevalence of DCTB as there was no significant difference in the amount of CF-dependent or Rpf-independent DCTB recovered between these groups (Figure 2C). Stratification of the participants based on CD4 counts showed no difference in the detection of DCTB (Figure 2D). In HIV-infected individuals, including those with CD4 counts <200 cells/mm^3, supplementation with CF (with or without Rpfs) did have a significant effect in the detection of DCTB compared with un-supplemented MPN assays (Supplementary Figures 2A, B). There was no difference in MGIT time to positivity or CFU between the RMR, MDR, and XDR groups (in all cases, p>0.5). Similarly, when the participants were stratified based on their HIV status, there was also no difference in MGIT time to positivity or CFU between HIV-positive and HIV-negative participants (in all cases, p>0.5).

Effects of drug treatment on the detection of DCTB

During treatment with second-line drugs, the disposition of the cohort changed as two participants died after 5 and 16 months of therapy, two others refused further participation after

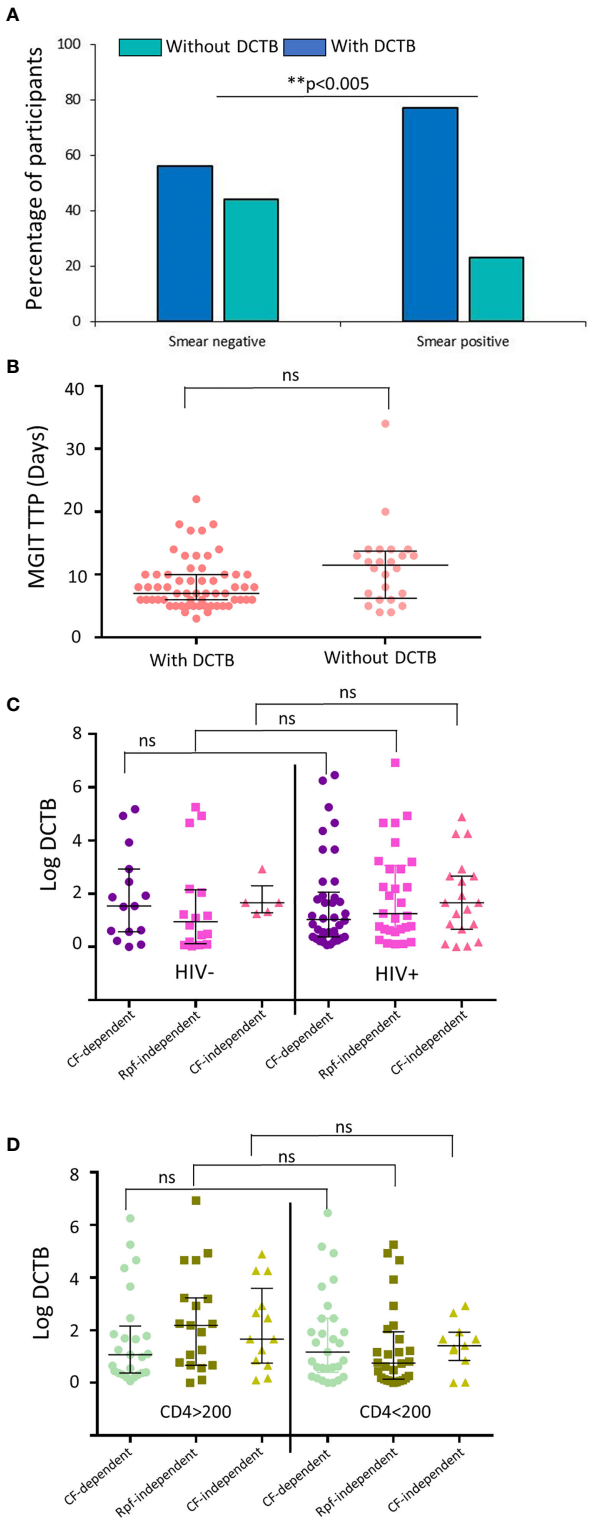


FIGURE 2
Prevalence of DCTB based on the laboratory diagnostics and HIV status of the participants. **(A)** Proportion of participants with (dark blue) and without (teal) DCTB based on their smear status. **(B)** Median mycobacterial growth indicator tube time to positivity (MGIT TTP) in specimens with and without DCTB. **(C)** Median DCTB (MPN/CFU) counts in specimens from participants stratified by HIV status. **(D)** Median DCTB (MPN/CFU) counts in specimens from HIV-infected participants stratified by CD4 counts. Values from specimens that had detectable levels of DCTB were used. Error bars depict the interquartile range. ** p-value <0.005 and ns, not significant.

3 and 6 months, respectively, while an additional two were terminated at months 5 and 9 as per the investigator's decision. The longitudinal DCTB data from the remaining participants were modeled up to week 16 (4 months) as beyond this treatment time point, insufficient levels of DCTB were detected to fit the model. The estimated probabilities from the unadjusted and adjusted model showed that the probability of detecting all forms of DCTB (CF-dependent, Rpf-independent, and CF-independent) reduced over time (Figure 3). On average, across all time points, using CF-dependent DCTB as reference, the odds of detecting bacteria reduced by 70%, 40%, and 20% for CFU, Rpf-independent DCTB, and CF-independent DCTB, respectively (Figure 3; Table S2).

At screening/enrolment, there was a 50% increase in the odds of bacterial detection by the CFU assay, while there was a 20% and 55% reduction for CF-independent and Rpf-independent DCTB, respectively, compared with CF-dependent DCTB (Figure 3; Table S2). However, 4 weeks after screening, there was a 63%, 5%, and 62% decrease in the odds of detecting CFU, CF-independent DCTB, and Rpf-independent DCTB, respectively, compared with CF-dependent DCTB. Six weeks post-treatment initiation, these odds were 90%, 30%, and 50% lower, respectively. While these odds remained lower than the CF-dependent DCTB at 8, 12, and 14 weeks post-treatment, the CFU comparisons showed statistical significance at weeks 8 and 12 (confidence intervals shown in Table S2). Smear-positive, XDR, and HIV-positive participants had 90%, 60%, and 60% higher odds of detecting bacteria, respectively, when compared with their counterpart groups; this difference was statistically significant (Table S2). There was no evidence of age, gender, and BMI influencing the detection of bacteria (Table S2). The inclusion of covariates in the model did not affect the effect of the CFs and their interaction with time.

The second-line drug regimen for 81% of the participants regardless of resistance profile consisted mainly of a combination of isoniazid, pyrizanamide, ethambutol, levofloxacin, ethionamide, clofazimine, bedaquiline, and linezolid (Table S3). This suggested that the inclusion of bedaquiline and linezolid, new or repurposed drugs, respectively, facilitated the clearance of both conventionally culturable and DCTB bacterial populations in individuals infected with DR-TB. However, in 19% of the participants, bedaquiline and linezolid were not administered and replaced primarily with kanamycin and moxifloxacin, respectively. Hence, we evaluated if treatment regimens lacking these drugs cleared DCTB after 4 weeks of treatment. Our analysis shows that the proportion of DCTB detected in participants on regimens without bedaquiline and linezolid was not significantly different compared with those whose regimens included these drugs (p= 0.3634, Fisher's exact test).

Discussion

Improving the efficiency of culture-based diagnostic tests to detect all organisms in sputum, such as DCTB, is imperative to

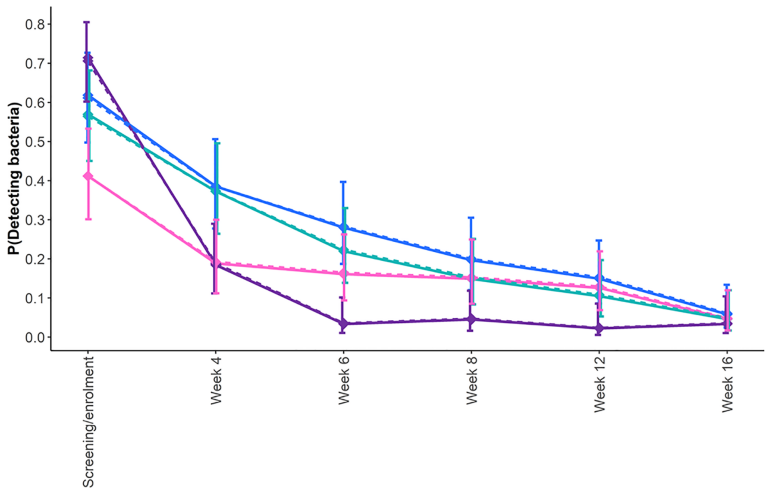


FIGURE 3
Estimated probability of detecting bacteria longitudinally in sputum specimens from participants with drug-resistant TB during treatment. Shown are the profiles of the probability of detecting DCTB within any of the MPN assays, CF-dependent DCTB (blue line); Rpf-independent DCTB (pink line); and CF-independent DCTB (teal line) and CFU (purple line) from screening/enrollment to 12 weeks after randomization. To determine the differences between the probabilities of detecting bacteria at each time point and across time points, we fitted a longitudinal generalized estimating equation (the results are presented in Table S2). The solid lines represent the adjusted model, and the dotted lines represent the unadjusted model.

enhance linkage to care and assess effectiveness of novel regimens for treatment shortening. Given that novel TB treatments should be active on currently circulating DR *M. tuberculosis* strains, we investigated the prevalence of DCTB in South African participants with DR-TB and also assessed the kinetics of bacterial clearance. In this work, DCTB populations were detected for a large proportion of participants infected with RMR, MDR, and XDR strains; however, it should be noted that the small sample size for XDR-TB limits definitive conclusions. We found that specimens containing MDR isolates displayed higher levels of Rpf-independent and CF-independent DCTB when compared with RMR isolates. Prior work demonstrated that a rifampicin-resistant isolate was limited in its ability to form DCTB during exposure to rifampicin, a phenomenon that was ascribed to the inability to inactivate the drug target (Saito et al., 2017). Considering this, the increased prevalence of DCTB in MDR isolates may be related to the inclusion of isoniazid resistance conferring mutations, where differences in mutational spectrum and drug modes of action could affect the establishment of DCTB populations. However, this observation requires further study for definitive conclusions.

Exclusion of Rpfs from the CF yielded more DCTB in specimens from participants with MDR-TB. As these molecules have been implicated in growth stimulation, this observation was counterintuitive. An inhibitory effect of CF supplementation in sputum culture assays has been noted in previous studies (Mukamolova et al., 2010; McIvor et al., 2021); however, whether this effect was specific to Rpfs in CF was not investigated. In addition, our prior work demonstrated that exclusion of Rpfs did not notably affect growth stimulatory capacity of CF derived from *Mycobacterium smegmatis*, suggesting that a combination of factors, specific to *M. tuberculosis*, are responsible for enhancing DCTB recovery (Gordhan et al., 2021). It is possible that MDR strains differ significantly in their capacity to detect growth stimulatory molecules or have inherent differences in growth that affect their recovery in MPN assays. Indeed, experiments in *ex vivo* models demonstrate that different MDR isolates have varying capacities to colonize macrophages and induce cytokines, which the authors ascribe to differences in growth rates (Yokobori et al., 2013).

Host immunity, together with drug treatment, is thought to drive the formation of, or select for, drug-tolerant populations during TB pathogenesis. In prior work with specimens from treatment-naïve individuals with drug-susceptible TB, comparison of the prevalence of DCTB in immunocompromised individuals (as defined by HIV infection and low CD4 counts) confirmed that a functional host immune response is associated with higher levels of DCTB (Chengalroyen et al., 2016). However, in our DR cohort, there was no difference in the amount of detectable DCTB between HIV-seropositive or -negative participants. In contrast to our findings with drug-susceptible TB, participants with CD4 counts of <200 cells/mm^3 showed a significant increase in the detection of Rpf-independent DCTB. This suggests that immunometabolism in individuals with MDR-TB could be distinct from drug-susceptible TB. Consistent with this, it has been demonstrated that MDR W-strains of *M. tuberculosis* overexpress cell wall lipids that facilitate bypasses of the interleukin 1 receptor type I (IL-1R1) signaling pathway, leading to reprogramming of macrophage metabolism (Howard et al., 2018). These data suggest that MDR isolates may be exposed to a distinct intracellular host environment that can affect the prevalence of DCTB in sputum, an effect that will most likely be influenced by HIV-infection status. Targeting the use of DCTB assays in these individuals may provide programmatic benefit in the diagnostic pickup of TB infection.

Patients treated with bedaquiline containing regimens have a 65%–100% culture conversion rate with reasonable treatment outcomes, and combination with linezolid is proposed to increase the effectiveness of bedaquiline (Li et al., 2019). The treatment regimen for 81% of the patients in our cohort contained bedaquiline and linezolid. Longitudinal assessment for growth during treatment revealed that most patients cleared all conventionally culturable bacteria by 2 weeks of treatment, as observed by the steep decline in the CFUs. However, the estimated probability of detecting DCTB after 4 weeks of treatment was high under all liquid culturing conditions. The magnitude of the estimated effect of CF on the probability of detecting bacteria was the highest with the CF-dependent supplement compared with the Rpf-independent, CF-independent, and CFU assessments. However, when measured by DCTB (MPN/CFU) assays, most of our participants had cleared DCTB after 3 months of therapy. These observations are consistent with previous reports in which DCTB in drug-susceptible and DR-TB were shown not to persist beyond 2–3 months of therapy (Almeida Junior et al., 2020; Zainabadi et al., 2021). However, the number of subjects used in these studies was small, and the treatment follow-up times were short, which limited a definitive longitudinal assessment. Based on our findings, it is clear that culture conversion for DR strains using routine tests, if negative after 2 months, should be interpreted with caution as the probability of the presence of non-culturable bacteria is still relatively high.

Collectively, our data highlight important differences in the prevalence of DCTB in individuals harboring rifampicin-resistant TB, with MDR isolates displaying a higher propensity to adopt the DCTB state in sputum. Detection of these appeared to be facilitated by use of CF devoid of Rpfs. Use of Rpf-deficient CF also enhanced the quantity of DCTB recovered in HIV-infected individuals with low CD4 counts. Detection of DCTB emerges as a promising new clinical endpoint to assess the durability of current cure readouts. These and other effects should be investigated in larger studies in programmatic settings.

Materials and methods

All methods were performed in accordance with the relevant guidelines and regulations for growth of *M. tuberculosis* and handling of human specimens. All procedures were conducted in a BioSafety Level III laboratory, registered with the South African Department of Agriculture Forestry and Fisheries (registration number: 39.2/NHLS-20/010). All procedures were approved by the Institutional BioSafety Committee of the University of the Witwatersrand (approval number: 20200502Lab).

Bacterial strains and culture conditions

The laboratory strain *M. tuberculosis* H37Rv and the corresponding mutant lacking all five *rpf* genes (Kana et al., 2008) was grown as previously described for the generation of culture filtrate (Chengalroyen et al., 2016).

Recruitment of participants to obtain sputum specimens

A randomized controlled clinical trial comparing the treatment success of a gene-derived individualized drug-resistant TB regimen with a standard TB regimen (INDEX study) based on South African National Tuberculosis guidelines was set up at CAPRISA, UKZN, South Africa. Ethics approval for participant recruitment was obtained from the UKZN Biomedical Research Ethics Committee (UKZN BREC), South Africa, with clearance number BFC 584/16. Individuals 18 years and older attending provincial satellite sites with drug-resistant TB testing facilities in the greater Durban area, South Africa, were approached for participation into the study and enrolled between 10 March 2018 and 4 March 2021. Participants were allowed not more than 20 days of treatment initiation before screening/enrolment. HIV-seronegative and -positive patients with drug-resistant TB, as determined by GeneXpert, were eligible for enrolment in the study. HIV-positive patients already on antiretroviral were also included in the study. Only patients with line probe assay (LPA) results consistent with MDR-TB, pre-XDR, and/or XDR-TB were included in the study. Patients willing to participate were approached, and written informed consent was obtained using Informed Consent Forms reviewed and approved by the UKZN, BREC. Patients were followed up for 25 months at various times during treatment. After written informed consent was granted, an additional spot sputum was collected for analysis by the most probable number (MPN) assay (otherwise referred to as limiting dilution assays) as described previously (Chengalroyen et al., 2016). The MPN assay is a limiting dilution series based on a Poisson distribution for the quantification of bacterial growth in liquid media, described in further detail in Supplementary Information. Sputum samples were serially diluted in a 48-well microtiter plate in media with culture filtrate (1:1 ratio) purified from wild-type *M. tuberculosis*. In addition, select dilutions of the sputum were spread on solid 7H11 plates to determine the CFUs. These assays are described in detail in Supplementary Information.

Data analysis

Statistical analysis was done using GraphPad Prism software, version 6; R software 4.2.1; and SAS 9.4. For assessing the effect of different CFs (and the no CF control) to recover DCTB, paired t-tests were used. All samples (including those that did not give DCTB with either CFs or in the no CF assay) were used for these comparisons. When comparing the quantity of DCTB recovered between categories (resistance categories, HIV status, or CD4 counts), unpaired t-tests were used and data from all specimens yielding no DCTB were excluded. For comparing proportions of individuals positive for DCTB and CFU during treatment, Fisher's exact test was used. The comparisons of the supplement effect were done with reference to the effect of the CF-dependent as it was anticipated to be more effective than the CF-independent, Rpf-independent, and the CFU. Longitudinal data were subjected to a generalized linear model that accounts for the repeated measurements per participant due to multiple time points and different CF supplementation to the probability of detecting bacteria. The parameters were estimated using a generalized estimating equations that assumed exchangeable working correlation. The time was treated as a categorical covariate. We further extended the model by adjusting for baseline covariates, thus adding the effect of gender (men or women), age (in years), HIV status (negative or positive), drug resistance (RMR, MDR, or XDR), and smear (negative or positive).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by University of Kwazulu Natal ethics number BFC 584/16. The patients/participants provided their written informed consent to participate in this study.

Author contributions

BK conceived the overall concept of the study. BG and AS executed the laboratory aspects of the study. NP, KN, NYZ and TC recruited the study participants. BG and BK wrote the first draft of the manuscript. ML assisted with data analysis. All authors contributed to the article and approved the submitted version.

Funding

The primary study was supported by funding from EDCTP, grant TMA2018SF to CAPRISA. The laboratory analysis of the specimens was supported by funding from the South African National Research Foundation (to BK), the South African Medical Research Council (to BK), and the National Health Laboratory Service Research Trust (to BG).

Acknowledgments

We thank CAPRISA for recruiting participants and collecting and transporting the sputum samples. We are thankful and grateful to the participants for providing their valuable specimens for this study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.949370/full#supplementary-material

References

Almeida Junior, P. S., Schmidt Castellani, L. G., Peres, R. L., Combadao, J., Tristao, T. C., Dietze, R., et al. (2020). Differentially culturable tubercle bacteria dynamics during standard anti-tuberculosis treatment: A prospective cohort study. *Tuberculosis (Edinb)* 124, 101945. doi: 10.1016/j.tube.2020.101945

Boehme, C. C., Nabeta, P., Hillemann, D., Nicol, M. P., Shenai, S., Krapp, F., et al. (2010). Rapid molecular detection of tuberculosis and rifampin resistance. *N Engl. J. Med.* 363, 1005–1015. doi: 10.1056/NEJMoa0907847

Bowness, R., Boeree, M. J., Aarnoutse, R., Dawson, R., Diacon, A., Mangu, C., et al. (2015). The relationship between *Mycobacterium tuberculosis* MGIT time to positivity and cfu in sputum samples demonstrates changing bacterial phenotypes potentially reflecting the impact of chemotherapy on critical sub-populations. *J. Antimicrob. Chemother.* 70, 448–455. doi: 10.1093/jac/dku415

Chengalroyen, M. D., Beukes, G. M., Gordhan, B. G., Streicher, E. M., Churchyard, G., Hafner, R., et al. (2016). Detection and quantification of differentially culturable tubercle bacteria in sputum from patients with tuberculosis. *Am. J. Respir. Crit. Care Med.* 194, 1532–1540. doi: 10.1164/rccm.201604-0769OC

Dartois, V., Saito, K., Warrier, T., and Nathan, C. (2016). New evidence for the complexity of the population structure of *Mycobacterium tuberculosis* increases the diagnostic and biologic challenges. *Am. J. Respir. Crit. Care Med.* 194, 1448–1451. doi: 10.1164/rccm.201607-1431ED

Dheda, K., Gumbo, T., Maartens, G., Dooley, K. E., Mcnerney, R., Murray, M., et al. (2017). The epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant, extensively drug-resistant, and incurable tuberculosis. *Lancet Respir. Med.* 5, 291–360. doi: 10.1016/S2213-2600(17)30079-6

Dookie, N., Rambaran, S., Padayatchi, N., Mahomed, S., and Naidoo, K. (2018). Evolution of drug resistance in *Mycobacterium tuberculosis*: a review on the molecular determinants of resistance and implications for personalized care. *J. Antimicrob. Chemother.* 73, 1138–1151. doi: 10.1093/jac/dkx506

Dusthackeer, A., Balasubramanian, M., Shanmugam, G., Priya, S., Nirmal, C. R., Sam Ebenezer, R., et al. (2019). Differential culturability of *Mycobacterium tuberculosis* in culture-negative sputum of patients with pulmonary tuberculosis and in a simulated model of dormancy. *Front. Microbiol.* 10, 2381. doi: 10.3389/fmicb.2019.02381

Gordhan, B. G., Peters, J. S., Mcivor, A., Machowski, E. E., Ealand, C., Waja, Z., et al. (2021). Detection of differentially culturable tubercle bacteria in sputum using mycobacterial culture filtrates. *Sci. Rep.* 11, 6493. doi: 10.1038/s41598-021-86054-z

Howard, N. C., Marin, N. D., Ahmed, M., Rosa, B. A., Martin, J., Bambouskova, M., et al. (2018). *Mycobacterium tuberculosis* carrying a rifampicin drug resistance mutation reprograms macrophage metabolism through cell wall lipid changes. *Nat. Microbiol.* 3, 1099–1108. doi: 10.1038/s41564-018-0245-0

Kana, B. D., Gordhan, B. G., Downing, K. J., Sung, N., Vostroktunova, G., Machowski, E. E., et al. (2008). The resuscitation-promoting factors of *Mycobacterium tuberculosis* are required for virulence and resuscitation from dormancy but are collectively dispensable for growth *in vitro*. *Mol. Microbiol.* 67, 672–684. doi: 10.1111/j.1365-2958.2007.06078.x

Li, Y., Sun, F., and Zhang, W. (2019). Bedaquiline and delamanid in the treatment of multidrug-resistant tuberculosis: Promising but challenging. *Drug Dev. Res.* 80, 98–105. doi: 10.1002/ddr.21498

Mcaulay, K., Saito, K., Warrier, T., Walsh, K. F., Mathurin, L. D., Royal-Mardi, G., et al. (2018). Differentially detectable *Mycobacterium tuberculosis* cells in sputum from treatment-naive subjects in Haiti and their proportionate increase after initiation of treatment. *MBio.* 9 1–12. doi: 10.1128/mBio.02192-18

Mcivor, A., Gordhan, B. G., Waja, Z., Otwombe, K., Martinson, N. A., and Kana, B. D. (2021). Supplementation of sputum cultures with culture filtrate to detect tuberculosis in a cross-sectional study of HIV-infected individuals. *Tuberculosis (Edinb)* 129, 102103. doi: 10.1016/j.tube.2021.102103

Melchionda, V., Wyatt, H., Capocci, S., Garcia Medina, R., Solamalai, A., Katiri, S., et al. (2013). Amikacin treatment for multidrug resistant tuberculosis: how much monitoring is required? *Eur. Respir. J.* 42, 1148–1150. doi: 10.1183/09031936.00184312

Mukamolova, G. V., Turapov, O., Malkin, J., Woltmann, G., and Barer, M. R. (2010). Resuscitation-promoting factors reveal an occult population of tubercle bacilli in sputum. *Am. J. Respir. Crit. Care Med.* 181, 174–180. doi: 10.1164/rccm.200905-0661OC

Mukamolova, G. V., Turapov, O. A., Young, D. I., Kaprelyants, A. S., Kell, D. B., and Young, M. (2002). A family of autocrine growth factors in *Mycobacterium tuberculosis*. *Mol. Microbiol.* 46, 623–635. doi: 10.1046/j.1365-2958.2002.03184.x

Saito, K., Warrier, T., Somersan-Karakaya, S., Kaminski, L., Mi, J., Jiang, X., et al. (2017). Rifamycin action on RNA polymerase in antibiotic-tolerant *Mycobacterium tuberculosis* results in differentially detectable populations. *Proc. Natl. Acad. Sci. U.S.A.* 114, E4832–E4840. doi: 10.1073/pnas.1705385114

Schaberg, T., Rebhan, K., and Lode, H. (1996). Risk factors for side-effects of isoniazid, rifampin and pyrazinamide in patients hospitalized for pulmonary tuberculosis. *Eur. Respir. J.* 9, 2026–2030. doi: 10.1183/09031936.96.09102026

Shleeva, M., Goncharenko, A., Kudykina, Y., Young, D., Young, M., and Kaprelyants, A. (2013). Cyclic AMP-dependent resuscitation of dormant mycobacteria by exogenous free fatty acids. *PLoS One* 8, e82914. doi: 10.1371/journal.pone.0082914

Turapov, O., O'connor, B. D., Sarybaeva, A. A., Williams, C., Patel, H., Kadyrov, A. S., et al. (2016). Phenotypically adapted *Mycobacterium tuberculosis* populations from sputum are tolerant to first-line drugs. *Antimicrob. Agents Chemother.* 60, 2476–2483. doi: 10.1128/AAC.01380-15

World Health Organization (2020). *WHO consolidated guidelines on tuberculosis, module 4: Treatment - drug-resistant tuberculosis treatment* (Geneva: World Health Organization).

Yokobori, N., Lopez, B., Geffner, L., Sabio Y Garcia, C., Schierloh, P., Barrera, L., et al. (2013). Two genetically-related multidrug-resistant *Mycobacterium tuberculosis* strains induce divergent outcomes of infection in two human macrophage models. *Infect. Genet. Evol.* 16, 151–156. doi: 10.1016/j.meegid.2013.01.007

Zainabadi, K., Walsh, K. F., Vilbrun, S. C., Mathurin, L. D., Lee, M. H., Saito, K., et al. (2021). Characterization of differentially detectable *Mycobacterium tuberculosis* in the sputum of subjects with drug-sensitive or drug-resistant tuberculosis before and after two months of therapy. *Antimicrob. Agents Chemother.* 65, e0060821. doi: 10.1128/AAC.00608-21

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 15 September 2022
DOI 10.3389/fcimb.2022.1008213

OPEN ACCESS

EDITED BY
Ben Gold,
Weill Cornell Medicine, United States

REVIEWED BY
Cynthia Dowd,
George Washington University, United States
William Jacobs,
Albert Einstein College of Medicine, United States

*CORRESPONDENCE
Matthew B. Neiditch
neiditmb@njms.rutgers.edu
Joel S. Freundlich
freundjs@rutgers.edu

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work and share last authorship

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 31 July 2022
ACCEPTED 24 August 2022
PUBLISHED 15 September 2022

CITATION
Rudraraju RS, Daher SS, Gallardo-Macias R, Wang X, Neiditch MB and Freundlich JS (2022) *Mycobacterium tuberculosis* KasA as a drug target: Structure-based inhibitor design. *Front. Cell. Infect. Microbiol.* 12:1008213.
doi: 10.3389/fcimb.2022.1008213

Mycobacterium tuberculosis KasA as a drug target: Structure-based inhibitor design

Reshma S. Rudraraju[1†], Samer S. Daher[2†], Ricardo Gallardo-Macias[2], Xin Wang[3], Matthew B. Neiditch[1*‡] and Joel S. Freundlich[2,4*‡]

[1]Department of Microbiology, Biochemistry and Molecular Genetics, New Jersey Medical School, Rutgers University, Newark, NJ, United States, [2]Department of Pharmacology, Physiology, and Neuroscience, New Jersey Medical School, Rutgers University, Newark, NJ, United States, [3]Department of Immunology and Infectious Diseases, Harvard University T.H. Chan School of Public Health, Boston, MA, United States, [4]Department of Medicine, Center for Emerging and Re-emerging Pathogens, New Jersey Medical School, Rutgers University, Newark, NJ, United States

Recent studies have reported the β-ketoacyl-acyl carrier protein KasA as a druggable target for *Mycobacterium tuberculosis*. This review summarizes the current status of major classes of KasA inhibitors with an emphasis on significant contributions from structure-based design methods leveraging X-ray crystal structures of KasA alone and in complex with inhibitors. The issues addressed within each inhibitor class are discussed while detailing the characterized interactions with KasA and structure-activity relationships. A critical analysis of these findings should lay the foundation for new KasA inhibitors to study the basic biology of *M. tuberculosis* and to form the basis of new antitubercular molecules of clinical significance with activity against drug-sensitive and drug-resistant infections.

KEYWORDS

***mycobacterium tuberculosis*, KasA, β-ketoacyl synthase, structure-based drug discovery, medicinal chemistry**

The cell wall of *Mycobacterium tuberculosis* plays a defining role in terms of its interactions with the host immune system and with antitubercular drugs (Rahlwes et al., 2019; Dulberger et al., 2020). Its cell wall is comprised of peptidoglycan (PG) covalently attached to the heteropolysaccharide arabinogalactan (AG) *via* phosphoryl-*N*-acetylglucosaminosyl-rhamnosyl linkage units (P-GlcNAc-Rha). AG is in turn esterified at its non-reducing ends to long α-alkyl β-hydroxy fatty acids known as mycolic acids (MAs). This PG-AG-MA triumvirate is viewed as the inner leaflet of a mycomembrane with the outer leaflet primarily comprised of non-covalently bound mycolic acids, trehalose monomycolate, and trehalose dimycolate (Zuber et al., 2008; Hoffmann et al., 2008; Marrakchi et al., 2014). MAs are synthesized by the fatty acid synthase-I (FAS-I) and fatty acid synthase-II (FAS-II) proteins (Figure 1) (Brennan and Nikaido, 1995; Cole et al., 1998).

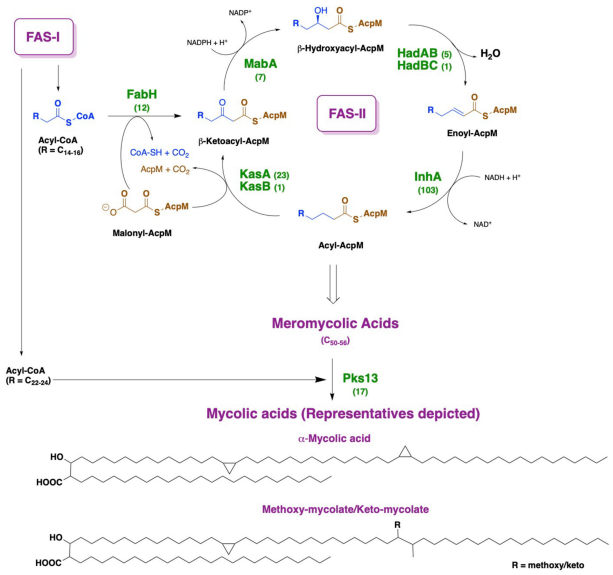


FIGURE 1
Depiction of mycolic acid biosynthesis through the FAS-I and FAS-II pathways. AcpM: mycobacterial acyl carrier protein. By each protein name, the number of corresponding X-ray crystal structures deposited in the Protein Data Bank is annotated.

The FAS-I cycle generates fatty acids (FAs) with shorter chain fragments ranging up to C_{16}-C_{18} and C_{24}-C_{26} (Takayama et al., 2005), in which the latter corresponds to the α-branch found in MAs. The FAS-II cycle further elongates the FAs to afford C_{50}-C_{56} meromycolates (Marrakchi et al., 2014). Pks13 condenses the FAS-I cycle fatty acid product and the FAS-II cycle meromycolic acid product to form the MA (Portevin et al., 2004; Leger et al., 2009; Gavalda et al., 2009). Despite the fact that the two cycles differ in the carrier protein, molecular organization, and substrates, they perform analogous reaction sequences with an iterative series of steps relying on consecutive additions of a two-carbon (acetate) unit ultimately from malonyl-Coenzyme A (CoA) to an acyl moiety. The malonyl group is transferred from malonyl-CoA to the mycobacterial acyl carrier protein (AcpM) by malonyl CoA-ACP transacylase (MtFabD, Rv2243) to form malonyl-AcpM. The condensation of malonyl-AcpM with acyl-CoA is catalyzed by the β-ketoacyl-ACP synthase III (MtFabH, Rv0533c) to form β-ketoacyl-AcpM and, thus, link the FAS-I and FAS-II cycles. Generally, there are four main enzymes involved in each cycle of elongation. The nicotinamide adenine dinucleotide phosphate-dependent 3-ketoacyl-acyl carrier protein reductase (MabA, Rv1483) reduces the β-keto group. Heterodimeric (3R)-hydroxyacyl-ACP dehydratase (HadAB, Rv0635 – Rv0636, and HadBC, Rv0636 – Rv0637) subsequently dehydrates the resulting β-hydroxy intermediate into enoyl-AcpM. Then, the nicotinamide adenine dinucleotide hydrogen (NADH)-dependent *trans*-2-enoyl-ACP reductase (InhA, Rv1484) reduces enoyl-AcpM into acyl-AcpM. Subsequent cycles of elongation are carried out by KasA (Rv2245) and KasB (Rv2246) (Figure 2) that elongate the acyl-AcpM by two carbons to form the β-ketoacyl-AcpM. KasA and KasB are 67% identical and 86% similar by protein sequence, and they exhibit differential substrate preferences (Bhatt et al., 2007; Bhatt et al., 2007).

MA synthesis inhibition has represented a fertile ground for antitubercular drug targeting (Abrahams and Besra, 2020; Fernandes et al., 2022; Dartois and Rubin, 2022), and this is undoubtedly due to the availability of X-ray crystal structures of many involved proteins (Figure 1) in the Protein Data Bank (www.rcsb.org). This strategy is highly favorable because developed inhibitors are expected to lack mechanism-based toxicity as mammals rely primarily on a FAS-I system (Bhatt et al., 2007). For instance, isoxyl and thioacetazone are known to target the HadAB/HadBC (3R)-hydroxyacyl-ACP dehydratase complex, whereas isoniazid and ethionamide prevent the reduction of enoyl-AcpM by targeting InhA (Banerjee et al., 1994; Vilcheze et al., 2006). We highlight the fact that an *M. tuberculosis* β-ketoacyl synthase is not currently the target of a tuberculosis drug. Thus, a therapeutic inhibiting KasA should be clinically useful versus both drug-sensitive and drug-resistant infections.

KasA is an essential and vulnerable drug target *in vitro*

kasA has been identified as an essential gene for the *M. tuberculosis* laboratory strain H37Rv under *in vitro* growth conditions *via* multiple transposon-based approaches,

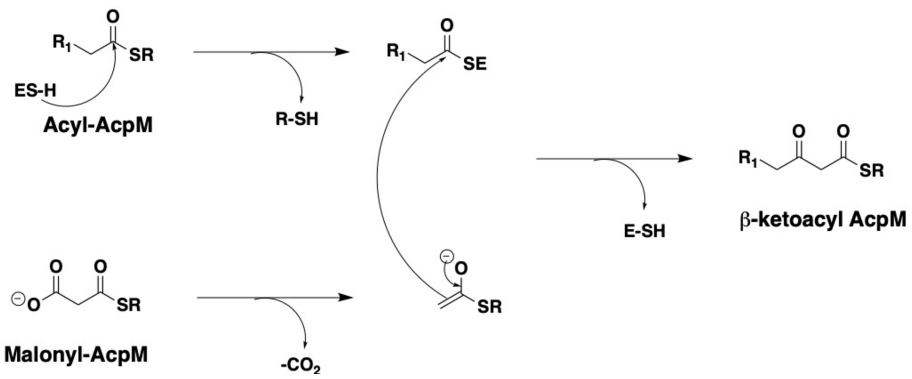


FIGURE 2
General catalytic mechanism of the KasA and KasB enzymes.

including the original transposon site hybridization method (TRaSH) (Sassetti et al., 2003), deep sequencing of transposon insertions (TnSeq) (Griffin et al., 2011; Zhang et al., 2012), and a more comprehensive TnSeq analysis (DeJesus et al., 2017) to account for TA insertability with a hidden Markov model. In contrast, the *in vitro* essentiality of *kasB* appears to be dependent on the experimental conditions (growth media and nature of genetic disruption) (Minato et al., 2019). Extensive studies show no evidence of *kasA* as a conditional non-essential gene, indicating that the *in vitro* requirement for *kasA* is independent of culture conditions. Consistent with these findings with laboratory strains, *kasA* was also revealed as essential for *in vitro* cultures of clinical isolates belonging to the most prevalent lineages: Euro-American, East Asian, and Indo-Oceanic (Carey et al., 2018). A gene vulnerability study based on the mycobacterial CRISPRi system (Rock et al., 2017) identified *kasA* as a vulnerable gene in both laboratory strain H37Rv and clinical isolate HN878 (Bosch et al., 2021). These studies indicate that *M. tuberculosis in vitro* growth is highly dependent on *kasA* and the FAS-II pathway in general. The data supporting the *in vitro* essentiality and vulnerability of *kasA* in *M. tuberculosis* have created a significant impetus to attain *in vivo* validation of this drug target.

Protein crystallography has provided key insights into KasA catalysis

X-ray crystallographic analyses of KasA alone (or apo) (Luckner et al., 2009; Kumar et al., 2018), KasA-C171Q in complex with fortuitously-bound phospholipid (Luckner et al., 2009; Schiebel et al., 2013), and KasA bound to small molecule inhibitors (Schiebel et al., 2013; Abrahams et al., 2016; Kumar et al., 2018; Cunningham et al., 2020; Inoyama et al., 2020) have provided atomic-level mechanistic insights into the KasA structure. In the crystals, KasA exhibits C2 symmetry and is composed of a five-layer αβαβα structure, a fingerprint of thiolases (Figure 3). Based on our understanding of KasA structure and function, and for the benefit of discussion, it can be divided into two regions – the core and cap domains. Within the core domain lies the catalytic triad residues (Cys171, His311, and His345) and the phoshopantetheine tunnel, which opens into the malonyl binding region. The proposed role of the catalytic triad in KasA acylation, decarboxylation and condensation is described in Figure 4. The acyl channel is a hydrophobic channel in the cap region formed by helices α2, α5, α9 and α5′. It can be accessed either through the malonyl binding site or from the opening of acyl channel in the cap region. It accommodates the growing acyl chain. The acyl chain binding site is traced by the phospholipid bound to KasA in PDB ID 4C6W (Schiebel et al., 2013). The cap domain is disordered in the apo structure but becomes ordered upon acylation of KasA Cys171 (Luckner et al., 2009; Kumar et al., 2018). The cap domain is proposed to be responsible for significant functional differences between KasA and KasB (Schiebel et al., 2013). How the core and cap domains function to condense the acyl donor and malonyl-AcpM substrates to form β-ketoacyl-AcpM products is outlined below.

In the apo state, the KasA active site residues are in a zwitterionic state, i.e., Cys171 is deprotonated and His311 is protonated. In step one, apo KasA binds to an acyl-AcpM, charging Cys171 with an acyl chain that is at least C_{16-18} and maximally C_{36-40} (Slayden and Barry, 2002). It is important to note that the acyl chain is covalently attached to AcpM *via* a phosphopantetheine group. Two models have been proposed to explain how the acyl chain accesses the KasA active site. In model one, it is hypothesized that both the acyl-AcpM and the malonyl-AcpM substrates are delivered through the phosphopantetheine tunnel mediated by the binding of acidic AcpM residues to basic residues on the KasA surface (namely

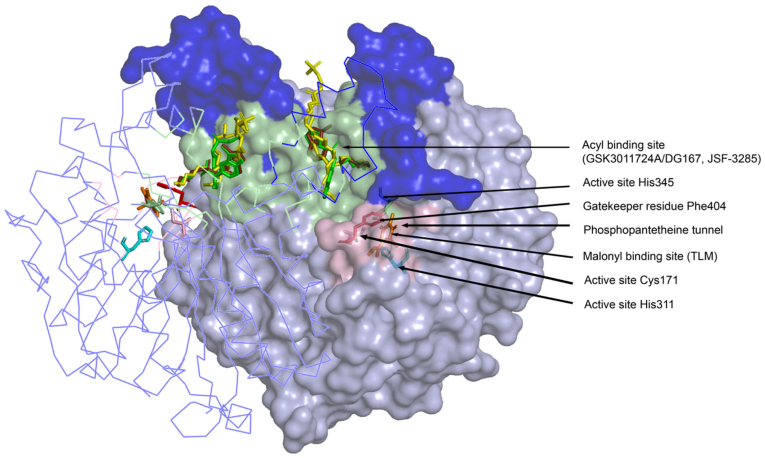


FIGURE 3
Crystal structure of the KasA dimer with inhibitors bound (GSK3011724A/DG167, JSF-3285, TLM) at different binding sites. The monomer on the left is represented as a ribbon tracing the alpha carbons and the monomer on the right is depicted as a surface. The dark blue surface indicates the cap region of KasA excluding the acyl channel whereas the light blue surface indicates the core domain; Light green surface, acyl binding site; pink surface, malonyl binding site; yellow sticks; phospholipid bound to KasA (PDB ID 4C6W); green sticks; DG167 (PDB ID 5W2P); brown sticks; JSF-3285 (PDB ID 6P9L); orange sticks; TLM (PDB ID 4C6U); pink sticks, Cys171; red sticks, Phe404; cyan sticks, His 311 and His 345. For clarity, only the KasA dimer from PDB ID 6P9L is shown.

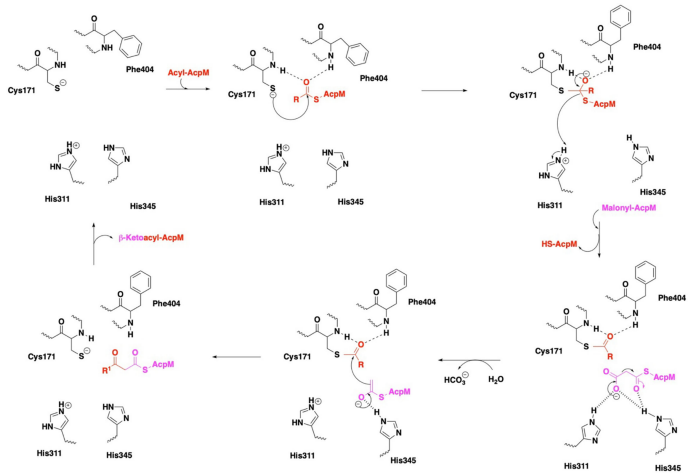


FIGURE 4
Mechanism of KasA catalysis with emphasis on the catalytic triad. In the KasA resting state, Cys171 and His311 are proposed to be deprotonated and protonated, respectively. Acylation of KasA occurs by nucleophilic attack of Cys171 on the acyl-AcpM. Several theories have been offered to explain the decarboxylation step, and it is unclear as to the protonation state of His311. The ensuing condensation of this enolate with the Cys171 bound acyl moiety occurs to elongate the acyl chain length by two carbons and then release the product.

Arg74, Arg 78, Arg79, Arg 135, Arg214 and Lys136) (Lee et al., 2011; Schiebel et al., 2013). In model two, it is proposed that KasA residues 115 – 147 in the flexible cap region of both monomers move in a scissor-like motion to allow direct access of the acyl chain into the hydrophobic cavity (Luckner et al., 2009). A structure of KasA in complex with acyl-AcpM may explain how the complex overcomes a variety of steric factors and the hydrophilic and hydrophobic nature of the acyl and phosphopantetheine channels. Structure-function studies do suggest that acylation of Cys171 likely induces a conformational change of gatekeeper residue Phe404 along with the additional gatekeeper residues Leu116 and Tyr126

(Schiebel et al., 2013). Acylated KasA adopts an open conformation, which facilitates the binding of malonyl-AcpM by widening the phosphopantethiene tunnel entrance and increasing the size of the malonyl binding site. Decarboxylation of malonyl-AcpM is then mediated by His311 and His345. It is important to note that different theories have been proposed to explain the protonation states of the catalytic triad residues during acylation and decarboxylation (Lee et al., 2011; Lee and Engels, 2014). Subsequent Claisen condensation occurs by a nucleophilic attack of the enolate on the thioester intermediate, yielding the product β-ketoacyl-AcpM (Figure 4).

The thiolactomycin chemotype as a malonyl binding site inhibitor

Thiolactomycin (TLM; Figure 5) is a thiolactone natural product, isolated from *Nocardia* spp., that initially was noted for its modest *in vitro* growth inhibitory efficacy versus a range of Gram-positive and Gram-negative bacteria (Oishi et al., 1982). TLM has been shown to exhibit low cytotoxicity to mammalian cells. Analysis of TLM serum levels in orally dosed rats evidenced rapid absorption and clearance (Miyakawa et al., 1982). Accordingly, TLM and its analogs have exhibited only modest *in vivo* efficacy in mouse models of infection with *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Serratia marcescens* (Miyakawa et al., 1982; Bommineni et al., 2016).

Slayden et al. demonstrated TLM *in vitro* efficacy versus *M. tuberculosis* albeit with racemic, synthetic material with a MIC = 120 μM on solid media (Slayden et al., 1996); the active enantiomer features a 5*R*-stereocenter (Kim et al., 2006). This group also showed that TLM afforded bactericidal efficacy against *M. tuberculosis* in an infected murine bone marrow-derived macrophage model and inhibition of mycolic biosynthesis within *M. smegmatis*. Knowledge of its inhibition of *E. coli* β-ketoacyl synthases (Nishida et al., 1986; Magnuson et al., 1993) hinted at its engagement of KasA and KasB which was evidenced through overexpression studies in *M. bovis* BCG (Kremer et al., 2000). Studies with the purified *M. tuberculosis* proteins showed that TLM inhibited all three annotated *M. tuberculosis* β–ketoacyl synthases in various functional assays. Whereas the metrics of inhibition vary according to the assay, in general, TLM inhibition followed the order of KasA > KasB > FabH (Choi et al., 2000; Schaeffer et al., 2001; Kim et al., 2006; Machutta et al., 2010).

In 2000, Kremer et al. reported a subset of TLM analogs designed without the initial guidance from an X-ray crystal structure (Kremer et al., 2000). Their assay results demonstrated the importance of the TLM isoprenyl group in maintaining whole-cell activity against *M. tuberculosis* as judged by MIC but showed potential disconnects between MIC and the respective inhibitions of mycolic acid biosynthesis and FAS-II biosynthesis in *M. smegmatis*. While the authors constructed a KasA homology model from *E. coli* FabF (PBD ID: 1KAS) (Huang et al., 1998), their analysis was limited to the observation of a hydrophobic pocket that could be engaged by the isoprenyl. This observation would later be supported by a 2009 reported X-ray crystallographic study (Luckner et al., 2009) that illustrated the orientation of the isoprenoid moiety toward an extended pocket where two water molecules were present stabilizing the loop from Asp272 to Pro280. Furthermore, a thorough examination of a range of substitutions for the isoprenyl moiety was performed by Kim and colleagues (Kim et al., 2006). In an assay quantifying KasA catalytic activity, the authors found linear or branched alkyl, cycloalkyl, and aryl moieties failed to afford TLM analogs with inhibition within six-fold of TLM. In fact, all but two compounds demonstrated IC_{50} values in excess of 100 μM. Importantly, all analogs failed to exhibit significant *M. tuberculosis* growth inhibitory efficacy versus the H37Rv strain.

The 2009 structural biology report (Luckner et al., 2009) was critical to elucidating the details of how TLM binds to KasA (Figures 3, 6). More specifically, it illustrated how TLM occupies the malonyl binding site. TLM interactions with the active site

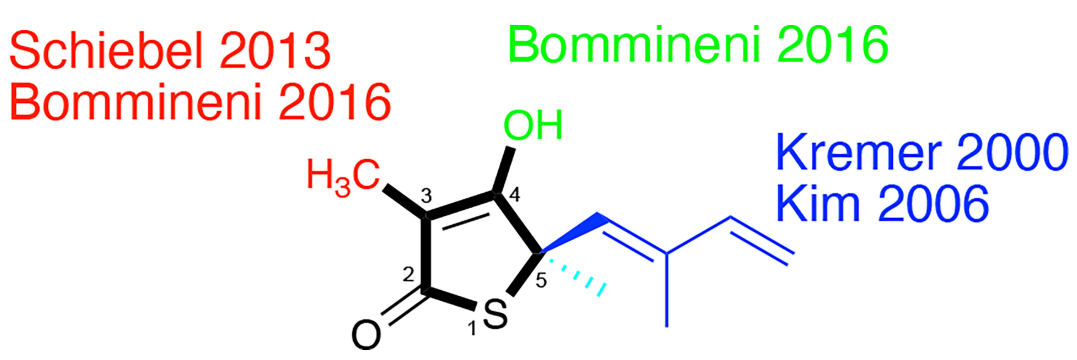


FIGURE 5
Thiolactomycin and its analogs. Noted are efforts, referenced within, regarding analogs at the specific positions of the thiophen-2(5H)-one ring system which are numbered.

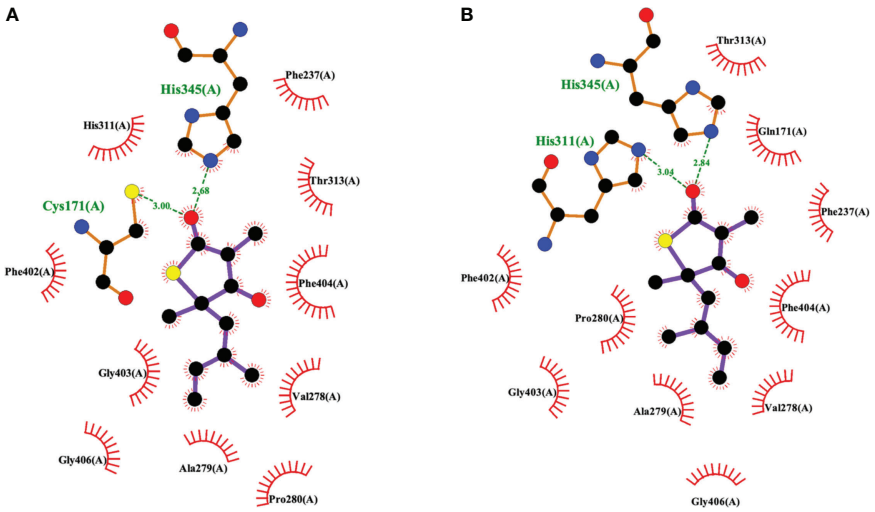


FIGURE 6
Interaction profiles for TLM bound to **(A)** wild type KasA (PDB ID 2WGE) and **(B)** KasA-C171Q (PDB ID 2WGG). Hydrogen bonds are shown as green dashed lines and distances are measured in Angstroms.

residues and the binding mode of TLM itself differ when KasA is in the closed (apo) or open (acylated) conformations (PDB IDs 2WGE and 2WGG, respectively). The isoprenoid chain and the double bond in the thiophen-2(5H)-one of TLM are critical for KasA inhibitory activity. In apo KasA, the TLM carbonyl oxygen formed two hydrogen bonds with the sulfhydryl group of Cys171 and sidechain N-H of His345, whereas in the C171Q mutant (that mimics acylated KasA) two hydrogen bonds were formed with the sidechain N-H moieties of His311 and His345. Furthermore, in the apo state, the KasA phenyl ring of gatekeeper residue Phe404 formed a face-to-face interaction with the thiolactone ring. With the KasA C171Q mutant, however, a conformational change shifted the Phe404-TLM interaction to edge-to-face. The orientation of the TLM isoprenoid tail in the lipophilic pocket also varied between the apo KasA and KasA-C171Q structures. Movement of Phe404 from the closed to open conformation led to a shift in residues Leu116, Val142, and Met146, and helix α9. This conformational change increased the size of the malonyl binding site and the phosphopantetheine tunnel. This explains the slow (Machutta et al., 2010) and preferential binding of TLM to acylated KasA as compared to apo KasA.

Schiebel and colleagues leveraged this structural information in the pursuit of TLM analogs (Schiebel et al., 2013). Attempting to mimic the alkyl portion of the acyl chain of bound acyl-AcpM, linear hydrophobic substituents (e.g, ethyl, *n*-propyl, and 4-azido-*n*-butyl) were introduced at the 3-position of the thiolactone ring (Figure 5). The TLM alkyl-AcpM mimics displayed a 4 – 18 fold reduction in KasA binding affinity for the C171Q mutant in comparison to TLM. However, they did maintain the TLM slow onset of binding phenotype. X-ray crystal structures demonstrated how the introduced alkyl chain oriented towards the aromatic cavity formed by Phe210, Phe237, Phe239, and His345 (PDB IDs 4C70, 4C6Z, 4C71; Figure 7). Interestingly, upon comparison to the whole-cell efficacy of TLM versus the *M. tuberculosis* H37Rv strain, the 3-ethyl analog was approximately equipotent while the 3-*n*-propyl derivative was about eightfold less active (Bommineni et al., 2016). Kapilashrami continued this effort and their *para*-substituted phenethyl and phenylbutyl analogs generally offered enhanced binding affinity for wild type KasA and an improved initial equilibrium binding constant for the C171Q mutant as compared to TLM, but failed to exhibit slow binding kinetics (Kapilashrami et al., 2013). Slow onset of binding has been demonstrated to be a key aspect of long residence time inhibitors and to their *in vivo* efficacy (Lu and Tonge, 2008; Lu and Tonge, 2010). These results serve as a reminder of the intricacies associated with inhibitor binding to KasA and the value of detailed kinetic measurements. Furthermore, an X-ray crystal structure of KasA bound to one of these 3-alkylphenyl analogs would help explain these observations.

TLM 3-substituted acyl analogs (e.g., acetyl and trifluoroacetyl) utilized the acyl moiety to mimic the diketo group of malonyl-AcpM. These derivatives offered 2 – 4 fold increased affinity for the C171Q mutant as compared to TLM while still maintaining slow onset kinetics. X-ray crystallographic studies again proved insightful (Figure 7). The structure of either 3-acyl analog is supportive of the increased binding affinity being due to the formation of a new hydrogen-bonding interaction between the acyl carbonyl and His345. A potentially favorable interaction between the trifluoromethyl group in the 3-trifluoroacetyl analog and the sidechain hydroxyl of Thr313 may also be present. In comparison to the whole-cell efficacy of TLM versus the *M. tuberculosis* H37Rv

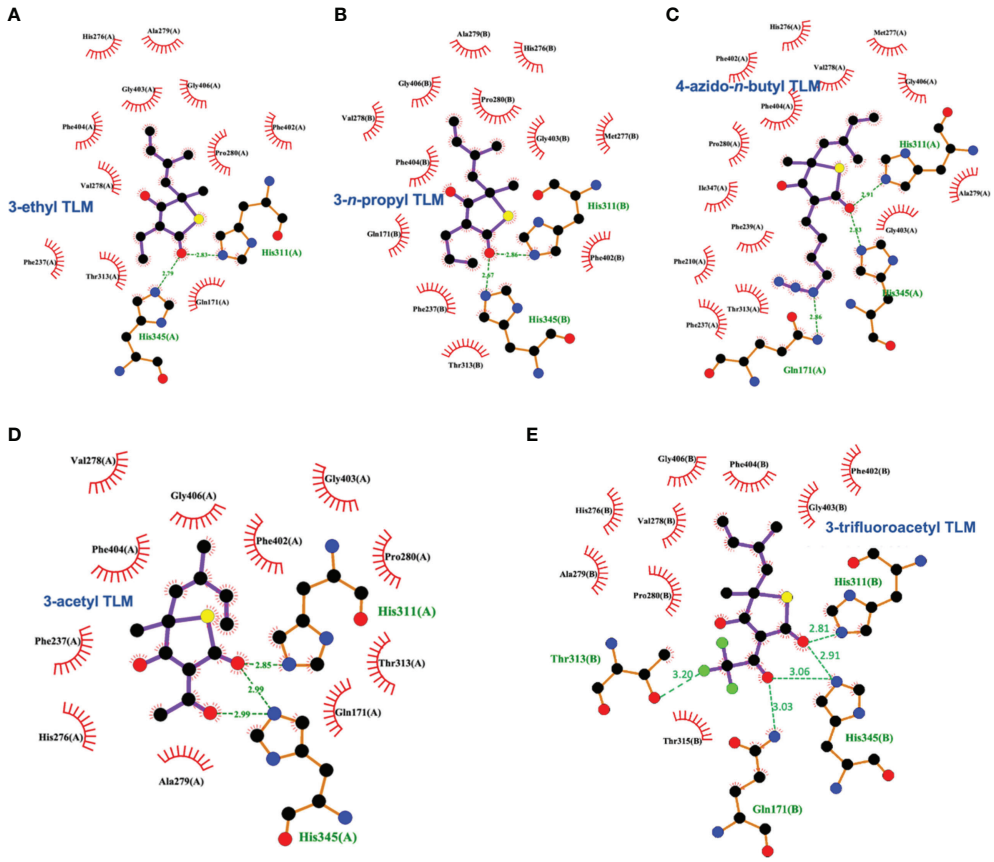


FIGURE 7
Interaction profiles for KasA C171Q bound to the following TLM analogs: **(A)** 3-ethyl (PDB ID 4C6Z), **(B)** 3-*n*-propyl (PDB ID 4C70), **(C)** 4-azido-*n*-butyl (PDB ID 4C71), **(D)** 3-acetyl (PDB ID 4C72), and **(E)** 3-trifluoroacetyl (PDB ID 4C73). Hydrogen bonds are shown as green dashed lines and distances are measured in Angstroms.

strain, the 3-acetyl and 3-trifluoroacetyl analogs were 4- and 32-fold less potent, respectively (Bommineni et al., 2016). Kapilashrami reported additional acyl analogs with a longer alkyl chain (i.e., *n*-Pr, $C_{15}H_{31}$, $(CH_2)_5(4\text{-}PhC_6H_4)$). These were not slow onset binders and they failed to offer improvements in binding affinity to wild type KasA or initial equilibrium binding constant for the C171Q mutant. These results further support the value of an X-ray crystal structure of KasA bound to malonyl-AcpM, or a non-reactive mimic.

Removal of the TLM 3-methyl group was also pursued and led to a decrease in whole-cell potency (MIC > 100 μM) while providing gains in the binding constants for the wild type and C171Q forms of KasA, respectively (Bommineni et al., 2016). The disconnect between enzyme binding and whole-cell efficacy could potentially be further explored by examining the effect of methyl group removal on compound accumulation and/or metabolism within *M. tuberculosis* (Wang et al., 2019; Wang et al., 2020). Furthermore, alkylation of the 4-OH of this desmethyl TLM abrogated not only growth inhibition of *M. tuberculosis* but also binding to wild type KasA or KasA-C171Q. An observed decrease in potency *via* removal of the hydroxy group may be consistent with the loss of a potential water-mediated hydrogen bond with the carbonyl oxygen of Val278. It is also possible that the newly introduced alkyl substituent makes unfavorable interactions with nearby KasA residues. While analysis of the relevant crystal structures does not reveal obvious unfavorable interactions, it is, however, possible that unfavorable interactions could occur when KasA adopts different conformations, e.g., when complexed with AcpM.

The indazole sulfonamide chemotype as an acyl channel inhibitor

GSK3011724A, an indazole sulfonamide (Figure 8), was first identified as an antitubercular by GlaxoSmithKline (GSK)

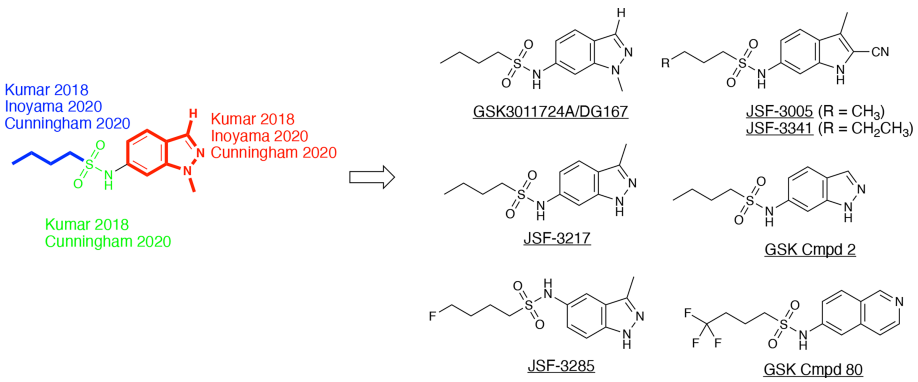


FIGURE 8
DG167 and its analogs. The derivatives, as referenced herein, are noted with regard to alterations in the heterocyclic core and sulfonamide moiety.

through a high-throughout whole-cell phenotypic screening campaign (Ballell et al., 2013; Rebollo-Lopez et al., 2015). This compound and other screening actives were made available to the research community to explore their mechanism of action and optimization. GSK and our laboratory (renaming the molecule as DG167; a convention that will be used throughout this review) each reported on their respective initial studies which characterized the property profile and mechanism of action of this indazole sulfonamide (Abrahams et al., 2016; Kumar et al., 2018). In our case, we were drawn to DG167 because of its signature as an inhibitor of cell wall biosynthesis through its induction of *iniBAC* (Wilson et al., 2013) that lacked cross-resistance with current front- and second-line tuberculosis drugs. While the two reports differ in terms of their exact values for different property metrics, in general, DG167 exhibited promising *in vitro* (i.e., sub-micromolar) potency versus the *M. tuberculosis* H37Rv strain, a lack of cytotoxicity to model mammalian cell lines, and good aqueous solubility. Both groups determined through the generation of spontaneous resistant mutants that DG167 targets KasA with acceptable frequencies of resistance for an antitubercular drug discovery molecule. The protein target was further confirmed *via* additional techniques, such as FAS-II thin layer chromatography (TLC) to evidence mycolic acid biosynthesis inhibition, binding constant quantification with respect to purified KasA, functional inhibition of purified KasA, and target pull-down with a bead-bound analog of DG167.

The X-ray crystal structure of this compound has been elucidated bound to KasA (Abrahams et al., 2016; Kumar et al., 2018). The GSK report established the initial crystal structure (PDB ID 5LD8) and our work had significant contributions in confirming and correcting the initial findings (PDB 5W2P). The GSK structure depicted the acyl channel of each KasA subunit to be occupied by one molecule of DG167 and one molecule of polyethylene glycol (PEG). The purification and crystallization conditions, however, excluded PEG. It is, therefore, unclear to us why PEG was modeled in this structure and continued to be modeled in the more recently determined structures of KasA (Cunningham et al., 2020). In contrast, the electron density maps of our published structure of KasA-DG167 enabled us to unambiguously build two molecules of DG167 ($DG167_A$ and $DG167_B$) in the acyl binding site (Figures 3, 9). $DG167_B$ was in the position modeled as PEG in the GSK publication. KasA-DG167 binding was stabilized by both hydrophobic and hydrogen bond interactions. For instance, the $DG167_A$ sulfonamide N-H formed a hydrogen bond with the Glu199 sidechain. An intermolecular hydrogen bond was observed between the $DG167_A$ sulfonamide oxygen and the $DG167_B$ sulfonamide N-H. Both indazole units of $DG167_A$ and $DG167_B$ contributed to hydrophobic interactions throughout the acyl channel. We observed the aliphatic moiety of $DG167_A$ mirrored the binding of the phospholipid acyl tail reported in previously determined structures of KasA (e.g., PDB ID 4C6W) (Schiebel et al., 2013). More specifically, this *n*-butyl moiety of the 6-sulfonamide was bound in a narrow hydrophobic channel lined by Gly200, Ile202, Pro206, Phe239, His345, and Ile347. Furthermore, the 1-methyl group fit into a shallow pocket defined by Pro201, Glu203, and Pro206, and engaged these amino acids through hydrophobic interactions. The $DG167_B$ indazole nitrogen formed water-mediated hydrogen bonds with Gly115, Asn148, and Ala170. Additionally, the $DG167_B$ indazole mediated hydrophobic interactions across the KasA/KasA′ dimer interface. It is important to note that in the KasA-DG167 structure, gatekeeper residue Phe404 was in the closed conformation where acyl chain access to the acyl channel was restricted. This was, to our knowledge, the first time a KasA ligand (such as an inhibitor) was demonstrated to bind KasA in the closed (nonacylated) conformation. It is remarkable that DG167 can access the acyl channel while Phe404 is in the closed conformation, defeating the elaborate mechanisms KasA has evolved to prevent the entry of cellular free fatty acids into the acyl channel.

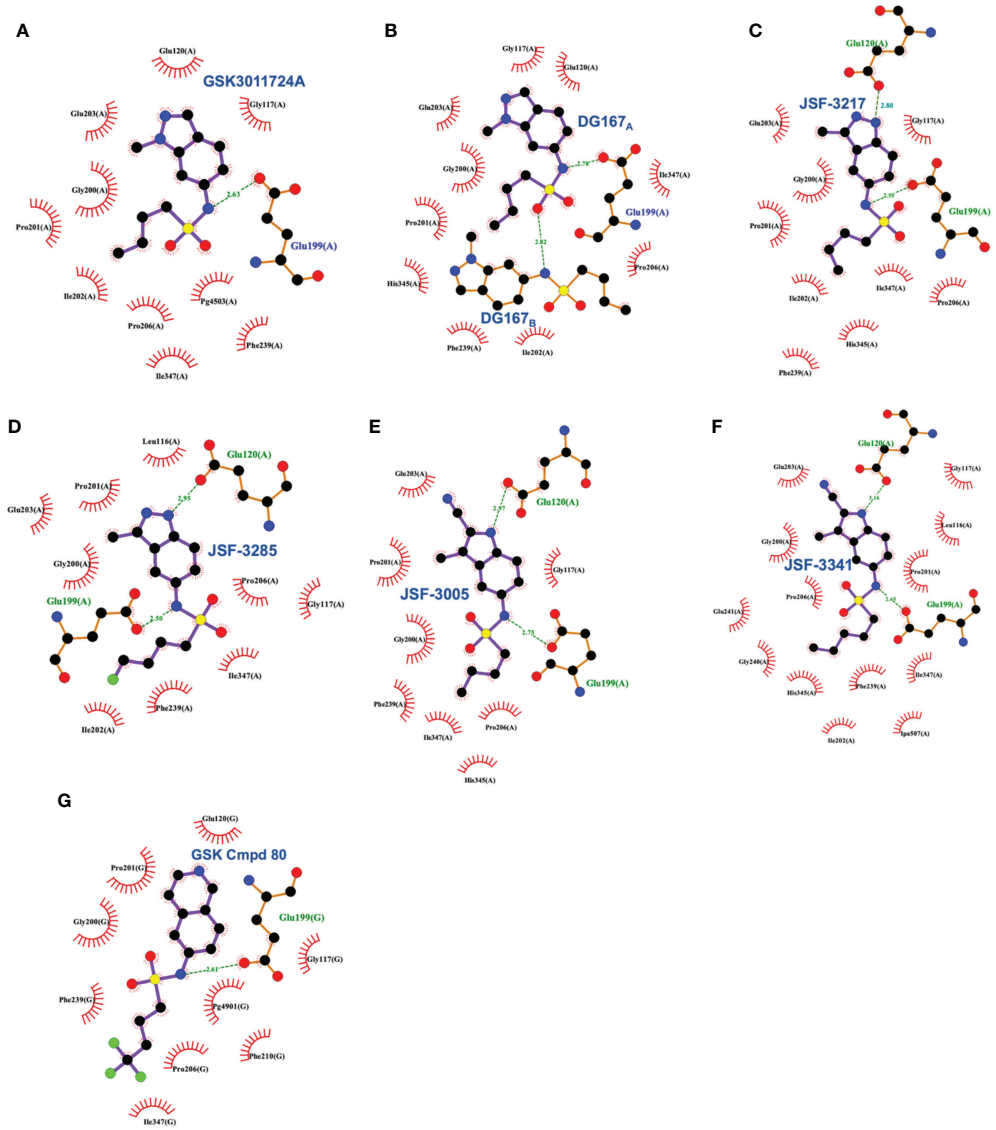


FIGURE 9
Interaction profiles for wild type KasA bound to the following sulfonamides: **(A)** GSK3011724A (PDB ID 5LD8), **(B)** DG167 (PDB ID 5W2P), **(C)** JSF-3217 (PDB ID 5W2S), **(D)** JSF-3285 (PDB ID 6P9L), **(E)** JSF-3005 (PDB ID 6P9K), **(F)** JSF-3341 (PDB ID 6P9M), and **(G)** GSK Cmpd 80 (PDB ID 6Y2J). Hydrogen bonds are shown as green dashed lines and distances are measured in Angstroms.

With the target of DG167 firmly established as KasA, the question as to its *in vivo* profile arose. Studies in both laboratories found the compound to exhibit a modest pharmacokinetic (PK) profile in mice with some evidence of toxicity at doses ≥300 mg/kg once-daily oral (qd po). We proposed that the oral exposure was limited by demethylation of the 1-methyl moiety (half-life $t_{1/2}$ = 10.1 min) to afford the whole-cell inactive 1H-indazole, as observed through incubation of DG167 in the presence of mouse liver microsomes (MLM). Consistent with its modest PK profile, we did not observe DG167 (100 mg/kg qd po) to exhibit the ability to reduce *M. tuberculosis* infection in a sub-acute model of infection when quantifying bacterial burden in the lungs of female BALB/c mice dosed for 2 weeks as compared to the lung bacterial burden at the outset of compound treatment. The GSK report first profiled DG167 in a "fast" and less conservative model (Rullas et al., 2010) with 8 d of drug treatment commencing 1 d post infection of C57BL/6 mice. Quantification of the reduction of lung bacterial burden utilized the level in mouse lung for the no-drug control arm at the end of treatment as the comparison. GSK reported cidal activity in this infection model as well as in a more typical chronic model of infection albeit with what we

would term a less conversative calculation of bacterial load reduction.

These *in vivo* results, in our minds, formed the basis of an optimization problem where we hypothesized that DG167 analogs with enhanced metabolic stability and mouse PK profile would demonstrate significant bactericidal efficacy *in vivo*. In our structure-based optimization reported in 2020, the central design hypothesis was to transpose the indazole 1-nitrogen of DG167 to afford 3-methyl-5-sulfonamide indazoles or indoles (Inoyama et al., 2020). It was hypothesized that this would maintain the hydrophobic interactions of the DG167 1-methyl moiety that our 2018 publication (Kumar et al., 2018) had demonstrated could not be replaced without losses in whole-cell potency and surprisingly also in MLM stability. We entertained a small number of potential alterations to the sulfonamide substituent, having learned from our earlier work (Kumar et al., 2018) that *n*-alkyl groups larger than pentyl and smaller than butyl afforded poorer MIC values. Furthermore, consistent with the relative narrowness of the hydrophobic channel recognizing the sulfonamide alkyl group, we were cognizant that substitutions on all but the terminal carbon of the *n*-butyl, or its replacement with carbocyclic or aromatic/heteroaromatic groups, were not tolerated. It should be noted that we had preliminarily reported on one transposed indazole (compound 5g or JSF-3217; Figure 8) in our 2018 publication that exhibited greater whole-cell activity (MIC = 0.2 μM) than DG167 but with only marginally better MLM stability ($t_{1/2}$ = 11.5 min). Critically, an X-ray structure of JSF-3217 bound to KasA (PDB ID 5W2S) demonstrated the protein-inhibitor interactions that were in our initial designs (Figure 9). Analysis of the X-ray crystal structure led us to postulate that a second molecule of JSF-3217 cannot bind in the $DG167_B$ site because it would force the indazole N(1)-H in close proximity to the hydrophobic surface associated with the KasA dimer interface. In addition to the sulfonamide N-H hydrogen bond with Glu199 similar to the one observed in the DG167 structure, the N-H group of the transposed indazole formed a new hydrogen bond with Glu120. Only one molecule of JSF-3217 bound to a KasA monomer and did not contact the other protomer in the KasA dimer. The KasA–JSF-3217 structure also showed that single molecule acyl channel occupancy was sufficient to stabilize the KasA flap (Luckner et al., 2009) (residues 115 – 147). Further optimization efforts with the transposed indazoles culminated in JSF-3285, which replaced the *n*-butyl of JSF-3217 with a 4-fluorobutyl. The main improvements realized with JSF-3285 as compared to DG167 were its improved mouse PK profile ($AUC_{0\text{-}5h}$ = 59323 vs. 1965 h*ng/mL), MLM stability ($t_{1/2}$ = 28.4 vs. 10.1 min), and kinetic aqueous solubility (S = 483 vs. 324 μM) while slightly enhancing *in vitro* potency (MIC = 0.20 vs. 0.39 μM). Critically, the *in vitro* efficacy of JSF-3285 versus the H37Rv laboratory strain was maintained versus a set of 48 drug-sensitive and drug-resistant clinical strains. This observation furthers our confidence that a KasA-targeting therapeutic will be of significant utility versus both drug-sensitive and drug-resistant infections. We determined the X-ray crystal structure of KasA complexed with JSF-3285 (PDB ID: 6P9L) (Figure 9). Overall, the JSF-3285 and JSF-3217 binding modes were similar. The JSF-3285 sulfonamide alkyl moiety, however, reached further into the hydrophobic channel, making different hydrophobic contacts than the JSF-3217 alkyl sulfonamide. Overall, we were gratified to have structural data to support our structure-based design hypothesis; removal of the metabolic instability of DG167 afforded an advanced compound for further study.

In addition to the transposed indazole optimization campaign, our analysis of the X-ray structure of the KasA-DG167 structure supported removal of the N(2) from the transposed indazole design to afford 3-methyl-5-sulfonamide indoles where we could explore additional interactions with KasA through modification of the 2-substituent. Key molecules in this campaign were JSF-3005 and JSF-3341 (Figure 8) which both featured a 2-cyano group and the sulfonamide moiety as *n*-butyl or *n*-pentyl, respectively. Their *in vitro* growth inhibitory potencies versus the *Mtb* H37Rv strain were 0.78 and 0.20 μM, respectively. Each indole was crystallized bound to the KasA (Figure 9). Both JSF-3005 and JSF-3341 utilized similar hydrogen bonding interactions between the indole N-H and KasA Glu120, as well as the sulfonamide N-H and KasA Glu199. However, a few differences were observed like additional electron density corresponding to an unknown molecule identified in the acyl channel of the KasA–JSF-3005 complex. The alkyl chain in JSF-3341 is longer by one carbon than in JSF-3005, extending 0.9 Å deeper into the hydrophobic channel. The sulfonamide group in JSF-3341 was slightly shifted by 0.5 Å when compared to JSF-3005. The additional methyl group on the sulfonamide of JSF-3341 also mediated additional hydrophobic contacts not observed in the KasA–JSF-3005 complex structure.

JSF-3005, JSF-3341, and JSF-3285 were studied thoroughly in an effort to proceed to *in vivo* efficacy assessment. Summarily, all three compounds were rigorously confirmed to primarily target KasA through the following methods in addition to the previously described X-ray crystallography with their KasA complex: quantification of KasA binding *via* microscale thermophoresis, fatty acid TLC, and drug-resistant mutant generation and sequencing. The three molecules were assessed in mouse models of sub-acute and chronic *M. tuberculosis* infection. In the sub-acute model with four weeks of compound treatment, JSF-3005 dosing (100 mg/kg qd po) led to increases in the lung bacterial burden and JSF-3341 dosing (200 mg qd po) afforded bacteriostatic activity. JSF-3285 was dosed at 100 mg/kg and 200 mg/kg qd po, in accordance with dose proportionality and tolerability studies, in the sub-acute infection model, and we were delighted to observe an ca. 2 $\log_{10}$ reduction in bacterial burden as quantified by the lung bacterial burden post 4 weeks of treatment as compared to the bacterial

burden at the beginning of compound treatment. The corresponding chronic infection assessment in BALB/c mice demonstrated JSF-3285 at 100 or 200 mg/kg to reduce the bacterial burden in the lungs >2 $\log_{10}$ colony-forming units (CFUs) after 4 weeks as compared to the bacterial burden at the start of the treatment. The addition of JSF-3285 (200 mg/kg) to INH or RIF (either at 10 mg/kg) improved efficacy of the front-line drug by about 1 $\log_{10}$ CFUs. Furthermore, the bactericidal efficacy of JSF-3285 at doses ranging from 200 – 20 mg/kg qd po was examined. At 20 mg/kg qd po, INH and RIF each afforded just more than a 2 $\log_{10}$ reduction in CFUs. JSF-3285 exhibited an approximately 1.5 – 2.0 $\log_{10}$ reduction in CFUs at doses of 20 – 200 mg/kg. These experiments provided rigorous validation for JSF-3285 as a preclinical tuberculosis drug candidate and critically established *in vivo* pharmacological validation of KasA with conservative quantification of cidal bacterial efficacy.

Building on their earlier work (Abrahams et al., 2016), a GSK team reported (Cunningham et al., 2020) on the results of their evolution of DG167 in a manuscript submitted four months after the submission of our report; both papers were published in March 2020. They verified the whole-cell potential of the transposed indazole chemotype found by us (Kumar et al., 2018; Inoyama et al., 2020), but mainly focused on their finding of the Ames mutagenicity of the amine metabolite of their initial hit or select transposed indazoles (e.g., GSK Cmpd 2 in Figure 8). Our efforts to address this concern will be published separately. While the amount of the amine metabolite found in the urine of a single Sprague Dawley rat dosed at 300 mg/kg po in each case was not quantified, the *in vivo* observation of an amine with an Ames positive signal was a significant concern in the absence of further studies.

The GSK team proposed several strategies to prevent the formation of a mutagenic amine, primarily by substituting for the sulfonamide with other moieties that would not suffer hydrolysis. Additionally, the steric and/or electronic environment was modified around the 6-membered ring of the core heterocycle or the 5-membered ring of the indazole was replaced with other ring systems; the goal was to eliminate the mutagenicity of the amine formed. These alterations failed to find afford a coalescence of antitubercular whole-cell efficacy with a lack of Ames mutagenicity associated with the related amine metabolite.

A significant factor in this outcome was the necessity of maintaining the sulfonamide, which, as described earlier, serves as a recognition element providing an appropriate straight-chain alkyl hydrophobe along with the hydrogen bond between its N-H to Glu199. Changes of the central heterocycle from indazole to indole, 2,3-dihydro-1*H*-indene, and isoquinoline (e.g., GSK Cmpd 80 in Figure 8) were achieved, but they do not appear to have offered sufficient advantages worthy of further exploration. The indole substitution was not surprising given that it involves the removal of the indazole nitrogen from DG167 to afford a 5-substituted indole. The corresponding N-(2,3-dihydro-1H-inden-5-yl)butane-1-sulfonamide exhibited modest efficacy (MIC = 16.3 μM) while lacking the N-H to proposedly hydrogen bond with Glu120. Finally, the GSK Cmpd 80 isoquinoline sulfonamide demonstrated modest potency (MIC = 15 μM) and was structurally characterized bound to KasA (PDB ID 6Y2J; Figure 9). While its *n*-butyl sulfonamide exhibited similar interactions as DG167, its basic nitrogen was shown to engage a water molecule through hydrogen-bonding; Glu120 did not interact with the inhibitor. These results led the authors to speculate on the need for the planarity of the heterocycle given the proximity of Gly200 and Pro201. However, it is not obvious that this would contribute significantly to the binding energy. We suspect that the loss of hydrogen-bonding to Glu120 is primarily responsible for the reduction in compound activity.

Summary

This review focuses on the promise of KasA as a tuberculosis drug target and, in particular, on the importance of structure-based design approaches to deliver KasA inhibitors of translational significance. Thus, we did not discuss in-depth earlier stage molecules, such as cerulenin (Schaeffer et al., 2001) and platensimycin (Brown et al., 2009), that have not been supported by published X-ray crystal structures in complex with KasA. We also have not described computational approaches (Puhl et al., 2020) involving docking and/or machine learning methods to design KasA binders. Most likely, X-ray crystal structures of these compounds bound to KasA will be necessary to enable their design optimization. Our analysis of the literature supports KasA as a highly valuable target for future study. A panoply of approaches has been utilized to vet KasA with regard to its essentiality and vulnerability. Two binding sites (i.e., the malonyl binding and acyl binding sites) have been targeted with small molecules and the relevant interactions have been discerned through X-ray crystallography. KasA acyl binding channel targeting with drug-like small molecules has been shown to be critical to significantly reduce the *M. tuberculosis* infection in mice, while efforts with malonyl binding site inhibitors have fallen short thus far of *in vivo* pharmacologic validation. While the current *in vivo* active molecules have limitations, we expect that ongoing studies with JSF-3285 and newer series from our laboratories, GSK, and others will find the requisite balancing of efficacy and toxicity profiles. We anticipate that structure-based design efforts, as summarized herein, will play a prominent role in these endeavors. Ultimately, we are optimistic that the sum total of efforts with *M. tuberculosis* KasA inhibitors will afford one or more molecules for clinical studies that will positively impact drug regimens for both drug-sensitive and drug-resistant infections.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

Funding

This work was supported at Rutgers University – New Jersey Medical School by the US National Institutes of Health grants U19AI142731 (MN and JF).

Acknowledgments

We want to thank Dr. Clifton E. Barry III (NIH/NIAID) and Dr. Alexander L. Perryman (Repare Therapeutics) for helpful discussions.

Conflict of interest

MN and JF are listed as inventors on patent filings pertinent to the indole and indazole compounds mentioned in this manuscript as employees of Rutgers University.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Abrahams, K. A., and Besra, G. S. (2020). Mycobacterial drug discovery. *RSC Med. Chem.* 11, 1354–1365. doi: 10.1039/D0MD00261E

Abrahams, K. A., Chung, C. W., Ghidelli-Disse, S., Rullas, J., Rebollo-Lopez, M. J., Gurcha, S. S., et al. (2016). Identification of KasA as the cellular target of an anti-tubercular scaffold. *Nat. Commun.* 7, 12581. doi: 10.1038/ncomms12581

Ballell, L., Bates, R. H., Young, R. J., Alvarez-Gomez, D., Alvarez-Ruiz, E., Barroso, V., et al. (2013). Fueling open-source drug discovery: 177 small-molecule leads against tuberculosis. *ChemMedChem* 8, 313–321. doi: 10.1002/cmdc.201200428

Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., et al. (1994). InhA, a gene encoding a target for isoniazid and ethionamide in *M. tuberculosis*. *Science* 263, 227–230. doi: 10.1126/science.8284673

Bhatt, A., Fujiwara, N., Bhatt, K., Gurcha, S. S., Kremer, L., Chen, B., et al. (2007). Deletion of kasB in Mycobacterium tuberculosis causes loss of acid-fastness and subclinical latent tuberculosis in immunocompetent mice. *Proc. Natl. Acad. Sci. U.S.A.* 104, 5157–5162. doi: 10.1073/pnas.0608654104

Bhatt, A., Molle, V., Besra, G. S., Jacobs, W. R.Jr., and Kremer, L. (2007). The Mycobacterium tuberculosis FAS-II condensing enzymes: Their role in mycolic acid biosynthesis, acid-fastness, pathogenesis and in future drug development. *Mol. Microbiol.* 64, 1442–1454. doi: 10.1111/j.1365-2958.2007.05761.x

Bommineni, G. R., Kapilashrami, K., Cummings, J. E., Lu, Y., Knudson, S. E., Gu, C., et al. (2016). Thiolactomycin-based inhibitors of bacterial beta-ketoacyl-ACP synthases with *in vivo* activity. *J. Med. Chem.* 59, 5377–5390. doi: 10.1021/acs.jmedchem.6b00236

Bosch, B., DeJesus, M. A., Poulton, N. C., Zhang, W., Engelhart, C. A., Zaveri, A., et al. (2021). Genome-wide gene expression tuning reveals diverse vulnerabilities of m. *tuberculosis Cell.* 184, 4579–92.e24. doi: 10.1016/j.cell.2021.06.033

Brennan, P. J., and Nikaido, H. (1995). The envelope of mycobacteria. *Annu. Rev. Biochem.* 64, 29–63. doi: 10.1146/annurev.bi.64.070195.000333

Brown, A. K., Taylor, R. C., Bhatt, A., Futterer, K., and Besra, G. S. (2009). Platensimycin activity against mycobacterial beta-ketoacyl-ACP synthases. *PLoS One* 4, e6306. doi: 10.1371/journal.pone.0006306

Carey, A. F., Rock, J. M., Krieger, I. V., Chase, M. R., Fernandez-Suarez, M., Gagneux, S., et al. (2018). TnSeq of Mycobacterium tuberculosis clinical isolates reveals strain-specific antibiotic liabilities. *PLoS Pathog.* 14, e1006939. doi: 10.1371/journal.ppat.1006939

Choi, K. H., Kremer, L., Besra, G. S., and Rock, C. O. (2000). Identification and substrate specificity of beta -ketoacyl (acyl carrier protein) synthase III (mtFabH) from Mycobacterium tuberculosis. *J. Biol. Chem.* 275, 28201–28207. doi: 10.1074/jbc.M003241200

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., et al. (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 393, 537–544. doi: 10.1038/31159

Cunningham, F., Esquivias, J., Fernandez-Menendez, R., Perez, A., Guardia, A., Escribano, J., et al. (2020). Exploring the SAR of the beta-Ketoacyl-ACP synthase inhibitor GSK3011724A and optimization around a genotoxic metabolite. *ACS Infect. Dis.* 6, 1098–1109. doi: 10.1021/acsinfecdis.9b00493

Dartois, V. A., and Rubin, E. J. (2022). Anti-tuberculosis treatment strategies and drug development: challenges and priorities. *Nat. Rev. Microbiol.* 20. doi: 10.1038/s41579-022-00731-y

DeJesus, M. A., Gerrick, E. R., Xu, W., Park, S. W., Long, J. E., Boutte, C. C., et al. (2017). Comprehensive essentiality analysis of the Mycobacterium tuberculosis genome *via* saturating transposon mutagenesis. *mBio* 8, e02133–e02116. doi: 10.1128/mBio.02133-16

Dulberger, C. L., Rubin, E. J., and Boutte, C. C. (2020). The mycobacterial cell envelope - a moving target. *Nat. Rev. Microbiol.* 18, 47–59. doi: 10.1038/s41579-019-0273-7

Fernandes, G. F. S., Thompson, A. M., Castagnolo, D., Denny, W. A., and Dos Santos, J. L. (2022). Tuberculosis drug discovery: Challenges and new horizons. *J. Med. Chem.* 65, 7489–7531. doi: 10.1021/acs.jmedchem.2c00227

Gavalda, S., Leger, M., van der Rest, B., Stella, A., Bardou, F., Montrozier, H., et al. (2009). The Pks13/FadD32 crosstalk for the biosynthesis of mycolic acids in Mycobacterium tuberculosis. *J. Biol. Chem.* 284, 19255–19264. doi: 10.1074/jbc.M109.006940

Griffin, J. E., Gawronski, J. D., Dejesus, M. A., Ioerger, T. R., Akerley, B. J., and Sassetti, C. M. (2011). High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. *PloS Pathog.* 7, e1002251. doi: 10.1371/journal.ppat.1002251

Hoffmann, C., Leis, A., Niederweis, M., Plitzko, J. M., and Engelhardt, H. (2008). Disclosure of the mycobacterial outer membrane: Cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. *Proc. Natl. Acad. Sci. U. S. A.* 105, 3963–3967. doi: 10.1073/pnas.0709530105

Huang, W., Jia, J., Edwards, P., Dehesh, K., Schneider, G., and Lindqvist, Y. (1998). Crystal structure of beta-ketoacyl-acyl carrier protein synthase II from e.coli reveals the molecular architecture of condensing enzymes. *EMBO J.* 17, 1183–1191. doi: 10.1093/emboj/17.5.1183

Inoyama, D., Awasthi, D., Capodagli, G. C., Tsotetsi, K., Sukheja, P., Zimmerman, M., et al. (2020). A preclinical candidate targeting Mycobacterium tuberculosis KasA. *Cell Chem. Biol.* 27, 560–570. doi: 10.1016/j.chembiol.2020.02.007

Kapilashrami, K., Bommineni, G. R., Machutta, C. A., Kim, P., Lai, C. T., Simmerling, C., et al. (2013). Thiolactomycin-based beta-ketoacyl-AcpM synthase a (KasA) inhibitors: Fragment-based inhibitor discovery using transient one-dimensional nuclear overhauser effect NMR spectroscopy. *J. Biol. Chem.* 288, 6045–6052. doi: 10.1074/jbc.M112.414516

Kim, P., Zhang, Y. M., Shenoy, G., Nguyen, Q. A., Boshoff, H. I., Manjunatha, U. H., et al. (2006). Structure-activity relationships at the 5-position of thiolactomycin: an intact (5R)-isoprene unit is required for activity against the condensing enzymes from Mycobacterium tuberculosis and escherichia coli. *J. Med. Chem.* 49, 159–171. doi: 10.1021/jm050825p

Kremer, L., Douglas, J. D., Baulard, A. R., Morehouse, C., Guy, M. R., Alland, D., et al. (2000). Thiolactomycin and related analogues as novel anti-mycobacterial agents targeting KasA and KasB condensing enzymes in Mycobacterium tuberculosis. *J. Biol. Chem.* 275, 16857–16864. doi: 10.1074/jbc.M000569200

Kumar, P., Capodagli, G. C., Awasthi, D., Shrestha, R., Maharaja, K., Sukheja, P., et al. (2018). Synergistic lethality of a binary inhibitor of Mycobacterium tuberculosis KasA. *mBio* 9, e02101–e02117. doi: 10.1128/mBio.02101-17

Lee, W., and Engels, B. (2014). The protonation state of catalytic residues in the resting state of KasA revisited: Detailed mechanism for the activation of KasA by its own substrate. *Biochemistry* 53, 919–931. doi: 10.1021/bi401308j

Lee, W., Luckner, S. R., Kisker, C., Tonge, P. J., and Engels, B. (2011). Elucidation of the protonation states of the catalytic residues in mtKasA: Implications for inhibitor design. *Biochemistry* 50, 5743–5756. doi: 10.1021/bi200006t

Leger, M., Gavalda, S., Guillet, V., van der Rest, B., Slama, N., Montrozier, H., et al. (2009). The dual function of the Mycobacterium tuberculosis FadD32 required for mycolic acid biosynthesis. *Chem. Biol.* 16, 510–519. doi: 10.1016/j.chembiol.2009.03.012

Luckner, S. R., Machutta, C. A., Tonge, P. J., and Kisker, C. (2009). Crystal structures of mycobacterium tuberculosis KasA show mode of action within cell wall biosynthesis and its inhibition by thiolactomycin. *Structure* 17, 1004–1013. doi: 10.1016/j.str.2009.04.012

Lu, H., and Tonge, P. J. (2008). Inhibitors of FabI, an enzyme drug target in the bacterial fatty acid biosynthesis pathway. *Acc. Chem. Res.* 41, 11–20. doi: 10.1021/ar700156e

Lu, H., and Tonge, P. J. (2010). Drug-target residence time: Critical information for lead optimization. *Curr. Opin. Chem. Biol.* 14, 467–474. doi: 10.1016/j.cbpa.2010.06.176

Machutta, C. A., Bommineni, G. R., Luckner, S. R., Kapilashrami, K., Ruzsicska, B., Simmerling, C., et al. (2010). Slow onset inhibition of bacterial beta-ketoacyl-acyl carrier protein synthases by thiolactomycin. *J. Biol. Chem.* 285, 6161–6169. doi: 10.1074/jbc.M109.077909

Magnuson, K., Jackowski, S., Rock, C. O., and Cronan, J. E.Jr. (1993). Regulation of fatty acid biosynthesis in escherichia coli. *Microbiol. Rev.* 57, 522–542. doi: 10.1128/mr.57.3.522-542.1993

Marrakchi, H., Laneelle, M. A., and Daffe, M. (2014). Mycolic acids: Structures, biosynthesis, and beyond. *Chem. Biol.* 21, 67–85. doi: 10.1016/j.chembiol.2013.11.011

Minato, Y., Gohl, D. M., Thiede, J. M., Chacon, J. M., Harcombe, W. R., Maruyama, F., et al. (2019). Genomewide assessment of mycobacterium tuberculosis conditionally essential metabolic pathways. *mSystems* 4, e00070–e00019. doi: 10.1128/mSystems.00070-19

Miyakawa, S., Suzuki, K., Noto, T., Harada, Y., and Okazaki, H. (1982). Thiolactomycin, a new antibiotic. IV. biological properties and chemotherapeutic activity in mice. *J. Antibiot. (Tokyo).* 35, 411–419. doi: 10.7164/antibiotics.35.411

Nishida, I., Kawaguchi, A., and Yamada, M. (1986). Effect of thiolactomycin on the individual enzymes of the fatty acid synthase system in escherichia coli. *J. Biochem.* 99, 1447–1454. doi: 10.1093/oxfordjournals.jbchem.a135614

Oishi, H., Noto, T., Sasaki, H., Suzuki, K., Hayashi, T., Okazaki, H., et al. (1982). Thiolactomycin, a new antibiotic. i. taxonomy of the producing organism, fermentation and biological properties. *J. Antibiot. (Tokyo).* 35, 391–395. doi: 10.7164/antibiotics.35.391

Portevin, D., De Sousa-D'Auria, C., Houssin, C., Grimaldi, C., Chami, M., Daffe, M., et al. (2004). A polyketide synthase catalyzes the last condensation step of mycolic acid biosynthesis in mycobacteria and related organisms. *Proc. Natl. Acad. Sci. U.S.A.* 101, 314–319. doi: 10.1073/pnas.0305439101

Puhl, A. C., Lane, T. R., Vignaux, P. A., Zorn, K. M., Capodagli, G. C., Neiditch, M. B., et al. (2020). Computational approaches to identify molecules binding to Mycobacterium tuberculosis KasA. *ACS Omega.* 5, 29935–29942. doi: 10.1021/acsomega.0c04271

Rahlwes, K. C., Sparks, I. L., and Morita, Y. S. (2019). Cell walls and membranes of actinobacteria. *Subcell. Biochem.* 92, 417–469. doi: 10.1007/978-3-030-18768-2_13

Rebollo-Lopez, M. J., Lelievre, J., Alvarez-Gomez, D., Castro-Pichel, J., Martinez-Jimenez, F., Papadatos, G., et al. (2015). Release of 50 new, drug-like compounds and their computational target predictions for open source anti-tubercular drug discovery. *PloS One* 10, e0142293. doi: 10.1371/journal.pone.0142293

Rock, J. M., Hopkins, F. F., Chavez, A., Diallo, M., Chase, M. R., Gerrick, E. R., et al. (2017). Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform. *Nat. Microbiol.* 2, 16274. doi: 10.1038/nmicrobiol.2016.274

Rullas, J., Garcia, J. I., Beltran, M., Cardona, P. J., Caceres, N., Garcia-Bustos, J. F., et al. (2010). Fast standardized therapeutic-efficacy assay for drug discovery against tuberculosis. *Antimicrob. Agents Chemother.* 54, 2262–2264. doi: 10.1128/AAC.01423-09

Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2003). Genes required for mycobacterial growth defined by high density mutagenesis. *Mol. Microbiol.* 48, 77–84. doi: 10.1046/j.1365-2958.2003.03425.x

Schaeffer, M. L., Agnihotri, G., Volker, C., Kallender, H., Brennan, P. J., and Lonsdale, J. T. (2001). Purification and biochemical characterization of the Mycobacterium tuberculosis beta-ketoacyl-acyl carrier protein synthases KasA and KasB. *J. Biol. Chem.* 276, 47029–47037. doi: 10.1074/jbc.M108903200

Schiebel, J., Kapilashrami, K., Fekete, A., Bommineni, G. R., Schaefer, C. M., Mueller, M. J., et al. (2013). Structural basis for the recognition of mycolic acid precursors by KasA, a condensing enzyme and drug target from Mycobacterium tuberculosis. *J. Biol. Chem.* 288, 34190–34204. doi: 10.1074/jbc.M113.511436

Slayden, R. A., and Barry, C. E.3rd. (2002). The role of KasA and KasB in the biosynthesis of meromycolic acids and isoniazid resistance in Mycobacterium tuberculosis. *Tuberculosis (Edinb).* 82, 149–160. doi: 10.1054/tube.2002.0333

Slayden, R. A., Lee, R. E., Armour, J. W., Cooper, A. M., Orme, I. M., Brennan, P. J., et al. (1996). Antimycobacterial action of thiolactomycin: An inhibitor of fatty acid and mycolic acid synthesis. *Antimicrob. Agents Chemother.* 40, 2813–2819. doi: 10.1128/AAC.40.12.2813

Takayama, K., Wang, C., and Besra, G. S. (2005). Pathway to synthesis and processing of mycolic acids in Mycobacterium tuberculosis. *Clin. Microbiol. Rev.* 18, 81–101. doi: 10.1128/CMR.18.1.81-101.2005

Vilcheze, C., Wang, F., Arai, M., Hazbon, M. H., Colangeli, R., Kremer, L., et al. (2006). Transfer of a point mutation in *Mycobacterium tuberculosis inhA* resolves the target of isoniazid. *Nat. Med.* 12, 1027–1029. doi: 10.1038/nm1466

Wang, X., Inoyama, D., Russo, R., Li, S. G., Jadhav, R., Stratton, T. P., et al. (2020). Antitubercular triazines: Optimization and intrabacterial metabolism. *Cell. Chem. Biol.* 27, 172–85.e11. doi: 10.1016/j.chembiol.2019.10.010

Wang, X., Perryman, A. L., Li, S. G., Paget, S. D., Stratton, T. P., Lemenze, A., et al. (2019). Intrabacterial metabolism obscures the successful prediction of an InhA inhibitor of Mycobacterium tuberculosis. *ACS Infect. Dis.* 5, 2148–2163. doi: 10.1021/acsinfecdis.9b00295

Wilson, R., Kumar, P., Parashar, V., Vilcheze, C., Veyron-Churlet, R., Freundlich, J. S., et al. (2013). Antituberculosis thiophenes define a requirement for Pks13 in mycolic acid biosynthesis. *Nat. Chem. Biol.* 9, 499–506. doi: 10.1038/nchembio.1277

Zhang, Y. J., Ioerger, T. R., Huttenhower, C., Long, J. E., Sassetti, C. M., Sacchettini, J. C., et al. (2012). Global assessment of genomic regions required for growth in Mycobacterium tuberculosis. *PLoS Pathog.* 8, e1002946. doi: 10.1371/journal.ppat.1002946

Zuber, B., Chami, M., Houssin, C., Dubochet, J., Griffiths, G., and Daffe, M. (2008). Direct visualization of the outer membrane of mycobacteria and corynebacteria in their native state. *J. Bacteriol.* 190, 5672–5680. doi: 10.1128/JB.01919-07

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 15 September 2022
DOI 10.3389/fcimb.2022.932556

OPEN ACCESS

EDITED BY
Ben Gold,
Weill Cornell Medicine, United States

REVIEWED BY
Carrie Leigh Shaffer,
University of Kentucky, United States
Tanya Parish,
Seattle Children's Hospital,
United States
Anna Upton,
Evotec, United States

*CORRESPONDENCE
Eric J. Rubin
erubin@hsph.harvard.edu

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 29 April 2022
ACCEPTED 17 August 2022
PUBLISHED 15 September 2022

CITATION
Tomasi FG and Rubin EJ (2022) Failing upwards: Genetics-based strategies to improve antibiotic discovery and efficacy in *Mycobacterium tuberculosis*. *Front. Cell. Infect. Microbiol.* 12:932556. doi: 10.3389/fcimb.2022.932556

Failing upwards: Genetics-based strategies to improve antibiotic discovery and efficacy in *Mycobacterium tuberculosis*

Francesca G. Tomasi and Eric J. Rubin*

Department of Immunology and Infectious Diseases, Harvard T. H. Chan School of Public Health, Boston, MA, United States

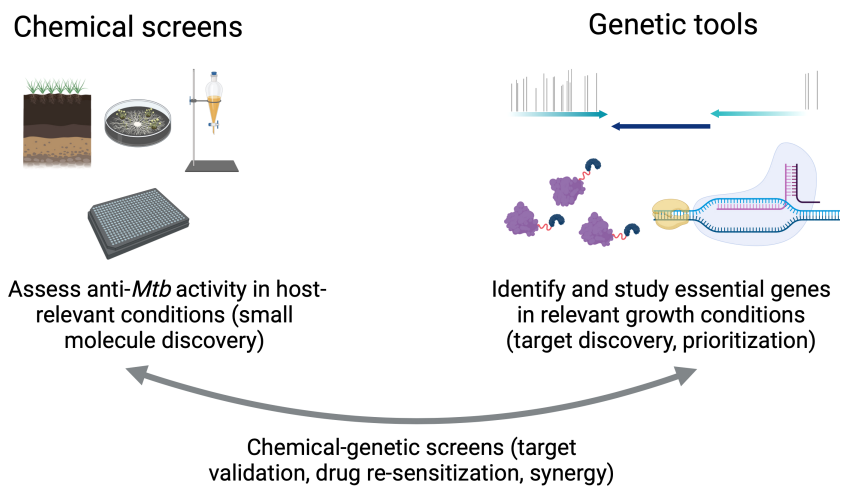
Therapeutic advances in the 20th century significantly reduced tuberculosis (TB) mortality. Nonetheless, TB still poses a massive global health challenge with significant annual morbidity and mortality that has been amplified during the COVID-19 pandemic. Unlike most common bacterial infectious diseases, successful TB treatment requires months-long regimens, which complicates the ability to treat all cases quickly and effectively. Improving TB chemotherapy by reducing treatment duration and optimizing combinations of drugs is an important step to reducing relapse. In this review, we outline the limitations of current multidrug regimens against TB and have reviewed the genetic tools available to improve the identification of drug targets. The rational design of regimens that sterilize diverse phenotypic subpopulations will maximize bacterial killing while minimizing both treatment duration and infection relapse. Importantly, the TB field currently has all the necessary genetic and analytical tools to screen for and prioritize drug targets *in vitro* based on the vulnerability of essential and non-essential genes in the *Mtb* genome and to translate these findings in *in vivo* models. Combining genetic methods with chemical screens offers a formidable strategy to redefine the preclinical design of TB therapy by identifying powerful new targets altogether, as well as targets that lend new efficacy to existing drugs.

KEYWORDS

antibiotic resistance, tuberculosis, bacterial genetics, chemical genetic profiling, CRISPRi, TnSeq, drug discovery

Current challenges in tuberculosis therapy

A persistent disease requires a persistent response. The 2021 Global Tuberculosis Report published by the World Health Organization (WHO) estimates that 1.5 million people died of TB in 2020 and that recent progress in reducing TB incidence has stalled globally during the COVID-19 pandemic (W.H.O, 2021). The social, political, and



GRAPHICAL ABSTRACT
Existing chemical and genetic tools can be used to improve antibiotic discovery against tuberculosis. Left: High-throughput screening of chemical libraries derived from natural products (such as soil and fungal extracts), as well as from synthetical small-molecule collections, can identify anti-tubercular compounds for further development into clinically available drugs. Right: Genetic tools including transposon sequencing (TnSeq; top), proteolytic degradation (bottom left), and CRISPR interference (CRISPRi; bottom right) can be used to identify and characterize genes whose products are required in relevant laboratory growth conditions (such as macrophage models) for target discovery and prioritization. Importantly, chemical and genetic tools can also be combined to validate drug targets, identify synergistic activities between existing molecules and hypothetical drug targets, and gain valuable insights while designing combination antibiotic therapies. Figure created on BioRender.com

economic challenges of TB control are exacerbated by the months-long regimens currently required to treat *Mycobacterium tuberculosis* (*Mtb*). Strengthening research programs to improve TB therapy will synergize with global health efforts to increase access to, as well as success of, treatment.

The first anti-tuberculous drug, streptomycin, was discovered by Selman Waksman, Elizabeth Bugie, and Albert Schatz in 1943. Patients who received streptomycin saw initial clinical improvement but eventually developed resistance; single-drug treatment did not significantly improve TB mortality in the long term (Gernez et al., 1948; Regniers et al., 1949; Tempel, 1949). These discoveries ushered in an era of small-molecule discovery against *Mtb* and the development of a multidrug therapy to minimize rates of acquired genetic resistance (Connolly et al., 2007). This multidrug therapy is administered for long periods of time (4–9 months with drug susceptible TB) to circumvent *Mtb*'s ability to develop phenotypic resistance, in which a subpopulation of metabolically altered cells tolerates antibiotic exposure and requires a longer time to eradicate (Connolly et al., 2007; Hicks et al., 2018). Longer treatment times also cause more frequent and serious side effects ranging from malaise to neuropathy. Adverse events during long-course antibiotic treatment have been major barriers to eliminating TB worldwide. When a drug regimen is taken fully as prescribed, relapse is still estimated to occur in about 5% of patients with drug-susceptible after 6 months of first-line treatment and in about 20% of patients after 4 months (Colangeli et al., 2018). *Mtb* is also intrinsically resistant to most of the antibiotics used to treat other bacterial infections, which requires TB-specific drug discovery programs and reduced overlap with other antibiotic discovery programs.

The emergence and spread of multidrug-resistant (MDR) TB pose an additional and massive medical and economic burden. MDR TB is defined by *Mtb* that is resistant to at least one first-line TB drug in addition to isoniazid or rifampicin (W.H.O, 2021). Extensively drug-resistant (XDR) TB is resistant to multiple first-line TB drugs and is even more complicated to treat. Recent advances in MDR and XDR TB therapy have significantly improved treatment outcomes and reflect ongoing progress in anti-tubercular drug discovery, including the discovery and implementation of pretomanid, bedaquiline, and linezolid (Conradie et al., 2020).

Long-term relapse-based experiments in mice established shorter and more effective regimens by combining pretomanid and bedaquiline with linezolid or moxifloxacin and pyrazinamide (Tasneen et al., 2011; Li et al., 2017; Xu et al., 2019). The Nix-TB trial on humans (ClinicalTrials.gov #NCT02333799) found that combining bedaquiline, pretomanid, and linezolid could treat patients with highly drug-resistant TB in 6 months (compared to traditional 20-month regimens), although not without significant adverse events (Conradie et al., 2020). The shift to an all-oral, more

effective, and shorter regimen for drug-resistant TB is a major success. Nonetheless, its duration and side effects warrant continued research, as access to simpler therapies remains a priority for drug susceptible, MDR, and XDR TB. Recent clinical trials have also seen promise in shorting treatment times for drug-susceptible TB (ClinicalTrials.gov #NCT02410772): a 4-month rifapentine-based regimen including moxifloxacin was determined to be non-inferior to the current standard 6-month regimen (Dorman et al., 2021). How can we continue to drive down treatment times?

In this review, we examine the tools available to identify new antibiotic targets in whole cells, to discover compound mechanisms of action, to search for off-target effects, and to use existing drugs in new ways. Identifying new antibiotic targets can be accomplished through high-throughput small-molecule screens against whole cells as well as target-based discovery using genetic tools. We can also apply these strategies to lend efficacy to existing drugs and quantify combinatorial effects between different experimental treatments. Historically, maximizing synergy between antibiotic targets was not a priority when establishing TB regimens. Here, we encourage a synergy-focused framework for designing new treatment courses that focus on well-tolerated combination antibiotics that maximize bacterial killing. This strategy is uniquely poised uniquely poised to improve both antibiotic discovery and efficacy of candidate treatments.

Identifying new antibiotic targets

Chemical screens

Systematic screens of soil, bacterial, or fungal extracts as well as synthetic chemical libraries have seen the most translational success so far in antibiotic discovery (Tommasi et al., 2015). A benefit to a chemical screen approach is the convenience of starting with small molecules with promising physiochemical properties that can be further modified. A major barrier to TB drug development is cell wall penetration, and chemical screens select for compounds that can already enter *Mtb* cells. Many first- and second-line TB drugs are derived from natural products including rifampicin and aminoglycosides such as streptomycin (Mdluli et al., 2015). These approaches work by testing compound libraries for their ability to block bacterial growth *in vitro*, usually under standardized aerobic growth conditions, and has enabled the identification of particularly accessible bacterial targets and pathways that are susceptible to existing compounds.

While historically effective, chemical library screening *in vitro* using standard laboratory growth conditions has now led to recurring targets (such as MmpL3) and redundancies in hits, with similar small molecules repeatedly being discovered in different libraries. In fact, the most-used antibiotics across bacterial infections block the same small subset of targets (Payne et al., 2007). This is also a reflection of library screen design: the same approaches to identifying compounds will yield the same results. In the era of antibiotic resistance, diversity of mechanism is crucial to bypass existing resistance mechanisms. Indeed, more recent chemical screens have been conducted in host-relevant growth conditions including low pH, different carbon sources, nitric oxide stress, hypoxia, and granuloma assays (Cho et al., 2007; Huang et al., 2018; Early et al., 2019).

Pyrazinamide is a breakthrough TB drug that underscores the value of testing multiple growth conditions and mimicking the host environment. On its own in a standard broth microdilution assay, pyrazinamide does not have appreciable growth-inhibiting effects on *Mtb*. However, when added under hypoxic conditions, pyrazinamide becomes highly sterilizing and has been critical to reducing TB treatment times from multiple years to several months (Wade and Zhang, 2004). This example shows the importance of evolving chemical screens to identify critical compounds that may not otherwise be uncovered using standard laboratory growth conditions.

Despite substantial efforts in systematic chemical library screens, treatment of drug-susceptible TB has remained largely unchanged since the mid-20th century, highlighting the need for creative new approaches. One such approach involves re-sensitization studies, in which compounds are screened for inhibition of stress responses instead of direct killing of bacterial cells. In one study, a small molecule was identified that inhibited *Mtb* tolerance to oxidative stress, acid stress, and isoniazid by inhibiting respiration (Flentie et al., 2019). In this case, use of this molecule with current TB treatment regimens could potentially reverse isoniazid resistance.

Library screens using specialized strains and other growth conditions described above such as macrophage screens, hypoxic media, or caseum-like environments are likely to improve translation from *in vitro* hits to *in vivo* success [reviewed in (Dartois and Barry, 2013)]. In 2009, Christophe et al., published a cell-based assay that used confocal fluorescence microscopy to screen for compounds that blocked *Mtb* replication within macrophages. Through this screen, they identified dinitrobenzamide derivatives with potent anti-tubercular activity and showed that these compounds inhibited the essential cell wall synthesis enzyme DprE1 (Christophe et al., 2009). At the same time, a study was published on the synthesis of benzothiazinones with anti-TB activity that used reverse genetics reveal that DprE1 is also the target of this compound (Makarov et al., 2009). Since these findings, DprE1 inhibitors with different types of chemical scaffolds have been developed and are being investigated as potential TB therapeutics [reviewed in (Chikhale et al., 2018)]. In subsequent sections of this review, we will highlight advances in maximizing the utility of compounds identified in chemical screens.

Genetic tools

Starting with a small-molecule and identifying the target for promising hits is the empirical, top-down approach to drug discovery. Recent progress in bacterial genetics, high-throughput sequencing, and medicinal chemistry such as fragment-based drug discovery (Scott et al., 2009) offers promise to dedicating increased efforts to the bottom-up approach of target-based drug development. In this strategy, a single-gene product or mechanism is identified as an effective drug target based on biological studies. Inhibition of this target is shown *in vitro* to be sufficient to confer a meaningful therapeutic effect. The identification of new targets and mechanisms of bacterial killing will also likely circumvent pre-existing resistance mechanisms and thus be applicable to drug susceptible, MDR, and XDR TB.

In the past, functional deletions such as genetic knockouts have been used to validate hits but with two major drawbacks. First, targets that are essential for bacterial viability cannot be knocked out; second, antibiotics do not always fully inhibit their targets. Instead, genetic knockdowns—titrating levels of gene expression or gene products—offer a more realistic simulation for potential inhibitors of essential and non-essential genes and offer insight on the "vulnerability" of a target: how much (or how little) target inhibition is required for a therapeutic effect. Three primary genetic approaches have been used in TB research to study individual targets and their vulnerability: transposon sequencing (TnSeq), proteolytic degradation systems, and CRISPR interference (CRISPRi).

TnSeq

TnSeq works by mapping random transposon integration sites in saturated mutant libraries: the ability or inability of a locus to sustain transposon insertion is indicative of that gene's requirement for growth in the condition tested. The predecessor to TnSeq, transposon site hybridization (TraSH), first identified the complete set of *Mtb* genes required for growth under different conditions. This tool combined high-density insertional mutagenesis using phage containing a *mariner*-based transposon with microarrays to map pools of mutants (Sassetti et al., 2001). It has since been replaced with TnSeq, which uses next-generation sequencing to quantify marked transposon insertions across the genome (Zhang et al., 2012; van Opijnen and Camilli, 2013; DeJesus et al., 2017).

In addition to providing insight on gene essentiality, TnSeq has been a powerful tool to identify drug targets, mechanisms of action, or antibiotic resistance and to characterize so-called "conditional essentiality". Although some genes may not be required for growth in standard laboratory media, they may no longer sustain transposon insertions in other conditions. Recent work has shed light on genes required for host-relevant conditions such as cholesterol catabolism (Griffin et al., 2011) and different genetic backgrounds *in vivo* using collaborative cross mouse panels (Smith et al., 2022). TnSeq has also been used to investigate phenotypic variation in clinical *Mtb* strains: certain genes are differentially required across clinical strains, including *katG*, which activate the pro-drug isoniazid, a first-line TB drug (Carey et al., 2018). This finding shows the ability for TnSeq to predict resistance mechanisms to antibiotics: a loss-of-function mutation in *katG* would confer isoniazid resistance by failing to activate this drug in cells.

Studies on the conditional essentiality of *Mtb* genes across strains and growth conditions have produced extensive amounts of data on the "druggable" genome in *Mtb*: genes that could be further explored as putative drug targets because they are indispensable for growth or confer increased susceptibility to antibiotics when inactivated (discussed below). Following up on hits of interest and transforming findings into phenotypic assays for both chemical screening and medicinal chemistry can help establish more productive strategies for TB drug discovery.

Genetic knockdowns

Proteolytic degradation systems and CRISPRi allow us to study essential genes by providing titratable ways to reduce their abundance in bacterial cells. Proteolytic degradation works by tagging a gene of interest with a degradation signal: inducible expression of an accessory molecule then shuttles tagged proteins to endogenous proteases (Kim et al., 2011). Different expression levels of this accessory molecule allow for a range of protein knockdown. CRISPRi instead uses targeted transcriptional repression. An inducible nuclease deactivated Cas9 (dCas9) is guided to a target gene by a single-guide RNA (sgRNA) molecule with complementarity to the target DNA sequence (Rock et al., 2017). The sgRNA molecule also contains a short protospacer-adjacent motif (PAM) for initial dCas9 recognition, which disrupts the target DNA and facilitates binding of sgRNA to its complement (Rock et al., 2017). This interaction physically blocks RNA polymerase from being able to initiate transcription or fully elongate mRNA. Different PAM sequences confer a range of dCas9 binding, which, in turn, modulates the level of knockdown (Rock et al., 2017).

Both tools facilitate target-based drug discovery by allowing researchers to define the vulnerability of specific genes or pathways. Chemicals can also be screened against hypomorph libraries to analyze potential off-target effects and to validate mechanisms of action. For instance, proteolytic degradation systems were recently used in a large chemical-genetic screen to combine small molecules with a pool of *Mtb* strains depleted in essential genes (Johnson et al., 2020). Because these hypomorphs are hypersensitive, this approach was found to

yield more hit compounds of interest than whole-cell chemical screens alone, and their effects on different strains shed light on compound mechanisms of action. More than 40 new compounds were found to target previously established *Mtb* targets including DNA gyrase, cell wall biosynthesis, and RNA polymerase. An inhibitor of a new target was also identified, which interfered with the essential *Mtb* efflux pump EfpA. This inhibitor, which was effective against an EfpA hypomorph, was then chemically modified for increased potency against a wild-type *Mtb* strain (Johnson et al., 2020). This example shows the utility of combining genetic knockdowns with whole-cell chemical screens to identify small molecules that would not be unearthed by exclusively screening wild-type cells.

Recently, a large-scale CRISPRi screen quantified the vulnerability of essential genes and pathways to predict *Mtb*'s susceptibility to an antibiotic targeting that gene or pathway (Bosch et al., 2021). A considerable challenge in target-based drug discovery is the development of a selective drug from scratch with suitable pharmacokinetic and toxicological profiles. Identifying highly vulnerable targets that require only small levels of inhibition for a clinically relevant phenotype would maximize the likelihood of success, because lower levels of target engagement would need to be achieved for compound efficacy. Advances in medicinal chemistry such as fragment-based drug design and dynamic combinatorial chemistry have the potential to further increase the likelihood of success for compound development (Scott et al., 2009) [reviewed in (Ladame, 2008)].

Multiple highly vulnerable targets have been identified that do not yet have inhibitors against them. Several promising pathways have emerged from recent work that were even more vulnerable than the targets of current TB antibiotics, such as protein folding and secretion, metabolism, DNA replication, cell division, and tRNA synthetases (Bosch et al., 2021). The latter group—aminoacyl tRNA synthetases (aaRS)—was found to be highly vulnerable regardless of the *Mtb* strain tested. Because aaRS have conserved active sites, compounds designed to target them could target multiple synthetases and reduce rates of antibiotic resistance (Kovalenko et al., 2019). Efforts are already underway to develop aaRS inhibitors against *Mtb* (Gudzera et al., 2016; Li et al., 2017; Soto et al., 2018; Kovalenko et al., 2019) [reviewed in (Kim et al., 2020)]. These enzymes are already the targets of the anti-malarial drug halofuginone and the antibiotic mupirocin.

These approaches have two other uses. One is that they can be used for validating chemical-genetic interactions. For compounds that have putative mechanisms of action, often based on *in vitro* biochemical assays, strains with depletions of possible targets or potential activators can be constructed using either targeted protein degradation or CRISPRi. This permits mapping to the true target in whole cells, as discussed below. Conversely, specifically constructed strains can aid in targeted drug discovery using a whole-cell approach. For example, Evans et al. used a complementary genetic approach to transcriptionally silence multiple genes in the panthothenate and coenzyme A biosynthesis pathways (Evans et al., 2016). These strains were then used with chemical screening to discover whole cell-active inhibitors.

Synergy: Using existing drugs in new ways

Target-based discovery has already offered new and promising insights to help simplify TB therapy and reduce treatment duration. By studying the interplay of drugs and specific targets, we can design more deliberate regimens with a focus on synergistic interactions. Synergy refers to a combined effect of multiple drugs that is greater than the sum of their individual effects. Although TB combination therapy was developed for several important reasons, synergy was not front of mind when designing current regimens. How can we incorporate a synergy-focused framework in the design of new TB treatments?

One way to do this is by screening genetic libraries against existing antibiotics to find genes whose depletion hypersensitizes *Mtb* to existing drugs that are either already used or currently ineffective against TB. Both deletion and depletion mutants can phenocopy antibiotics targeting the mutated protein, allowing us to screen for synergistic "drugs" without yet having a molecule in hand. In a recent TnSeq screen, transposon mutant libraries were screened in the presence of different antibiotics with diverse mechanisms of action (Xu et al., 2017). Multiple genetic determinants of antibiotic susceptibility were involved in synthesis and maintenance of *Mtb*'s cell envelope. For instance, deletion of the gene *fecB*, which mediates cell wall integrity, conferred susceptibility to every antibiotic tested. Findings like this suggest that efforts directed toward increasing permeability of *Mtb* cells would expand the arsenal of antibiotics that could potentially be used to treat TB by exploiting synergy between cell wall inhibitors and intracellular antibiotics. Similarly, another TnSeq screen performed in mice treated with the first-line TB drugs rifampicin, isoniazid, ethambutol, and pyrazinamide found that the bottleneck for rifampicin efficacy is permeability to the drug, whereas isoniazid susceptibility is predominantly affected by replication rates (Bellerose et al., 2020). These findings were also recently corroborated by observations that growth on cholesterol—a host-relevant carbon source—decreased susceptibility to rifampicin by causing lipid composition changes on *Mtb*'s cell envelope: efforts targeting cell wall synthesis pathways would enhance killing by rifampicin during infection (Koh et al., 2022).

Another chemical-genetic screen was recently performed using CRISPRi libraries. Expression levels of most *Mtb* genes were titrated and bacterial fitness quantified in the presence of

different antibiotics (Li et al., 2021). A putative aminoglycoside transporter was identified when decreased levels of Rv1819c (*bacA*, an ABC importer of hydrophilic solutes) conferred resistance to streptomycin, showing once again the importance of understanding determinants of drug resistance and the promise of increasing cell wall permeability to improving *Mtb* susceptibility to antibiotics.

In addition to chemical-genetic screens to identify mechanisms of synergy between putative drug targets and existing antibiotics, combinatorial drug screening offers an approach to quantify synergy and prioritize drug combinations to test *in vivo*. New experimental and analytical methodologies have strengthened our ability to measure drug interactions. Checkerboard assays have historically been the gold standard for measuring pairwise drug interactions. These assays test antibiotics in double serial dilutions and compare their combined effect with each drug's individual effect in a microtiter plate. The recent development of the more efficient and less expensive DiaMOND (Diagonal Measurement Of N-way Drug interactions) offers a relatively simple way to measure interactions between any number of drugs (Cokol et al., 2017). This technique compares dose responses for mixtures of drugs with dose responses of each individual drug using a Loewe additivity model. DiaMOND's efficiency comes from applying geometric models to factor high-order drug interactions into lower-order components, creating a framework to predict higher-order interactions. Synergy studies *in vitro* have been extended to prioritize experimental drug combinations *in vivo*. Recent work by Larkins-Ford et al. identified signatures of drug potency and interactions in *in vitro* models that were predictive of efficacy in preclinical mouse models of TB (Larkins-Ford et al., 2021). The ability to identify synergy and antagonism between different drugs *in vitro* will be extremely helpful in designing future combination drug regimens against TB, especially as more drug targets enter the discovery pipeline. Drugs that bolster each other in combination will likely be more effective in clearing infections and have fewer side effects in patients due to reducing both dosage and treatment time.

Prioritizing candidate regimens

An obvious limitation to the design of new combination therapies is the financial and time cost of large-scale clinical trials to assess their efficacy. Thus, preclinical testing of TB therapies will need to address multiple important factors to prioritize regimens for clinical assessment: (1) *in vitro* efficacy on total killing in different bacterial subpopulations and growth conditions and (2) their efficacy in animal models of TB relapse.

A major challenge to TB treatment is the heterogeneity of bacterial populations within a host and host environments. As described above, TB therapy is administered for several months because *Mtb* creates phenotypically diverse subpopulations with varying levels of drug tolerance. This is a result both of *Mtb*'s metabolic adaptation to different microenvironments within a host such as different carbon sources and other changes between granulomas, as well as *Mtb*'s asymmetric growth and division pattern [reviewed in (Chung et al., 2022)]. For instance, genetic knockdown of *lamA*, which influences cell size variation in mycobacteria, increases *Mtb* susceptibility to rifampicin and vancomycin (Rego et al., 2017). Assays that measure total bacterial killing or the rate of bacterial killing—as opposed to minimal inhibitory concentrations, the concentration at which >90% of bacterial growth is inhibited in a broth microdilution assay—might be more informative about the bactericidal dynamics of small molecules. Recent progress with animal models has also increased our ability to assess applicability of *in vitro* findings to *in vivo* models. Infection relapse models, in which mice are monitored for culturable bacteria after stopping drug treatment, are an important way to test the total sterilizing activity of a potential therapy (Larkins-Ford et al., 2021). Work on understanding the metabolic changes that *Mtb* undergoes in different microenvironments demonstrates the importance of testing compounds in different growth conditions including various carbon sources, pH, and hypoxia (Hicks et al., 2018; Gouzy et al., 2021). Future work continuing to understand how these subpopulations form will facilitate the discovery of putative drug targets that synergize to fully sterilize all infected microenvironments.

Conclusion

In this review, we have outlined the limitations of current multidrug regimens against tuberculosis in the era of antibiotic resistance and the genetic tools available to improve the identification of drug targets and assess their vulnerability in *Mtb*. The rational design of regimens that sterilize diverse phenotypic subpopulations will maximize bacterial killing while minimizing both treatment duration and infection relapse. Importantly, the TB field currently has all the necessary genetic and analytical tools to screen for and prioritize drug targets *in vitro* based on the vulnerability of essential and non-essential genes in the *Mtb* genome and to translate these findings in *in vivo* models. Combining genetic methods with chemical screens offers a formidable strategy to redefine the preclinical design of TB therapy by identifying powerful new targets altogether, as well as targets that lend new efficacy to existing drugs.

Author contributions

Conceptualization and outline – FGT and EJR. Writing – Original Draft: FGT. Writing – Review and Editing: FGT, EJR.

All authors contributed to the article and approved the submitted version.

Funding

This work was supported in part by NIH/NIAID through award P01AI095208, and by the Office of the Assistant Secretary of Defense for Health Affairs, through the Peer Reviewed Medical Research Program, Focused Program Award under Award No. W81XWH-17-1-0692. Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the Department of Defense.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Bellerose, M. M., Proulx, M., Smith, C., Baker, R., Ioerger, T., Sassetti, C., et al. (2020). Distinct bacterial pathways influence the efficacy of antibiotics against mycobacterium tuberculosis. *mSystems* 5 (4), e00396-20. doi: 10.1128/mSystems.00396-20

Bosch, B., DeJesus, M., Poulton, N., Zhang, W., Engelhart, C., Zaveri, A., et al. (2021). Genome-wide gene expression tuning reveals diverse vulnerabilities of m. tuberculosis. *Cell* 184 (17), 4579–4592.e24. doi: 10.1016/j.cell.2021.06.033

Carey, A. F., Rock, J., Krieger, I., Chase, M., Fernandez-Suarez, M., Gagneux, S., et al. (2018). TnSeq of mycobacterium tuberculosis clinical isolates reveals strain-specific antibiotic liabilities. *PloS Pathog.* 14 (3), e1006939. doi: 10.1371/journal.ppat.1006939

Chikhale, R. V., Barmade, M. A., Murumkar, P. R., and Yadav, M. R. (2018). Overview of the development of DprE1 inhibitors for combating the menace of tuberculosis. *J. Med. Chem.* 61 (19), 8563–8593. doi: 10.1021/acs.jmedchem.8b00281

Cho, S., Warit, S., Wan, B., Hwang, C., Pauli, G., Franzblau, S., et al. (2007). Low-oxygen-recovery assay for high-throughput screening of compounds against nonreplicating mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 51 (4), 1380–1385. doi: 10.1128/AAC.00055-06

Christophe, T., Jackson, M., Jeon, H., Fenistein, D., Contreras-Dominguez, M., Kim, , et al. (2009). High content screening identifies decaprenyl-phosphoribose 2' epimerase as a target for intracellular antimycobacterial inhibitors. *PloS Pathog.* 5 (10), e1000645. doi: 10.1371/journal.ppat.1000645

Chung, E. S., Johnson, W. C., and Aldridge, B. B. (2022). Types and functions of heterogeneity in mycobacteria. *Nat. Rev. Microbiol* 20, 529–41. doi: 10.1038/s41579-022-00721-0

Cokol, M., Kuru, N., Bicak, E., Larkin-Ford, J., and Aldridge, B. B. (2017). Efficient measurement and factorization of high-order drug interactions in mycobacterium tuberculosis. *Sci. Adv.* 3 (10), e1701881.

Colangeli, R., Jedrey, H., Kim, R., Connell, R., Ma, S., Venkata, U., et al. (2018). Bacterial factors that predict relapse after tuberculosis therapy. *N Engl. J. Med.* 379 (9), 823–833. doi: 10.1056/NEJMoa1715849

Connolly, L. E., Edelstein, P. H., and Ramakrishnan, L. (2007). Why is long-term therapy required to cure tuberculosis? *PloS Med.* 4 (3), e120.

Conradie, F., Diacon, A., Ngubane, N., Howell, P., Everitt, D., Crook, A., et al. (2020). Treatment of highly drug-resistant pulmonary tuberculosis. *N Engl. J. Med.* 382 (10), 893–902. doi: 10.1056/NEJMoa1901814

Dartois, V., and Barry, C. E.3rd (2013). A medicinal chemists' guide to the unique difficulties of lead optimization for tuberculosis. *Bioorg Med. Chem. Lett.* 23 (17), 4741–4750. doi: 10.1016/j.bmcl.2013.07.006

DeJesus, M. A., Gerrick, E., Xu, W., Park, S., Long, J., Boutte, C., et al. (2017). Comprehensive essentiality analysis of the mycobacterium tuberculosis genome *via* saturating transposon mutagenesis. *mBio* 8 (1), e02133–16. doi: 10.1128/mBio.02133-16

Dorman, S. E., Nahid, P., Kurbatova, E., Phillips, P., Bryant, K., Dooley, K., et al. (2021). Four-month rifapentine regimens with or without moxifloxacin for tuberculosis. *N Engl. J. Med.* 384 (18), 1705–1718. doi: 10.1056/NEJMoa2033400

Early, J. V., Mullen, S., and Parish, T. (2019). A rapid, low pH, nutrient stress, assay to determine the bactericidal activity of compounds against non-replicating mycobacterium tuberculosis. *PloS One* 14 (10), e0222970. doi: 10.1371/journal.pone.0222970

Evans, J. C., Trujillo, C., Wang, Z., Eoh, H., Ehrt, S., Schnappinger, D., et al. (2016). Validation of CoaBC as a bactericidal target in the coenzyme a pathway of mycobacterium tuberculosis. *ACS Infect. Dis.* 2 (12), 958–968. doi: 10.1021/acsinfecdis.6b00150

Flentie, K., Harrison, G., Tükenmez, H., Livny, J., Good, J., Sarkar, S., et al. (2019). Chemical disarming of isoniazid resistance in mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. U.S.A.* 116 (21), 10510–10517. doi: 10.1073/pnas.1818009116

Gernez, R., and Breton,. (1948). [Treatment of pulmonary tuberculosis in children with streptomycin]. *Sem Hop* 24 (89), 2916.

Gouzy, A., Healy, C., Black, K. A., Rhee , K. Y., and Ehrt, S. (2021). Growth of mycobacterium tuberculosis at acidic pH depends on lipid assimilation and is accompanied by reduced GAPDH activity. *Proc. Natl. Acad. Sci. U. S. A.* 118 (32), e2024571118. doi: 10.1073/pnas.2024571118

Griffin, J. E., Gawronski, J., DeJesus, M., Ioerger, T., Akerley, B., Sassetti, C., et al. (2011). High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. *PloS Pathog.* 7 (9), e1002251. doi: 10.1371/journal.ppat.1002251

Gudzera, O. I., Golub, A., Bdzhola, V., Volynets, G., Kovalenko, O., Boyarshin, K., et al. (2016). Identification of mycobacterium tuberculosis leucyl-tRNA synthetase (LeuRS) inhibitors among the derivatives of 5-phenylamino-2H-[1,2,4]triazin-3-one. *J. Enzyme Inhib Med. Chem.* 31 (sup2), 201–207. doi: 10.1080/14756366.2016.1190712

Hicks, N. D., Yang, J., Zhang, X., Zhao, B., Grad, Y., Liu, L., et al. (2018). Clinically prevalent mutations in mycobacterium tuberculosis alter propionate metabolism and mediate multidrug tolerance. *Nat. Microbiol.* 3 (9), 1032–1042. doi: 10.1038/s41564-018-0218-3

Huang, L., Kushner, N., Theriault, M., Pisu, D., Tan, S., McNamara, C., et al. (2018). The deconstructed granuloma: A complex high-throughput drug screening platform for the discovery of host-directed therapeutics against tuberculosis. *Front. Cell Infect. Microbiol.* 8, 275. doi: 10.3389/fcimb.2018.00275

Johnson, E. O., Office, E., Kawate, T., Orzechowski, M., and Hung, D. T. (2020). Large-Scale chemical-genetic strategy enables the design of antimicrobial combination chemotherapy in mycobacteria. *ACS Infect. Dis.* 6 (1), 56–63. doi: 10.1021/acsinfecdis.9b00373

Kim, S. H., Bae, S., and Song, M. (2020). Recent development of aminoacyl-tRNA synthetase inhibitors for human diseases: A future perspective. *Biomolecules* 10 (12), 1625. doi: 10.3390/biom10121625

Kim, J. H., Wei, JR, Wallach, JB, Robbins, RS, Rubin, EJ, Schnappinger, D., et al. (2011). Protein inactivation in mycobacteria by controlled proteolysis and its application to deplete the beta subunit of RNA polymerase. *Nucleic Acids Res.* 39 (6), 2210–2220. doi: 10.1093/nar/gkq1149

Koh, E. I., Oluoch, P., Ruecker, N., Proulx, M., Soni, V., Murphy, K., et al. (2022). Chemical-genetic interaction mapping links carbon metabolism and cell wall

structure to tuberculosis drug efficacy. *Proc. Natl. Acad. Sci. U. S. A.* 119 (15), e2201632119. doi: 10.1073/pnas.2201632119

Kovalenko, O. P., Volynets, G., Rybak, M., Starosyla, S., Gudzera, O., Lukashov, S., et al. (2019). Dual-target inhibitors of mycobacterial aminoacyl-tRNA synthetases among n-benzylidene-N'-thiazol-2-yl-hydrazines. *Medchemcomm* 10 (12), 2161–2169. doi: 10.1039/C9MD00347A

Ladame, S. (2008). Dynamic combinatorial chemistry: on the road to fulfilling the promise. *Org Biomol. Chem.* 6 (2), 219–226. doi: 10.1039/B714599C

Larkins-Ford, J., Greenstein, T., Van, N., Degefu, Y., Olson, M., Sokolov, A., et al. (2021). Systematic measurement of combination-drug landscapes to predict *in vivo* treatment outcomes for tuberculosis. *Cell Syst.* 12 (11), 1046–1063 e7. doi: 10.1016/j.cels.2021.08.004

Li, S. Y., et al. (2017). Bactericidal and sterilizing activity of a novel regimen with bedaquiline, pretomanid, moxifloxacin, and pyrazinamide in a murine model of tuberculosis. *Antimicrob. Agents Chemother.* 61 (9). doi: 10.1128/AAC.00913-17

Li, X., et al. (2017). Discovery of a potent and specific m. tuberculosis leucyl-tRNA synthetase inhibitor: (S)-3-(Aminomethyl)-4-chloro-7-(2-hydroxyethoxy) benzo[c][1,2]oxaborol-1(3H)-ol (GSK656). *J. Med. Chem.* 60 (19), 8011–8026.

Li, S., Poulton, N., Chang, J., Azadian, Z., DeJesus, M., Ruecker, N., et al. (2021). A chemical-genetic map of the pathways controlling drug potency in mycobacterium tuberculosis. *BioRxiv* 2021.11.27.469863. doi: 10.1101/2021.11.27.469863

Makarov, V., Manina, G., Mikusova, K., Möllmann, U., Ryabova, O., Saint-Joanis, B., et al. (2009). Benzothiazinones kill mycobacterium tuberculosis by blocking arabinan synthesis. *Science* 324 (5928), 801–804.

Mdluli, K., Kaneko, T., and Upton, A. (2015). The tuberculosis drug discovery and development pipeline and emerging drug targets. *Cold Spring Harb. Perspect. Med.* 5 (6), a021154. doi: 10.1101/cshperspect.a021154

Payne, D. J., Gwynn, M. N., Holmes, D. J., and Pompliano, D. L. (2007). Drugs for bad bugs: Confronting the challenges of antibacterial discovery. *Nat. Rev. Drug Discov.* 6 (1), 29–40. doi: 10.1038/nrd2201

Regniers, P., Beerens, J., and De Landsheere, B. (1949). Streptomycin treatment of pulmonary tuberculosis in adults. *Belg Tijdschr Geneesk* 5 (1), 2–20.

Rego, E. H., Audette, R. E., and Rubin, E. J. (2017). Deletion of a mycobacterial divisome factor collapses single-cell phenotypic heterogeneity. *Nature* 546 (7656), 153–157.

Rock, J. M., Hopkins, F., Chavez, A., Diallo, M., Chase, M., Gerrick, E., et al. (2017). Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform. *Nat. Microbiol.* 2, 16274. doi: 10.1038/nmicrobiol.2016.274

Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2001). Comprehensive identification of conditionally essential genes in mycobacteria. *Proc. Natl. Acad. Sci. U. S. A.* 98 (22), 12712–12717. doi: 10.1073/pnas.231275498

Scott, D. E., Dawes, G. J., Ando, M., Abel, C., and Ciulli, A. (2009). A fragment-based approach to probing adenosine recognition sites by using dynamic combinatorial chemistry. *Chembiochem* 10 (17), 2772–2779. doi: 10.1002/cbic.200900537

Smith, C. M., Baker, R., Proulx, M., Mishra, B., Long, J., Park, S., et al. (2022). Host-pathogen genetic interactions underlie tuberculosis susceptibility in genetically diverse mice. *Elife* 11, 74419. doi: 10.7554/eLife.74419.sa2

Soto, R., Perez-Herran, E., Rodriguez, B., Duma, B., Cacho-Izquierdo, M., Mendoza-Losana, A., et al. (2018). Identification and characterization of aspartyl-tRNA synthetase inhibitors against mycobacterium tuberculosis by an integrated whole-cell target-based approach. *Sci. Rep.* 8 (1), 12664. doi: 10.1038/s41598-018-31157-3

Tasneen, R., Li, SY, Peloquin, C., Taylor, D., Williams, K., Andries, K., et al. (2011). Sterilizing activity of novel TMC207- and PA-824-containing regimens in a murine model of tuberculosis. *Antimicrob. Agents Chemother.* 55 (12), 5485–5492. doi: 10.1128/AAC.05293-11

Tempel, C. W. (1949). The place of streptomycin in the treatment of pulmonary tuberculosis. *Bull. US Army Med. Dep.* 9 (1), 25–34.

Tommasi, R., Brown, D. G., Walkup, G. K., Manchester, J. I., and Miller, A. A. (2015). ESKAPEing the labyrinth of antibacterial discovery. *Nat. Rev. Drug Discov.* 14 (8), 529–542. doi: 10.1038/nrd4572

van Opijnen, T., and Camilli, A. (2013). Transposon insertion sequencing: a new tool for systems-level analysis of microorganisms. *Nat. Rev. Microbiol.* 11 (7), 435–442. doi: 10.1038/nrmicro3033

Wade, M. M., and Zhang, Y. (2004). Anaerobic incubation conditions enhance pyrazinamide activity against mycobacterium tuberculosis. *J. Med. Microbiol.* 53 (Pt 8), 769–773. doi: 10.1099/jmm.0.45639-0

W.H.O. (2021). *Global tuberculosis report 2021.*

Xu, W., DeJesus, M., Rücker, N., Engelhart, C., Wright, M., Healy, C., et al. (2017). Chemical genetic interaction profiling reveals determinants of intrinsic antibiotic resistance in mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 61 (12), AAC.01334-17 . doi: 10.1128/AAC.01334-17

Xu, J., Li, SY, Almeida, D., Tasneen, R., Barnes-Boyle, K., Converse, P., et al. (2019). Contribution of pretomanid to novel regimens containing bedaquiline with either linezolid or moxifloxacin and pyrazinamide in murine models of tuberculosis. *Antimicrob. Agents Chemother.* 63 (5), AAC.00021-19. doi: 10.1128/AAC.00021-19

Zhang, Y. J., Ioerger, T., Huttenhower, C., Long, J., Sassetti, C., Sacchettini, J., et al. (2012). Global assessment of genomic regions required for growth in mycobacterium tuberculosis. *PloS Pathog.* 8 (9), e1002946. doi: 10.1371/journal.ppat.1002946

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 23 September 2022
DOI 10.3389/fcimb.2022.943545

OPEN ACCESS

EDITED BY
Ben Gold,
Weill Cornell Medicine, United States

REVIEWED BY
Ritesh Thakare,
Brown University, United States
Babak Javid,
University of California, San Francisco, United States

*CORRESPONDENCE
Bavesh Davandra Kana
bavesh.kana@wits.ac.za

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 13 May 2022
ACCEPTED 06 September 2022
PUBLISHED 23 September 2022

CITATION
Liebenberg D, Gordhan BG and Kana BD (2022) Drug resistant tuberculosis: Implications for transmission, diagnosis, and disease management.
Front. Cell. Infect. Microbiol. 12:943545.
doi: 10.3389/fcimb.2022.943545

Drug resistant tuberculosis: Implications for transmission, diagnosis, and disease management

Dale Liebenberg†, Bhavna Gowan Gordhan† and Bavesh Davandra Kana*

Department of Science and Innovation/National Research Foundation (DSI/NRF) Centre of Excellence for Biomedical Tuberculosis Research (TB), Faculty of Health Sciences, University of the Witwatersrand, National Health Laboratory Service, Johannesburg, South Africa

Drug resistant tuberculosis contributes significantly to the global burden of antimicrobial resistance, often consuming a large proportion of the healthcare budget and associated resources in many endemic countries. The rapid emergence of resistance to newer tuberculosis therapies signals the need to ensure appropriate antibiotic stewardship, together with a concerted drive to develop new regimens that are active against currently circulating drug resistant strains. Herein, we highlight that the current burden of drug resistant tuberculosis is driven by a combination of ongoing transmission and the intra-patient evolution of resistance through several mechanisms. Global control of tuberculosis will require interventions that effectively address these and related aspects. Interrupting tuberculosis transmission is dependent on the availability of novel rapid diagnostics which provide accurate results, as near-patient as is possible, together with appropriate linkage to care. Contact tracing, longitudinal follow-up for symptoms and active mapping of social contacts are essential elements to curb further community-wide spread of drug resistant strains. Appropriate prophylaxis for contacts of drug resistant index cases is imperative to limit disease progression and subsequent transmission. Preventing the evolution of drug resistant strains will require the development of shorter regimens that rapidly eliminate all populations of mycobacteria, whilst concurrently limiting bacterial metabolic processes that drive drug tolerance, mutagenesis and the ultimate emergence of resistance. Drug discovery programs that specifically target bacterial genetic determinants associated with these processes will be paramount to tuberculosis eradication. In addition, the development of appropriate clinical endpoints that quantify drug tolerant organisms in sputum, such as differentially culturable/detectable tubercle bacteria is necessary to accurately assess the potential of new therapies to effectively shorten treatment duration. When combined, this holistic approach to addressing the critical problems associated with drug resistance will support delivery of quality care to patients suffering from tuberculosis and bolster efforts to eradicate this disease.

KEYWORDS

acquired drug resistance, transmitted drug resistance, health systems strengthening, persisters, tolerance

Introduction

The growing spread of antimicrobial resistance (AMR) will most certainly undermine delivery of effective healthcare globally, necessitating a multipronged approach that accelerates development of new antimicrobials, together with careful deployment of these life-saving treatments in a more regulated manner (Nathan, 2020). Effective stewardship of antibiotics in plants and animals through a One-Health approach is also central to future efforts for combating AMR. Whilst antibiotic resistant bacteria and other pathogens comprise a notable proportion of resistance, drug resistant tuberculosis (TB) accounts for a disproportionately large amount of the global AMR burden. Much of this comprises circulation of *Mycobacterium tuberculosis* strains resistant to rifampicin and isoniazid (commonly referred to as multidrug resistance, MDR, Box 1) or strains that are MDR with added resistance to second line agents including fluoroquinolones and aminoglycosides (extensively drug resistant, XDR-TB, Box 1). Given that many health systems have opted to discontinue use of aminoglycosides, this definition of XDR tuberculosis will soon be outdated and possibly replaced by one that refers to the WHO drug category and resistance pattern (Dheda et al., 2019). Currently available agents for drug resistant TB, classified into groups A-C are shown in Box 1. Monoresistance to rifampicin or isoniazid is also a growing problem in TB endemic regions (Variava and Martinson, 2018). In 2020, 71% (2.1 of 3.0 million) of people diagnosed with bacteriologically confirmed pulmonary TB were also tested for rifampicin resistance (RR). Among these, 132 222 cases of MDR/RR-TB and 25 681 cases of pre-XDR-TB (MDR TB plus resistance to any fluoroquinolone or an injectable, Box 1) or XDR-TB were reported (World Health Organization, 2021a). In addition to drug resistance, HIV co-infection is another primary driver of poor TB outcomes with 214 000 deaths occurring among people living with HIV (PLWHIV), in addition to the 1.3 million worldwide TB deaths in 2020 (World Health Organization, 2021a). Moreover, any gains that global TB programs made during the last decade have been severely undermined by Covid-19, the most obvious being an 18% drop (7.1 million to 5.8 million) globally in the number of people newly diagnosed with TB in 2020, compared with 2019 (World Health Organization, 2021a). While the mandated wearing of masks and widespread lockdowns to limit the spread of SARS-CoV-2 might have helped decrease the

BOX 1 Definitions, WHO MDR-TB drug categorization and new TB drug development pipeline.

Definitions	
Resistant Bacteria	A bacterial population with a genetic mutation that facilitates survival against antibiotic treatment
Bacterial Tolerance	Bacteria that are able to phenotypically survive antibiotic challenge, with no genetic mutations directly associated with drug resistance
Persistent Bacteria	A subpopulation of bacterial cells that display a tolerant phenotype towards antibiotics
Acquired Resistance	Bacteria that develop drug resistance due to host or antibiotic pressure while infecting the lung
Transmitted Resistance	The infection of naïve lungs with bacteria already having a resistance genotype
Multidrug Resistant TB (MDR-TB)	Infection with tubercle bacilli genetically resistant to rifampicin and isoniazid
Pre-Extensively Drug Resistant TB (pre-XDR-TB)	MDR-TB with additional resistance to any fluoroquinolone OR a second line injectable
Extensively Drug Resistant TB (XDR-TB)	MDR-TB with resistance to any fluoroquinolone AND a further Group A drug

WHO TB Drug Classification for use in the treatment of MDR-TB			
Group A:	Group B:	Group C:	
Levofloxacin/moxifloxacin Bedaquiline Linezolid	Clofazimine Cycloserine/terizidone	Ethambutol Delamanid Pyrazinamide Imipenem-cilastatin/meropenem	Amikacin/streptomycin Ethionamide/prothionamide P-aminosalicylic acid Other agents

Candidate Pipeline for New Anti-TB Drugs					
Phase I:		Phase II:		Phase III: (drug regimen human trials)	
BVL-GSK098 GSK-286 PBTZ-169	TBAJ-587 TBAJ-876 TBI-223	BTZ-043 Delpazolid (LCB01-0371) GSK 3036656 OPC-167832 Pyrifazimine (TBI-166) SPR720 (Fobrepodacin)	SQ109 Sudapyridine (WX-081) Sutezolid TBA-7371 Telacebec (Q203)	Bedaquiline Clofazimine Delamanid Levofloxacin	Linezolid Moxifloxacin Pretomanid Rifapentine

tuberculosis burden, there are multiple Covid-19-derived factors that have had a negative impact on TB control. These include delayed treatment due to reduced access to public transport and health care facilities, misdiagnosis given symptom similarities between TB and Covid-19, disruptions of medical stocks and laboratory services, and the desire to avoid the stigma of disease (Dheda et al., 2022). During the same time, the number of people provided with treatment for drug-resistant TB reduced by 15%, from 177 100 to 150 359, about 1 in 3 of those in need (World Health Organization, 2021a).

Transmitted and acquired resistance

For more than 10 years, estimates of the proportion of people diagnosed for the first time with MDR/RR-TB has remained at about 3–4% and for those previously treated for TB has stayed at about 18–21% (World Health Organization, 2021a). This indicates that a substantive burden of drug resistant TB is driven by on-going transmission. Given this, the quest to eradicate drug resistant TB needs bolstering using approaches that address primary drivers including (I) deployment of transmission blocking strategies, together with novel approaches to case finding, contact tracing and related public health measures and (II) fast-tracking development of new therapeutics with novel modes of action that shorten the duration of treatment, limit emergence of resistance and are active on contemporaneous resistant strains, Figure 1. Drug resistant TB can arise either through direct transmission of genetically resistant bacteria (transmitted resistance, Figure 2) or intra-patient evolution of resistance (acquired resistance, Figure 2). Consequently, strategies to eliminate TB need to address health systems strengthening in a

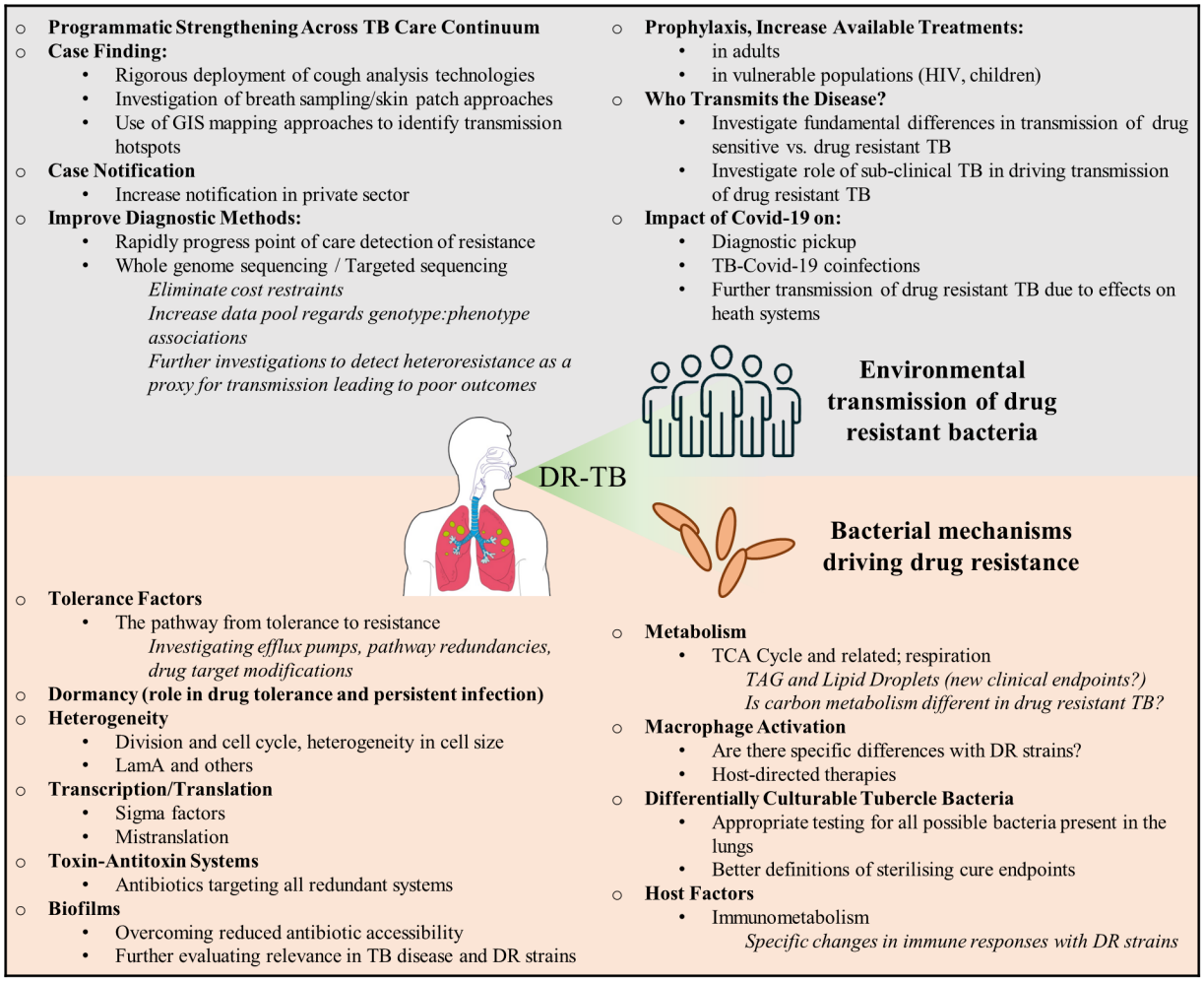


FIGURE 1
Drivers of drug resistance in TB. Key issues that need to be addressed in the fight against transmitted and acquired drug resistance. These primarily relate to strengthening health care systems to understanding how drug resistance develops.

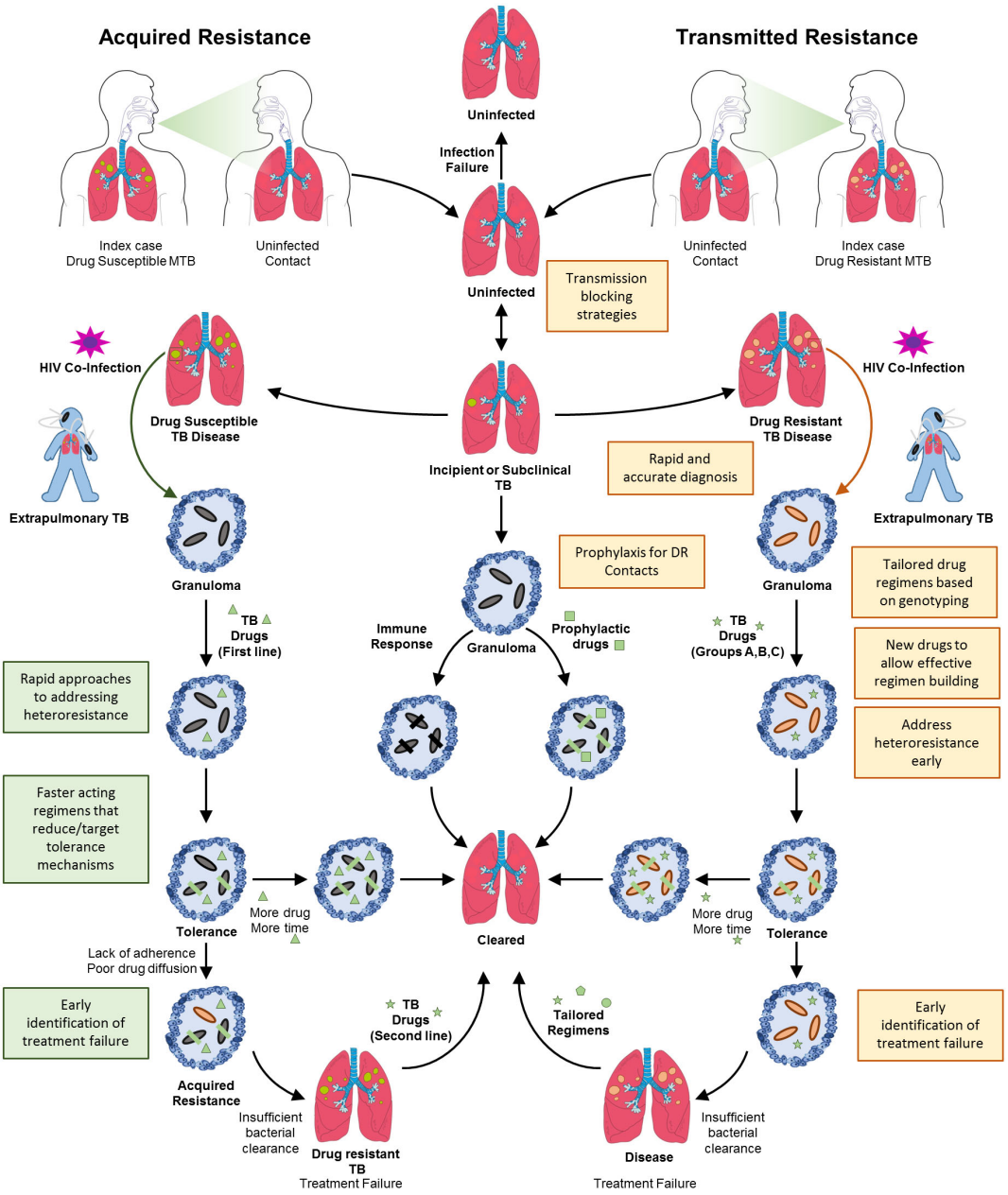


FIGURE 2
Transmitted and acquired drug resistant TB. Drug resistant TB can arise by two mechanisms, acquired resistance or directly through transmitted resistance. Shown is the natural progression of TB infection and disease, initiated by transmission of bacteria, followed by the development of incipient or subclinical TB. If identified, incipient or subclinical TB can be resolved by administration of prophylaxis or spontaneously. A small proportion of these cases will progress to full blown TB disease in the lungs wherein granulomas necrotize and rupture. Concomitant HIV infection will also result in extrapulmonary TB. In the case of infection with drug sensitive TB, the bacterial population will expand and drug treatment will result in the survival of tolerant organisms that will eventually be cleared with protracted treatment. The phenomenon of drug tolerance highlights the need for additional and faster acting drug treatments. Lack of adherence or poor drug diffusion will result in these tolerant organisms eventually acquiring stable drug resistance, resulting in treatment failure and drug resistant TB. This will necessitate administration of an alternate regimen directed at eliminating resistant organisms. Transmitted resistance occurs when an individual is infected with organisms that are genetically drug resistant. In the absence of diagnostic testing capable of identifying drug resistance mutations for most drugs except rifampicin, the treatment of these patients with first-line drugs will unfortunately result in failure. If identified, together with appropriate susceptibility testing, treatment of drug resistant TB follows a similar sequence of events to that for drug susceptible TB. Given the longer durations of treatment, the chances for evolution of further resistance are high. Hence, the requirement for the correct choice of regimens and development of novel drugs to eliminate these resistant organisms. Shaded boxes indicate the need for new interventions or health system strengthening.

manner that limits community wide TB transmission. Furthermore, intense research is required to yield new therapies that limit the acquisition of resistance and reduce treatment duration. Herein, we discuss the possible approaches for addressing these issues, arguing that multifaceted approaches are required, ranging from health systems strengthening for delivering effective diagnosis and patient care to fundamental research on new drug targets.

Effective health systems for interrupting the transmission of drug resistant TB

Continued transmission will be the key driver to any future burden of TB, with several recent studies pointing to rampant spread of MDR and XDR-TB through deficiencies in the TB care continuum, which include delays in diagnosis, poor linkage to care and lack of retention in the health system (Dheda et al., 2017; Shah et al., 2017; Atre et al., 2022). To address these drivers, health systems require new ways of case finding, faster and cheaper diagnostic tests, together with prophylaxis of contacts to minimise further spread of disease.

Case finding

Transmission of most infectious diseases is estimated to occur prior to case detection in the health system. Our experience with the management of the recent Covid-19 pandemic has demonstrated that rapid and active case finding approaches were critical to curb further spread of disease (Hossain et al., 2022). Hence, active case finding, rapid diagnosis (including drug resistance profiling) and prompt effective therapy are recognized as the most important interventions to stop transmission (Yuen et al., 2015). Several countries have adopted the FAST (Find cases Actively by cough surveillance and rapid molecular sputum testing, Separate safely, and Treat effectively based on rapid drug susceptibility testing) approach to actively screen for undetected TB and identification of drug resistance, followed by appropriate effective therapy to interrupt transmission. However, for these screening approaches to have a widespread application, cost effective novel methods that do not require sputum but alternative approaches such as active screening through cough surveillance, digital radiology and breath tests need urgent development. Examples of such innovations include the development of machine learning programmes that can successfully distinguish between the cough of a patient with TB or other respiratory illnesses, at a level exceeding the minimum target product profile requirements for a WHO triage test (Pahar et al., 2021). There are already several smartphone applications in development that can analyse coughs and overall coughing trends to detect incidences of respiratory disease (Gabaldon-Figueira et al., 2021; Moschovis et al., 2021). Such approaches need rapid scaling, together with clear processes for accelerating the WHO's approval and widespread deployment. Recently there has been a renewed focus on non-invasive sampling and using alternate clinical specimens such as oral swabs in diagnosis. For example, the use of cheek swabs have been assessed in adults living with HIV (LaCourse et al., 2022). While tongue swabs were evaluated in young children, a population were traditional sputum samples are not easily obtained (Ealand et al., 2021).

Case notification

In some countries, TB cases are often not notified, especially those managed in private care where facilities are not linked to national tuberculosis programs (Rupani et al., 2021). As a result, significant under-notification of cases undermines important processes such as disease surveillance and contact tracing. To find more cases, control programs need to expand TB case-detection to populations with increasingly low prevalence of disease. Caution needs to be taken as mathematical modelling demonstrates that poor diagnostic-specificity can result in a high number of false positive diagnoses, giving a skewed picture of program performance leading to inappropriate policy decisions (Lalli et al., 2018). Current diagnostic tests are not suitable for these mass testing approaches. Household contact tracing of index TB cases is promising for TB control but has not been widely implemented, particularly in low-resource settings because of the lack of high quality evidence for effectiveness or due to cost limitations. This would require reallocation of the already insufficient resources available for TB diagnosis and treatment (MacPherson et al., 2019). Dowdy et al. (2017) provided a roadmap for designing, evaluating, and modelling interventions to interrupt transmission in a diverse array of tuberculosis epidemics worldwide. They proposed a three-prong approach for effectively reducing TB transmission that involves (I) preventative therapy to reduce the reservoir of latent infections, (II) diagnosis and case finding to shorten the time from disease onset to initiation of treatment and (III) infection control in certain settings. Although synergistic public health interventions can halt tuberculosis transmission, knowledge and understanding of the local epidemiology is another crucial factor for success (Dowdy et al., 2017).

Point of care diagnostics

It is imperative that rapid point-of-care (POC) diagnostic tests are developed for early and rapid diagnosis of *M.*

tuberculosis. Since 2007, several new tests and diagnostic approaches have been endorsed by the WHO, including: liquid culture with rapid speciation as the reference standard for bacteriological confirmation; molecular line probe assays for rapid diagnosis of multidrug-resistant TB (Hain Lifescience LPA); non-commercial culture and drug-susceptibility testing methods; light-emitting diode fluorescence microscopes; and nucleic acid amplification tests for rapid and simultaneous diagnosis of TB and rifampicin-resistance (Cepheid, GeneXpert; Molbio Diagnostics, TrueNat) (World Health Organization, 2021b). More recently, the Deeplex Myc-TB (Genoscreen) is a culture-free assay based on targeted deep sequencing that is able to provide information on resistance to 15 anti-TB drugs within 48 hours. The Deeplex has 3% sensitivity for the detection of heteroresistance and is also able to identify non-tuberculous mycobacteria, as well as lineages and spoligotypes (Feuerriegel et al., 2021). Despite this progress, an accurate and rapid POC test for drug resistant TB that is usable under field conditions is still unavailable, suggesting that greater research effort is required to convert these often-complex laboratory technologies into robust, accurate and cost-effective POC programs (Abdulgader et al., 2022). The Covid-19 pandemic has expanded the framework for self-testing to enable greater diagnostic pickup of infection, such approaches should be considered and developed for rapid TB diagnosis.

Whole genome sequencing

The currently available nucleic acid amplification tests detect specific regions of the TB genome to identify probable drug resistance, but new mutations are constantly evolving. Whole genome sequencing (WGS) has the capability to identify all genetic mutations in a sample (Katale et al., 2020), but the use of such technology needs to be refined before it can be implemented in a diagnostic setting. WGS testing would need to be performed directly on sputum in a form that is cheap, fast and accessible but thus far, no such technique that can be deployed at programmatic levels has been reported (Dookie et al., 2022). An additional consideration is that the use of WGS for clinical decision making requires accurate information about genotype to phenotype correlations (Faksri et al., 2019). In the absence of definitive information in this regard, a combination of genotypic and phenotypic approaches will most likely be needed (Dookie et al., 2022). An alternative to WGS is targeted sequencing, which also makes use of next-generation technology to provide information on drug resistance loci directly from clinical samples (MacLean et al., 2020; Dookie et al., 2022). Despite the fact that this technique provides simpler data than WGS, it has the ability to detect low frequency variants (MacLean et al., 2020; Dookie et al., 2022).

Bacterial culturability

Major limitations of traditional culture methods are slow turn-around times, suboptimal sensitivity, and the prohibitive cost of using automated liquid broth systems in endemic countries. Hence, there is a dire need for improved culture techniques to enhance diagnosis, particularly in certain vulnerable populations. Microbiological confirmation of TB in PLWHIV and in children is challenging due to the paucibacillary nature of disease presentation. Collection of specimens such as induced sputum or gastric aspirate from these groups is complex and invasive often with poor diagnostic confirmation due to limited retrieval of bacteria (Connell et al., 2011). A few novel approaches have been evaluated to improve recovery of *M. tuberculosis* from these limited specimens. These include the colorimetric TK medium assay (Salubris) that changes colour from red to yellow, indicating early positive growth of mycobacteria before visible bacterial colonies appear and also has the capacity for drug susceptibility testing (Kocagoz et al., 2012). The microscopic observation drug susceptibility assay (MODS) which uses an inverted light microscope and Middlebrook 7H9 broth culture containing antimicrobial drugs for susceptibility testing is able to detect early mycobacterial growth as "strings and tangles" of bacterial cells in the media in a shorter time (average of 8 days) compared with Lowenstein–Jensen culture (Moore et al., 2004). Due to limited accessibility of the MODS media, evaluation of two alternative culture media (powder and lyophilized forms) showed equivalent bacterial growth, suggesting that MODS can be implemented in resource limited settings (Sheen et al., 2022). Commercially available bacteriophage-based kits have been developed to detect mycobacteria and identify rifampicin resistance directly in sputum specimens within 2-3 days (Pai et al., 2005). Although TK Medium, MODS and bacteriophage based tests are promising, practical and inexpensive tools, their utility for diagnosis of TB in children and PLWHIV is unknown (Kumar et al., 2015).

The performance and accuracy of culture-based TB diagnostic tests is based on the premise that all bacterial populations have equivalent culturability in routine diagnostic media. However, there is a growing body of evidence that points to bacterial populations adopting a spectrum of physiological states associated with altered culturability. The description of "viable but nonculturable" (VBNC) *Vibrio cholerae* cells more than thirty years ago (Colwell et al., 1985) was just one example of a sub-population of bacterial cells that are incapable of growing on solid medium but can be detected in liquid media. *M. tuberculosis* cells that display the same behaviour are described in the literature, as either differentially culturable tubercle bacteria (DCTB) (Chengalroyen et al., 2016) or differentially detectable (DD) bacteria (Saito et al., 2017). The relative proportion of DCTB in a sample is quantified by

comparing the number of culturable bacteria in liquid-limiting dilution assays with that of plateable bacteria on solid media.

Initial descriptions of these cells in mycobacteria were associated with resuscitation promoting factors (Rpfs), originally described in *Micrococcus luteus* as being able to promote the resuscitation of dormant bacteria (Mukamolova et al., 1998). There are five *rpf* genes in the *M. tuberculosis* genome and the associated proteins, which are present in the culture filtrate (CF) of *M. tuberculosis*, have been shown to activate the growth of non-culturable bacteria (Mukamolova et al., 2002; Kana et al., 2008). Both liquid culture dependent and plateable populations of bacteria have been identified in sputum of patients with TB disease, including DCTB populations that can be classed as CF-dependent and CF-independent (Mukamolova et al., 2010; Dhillon et al., 2014; Chengalroyen et al., 2016). Furthermore, the identification of a subpopulation of bacteria able to grow with CF from a *M. tuberculosis* mutant lacking Rpfs suggests involvement of other growth stimulatory factors (Chengalroyen et al., 2016). Individually cAMP and fatty acids appear to offer no benefit in DCTB recovery from sputum samples, contrary to their effectiveness in an *in vitro* model (Shleeva et al., 2013; Gordhan et al., 2021). The presence of these differentially culturable organisms in clinical specimens suggests that approaches to enhance their growth may allow for greater diagnostic pick-up. Indeed, the use of CF and Rpfs allows for the detection of viable *M. tuberculosis* in clinical samples with negative results by standard tests (Dusthackeer et al., 2019). Furthermore, the inclusion of CF is also beneficial for the detection of DCTB in culture negative samples from PLWHIV (McIvor et al., 2021). Recently, *M. tuberculosis* was recovered in a subpopulation of culture negative clinical specimens using lipid-rich media, instead of the standard glycerol-based media (Mesman et al., 2021). DCTB populations in sputum have been shown to be tolerant to a range of anti-TB drugs (Turapov et al., 2016). The number of DCTB in sputum samples increased following the first two weeks of TB treatment, either as a consequence of the antibiotics enriching the number of these bacteria in the samples or by stimulating the generation of new DCTB (McAulay et al., 2018; Zainabadi et al., 2021). However, in patients with drug-resistant TB, the proportion of DCTB did not change significantly following two weeks of treatment, perhaps as a consequence of the administered treatment regimens that excluded rifampicin (Zainabadi et al., 2021).

The presence of co-infections, such as HIV, need to be considered when diagnosing and treating patients with clinical TB. PLWHIV and TB, with CD4 counts >200 cells/ml, are more likely to display higher levels of CF-dependent DCTB then people with CD4 counts <200 cells/ml, suggesting that the host immune system has an effect on the production on these differentially culturable bacteria (Chengalroyen et al., 2016). It has been shown that DCTB are present in the sputum from individuals clinically cured of TB (Beltran et al., 2020). These data underscore that multiple diagnostic tests that account for bacterial phenotypic diversity in sputum are likely required to ensure a true estimation of bacteriological cure rates. This is particularly important when discussing possible alterations to treatment length. Stopping the administration of TB drugs when an individual appears to be cured, and then restarting treatment following relapse of disease, creates the ideal conditions for the emergence of drug tolerant and resistant bacterial populations.

Addressing heteroresistance

Heteroresistance occurs when only a subpopulation of the bacteria displays the resistance phenotype, or if multiple resistance genotypes occur together. This phenomenon is often unstable, with the resultant higher drug minimal inhibitory concentration (MIC) values being transient and difficult to assess consistently (Andersson et al., 2019). Diagnostic testing of such mixed infections may only identify the one strain, resulting in treatment with inappropriate drugs that would ultimately select for DR strains. There is conflicting data about the extent that heteroresistance plays in treatment outcomes, with some studies showing poor outcomes whilst others show no association (Kargarpour Kamakoli et al., 2017; Shin et al., 2018; Kargarpour Kamakoli et al., 2020; Chen et al., 2021). Development of deep sequencing algorithms to identify minority strain genotypes in mixed infections will be important for early diagnosis of heteroresistance. Whether this facilitates better patient management will require extensive clinical studies which should be prioritized as heteroresistance remains a clear and present threat to the elimination of drug resistant TB.

Treatment and prophylaxis

Drug-susceptible TB disease can be treated with antibiotic regimens that take 4 to 9 months to complete. A newly proposed 4-month treatment course consists of 2 months of intense rifapentine, moxifloxacin, isoniazid and pyrazinamide therapy, followed by 9 weeks of rifapentine, moxifloxacin and isoniazid (Carr et al., 2022). Alternatively, rifampicin, isoniazid, pyrazinamide and ethambutol can be administered for 2 months, followed by rifampicin and isoniazid for 4 months. The treatment of drug-resistant TB is more complicated and is dictated by the resistance-causing mutations present. The WHO recommended in 2020 that isoniazid-resistant, but rifampicin-susceptible TB, be treated with rifampicin, ethambutol, pyrazinamide and levofloxacin for 6 months. RR-TB and MDR-TB treatment regimens during an intensive 4-month phase consist of levofloxacin/moxifloxacin, clofazimine, ethionamide, ethambutol, isoniazid (high dose), pyrazinamide and bedaquiline (for 6 months). This is followed by a continuation phase with treatment using levofloxacin/moxifloxacin, clofazimine, ethambutol and pyrazinamide for 5

months (World Health Organization, 2020). Individuals infected with TB resistant to any additional antibiotic require individualised treatment regimens that last for 18 months. Potentially new anti-TB compounds currently undergoing clinical trials are listed in Box 1 (Dartois and Rubin, 2022; Working Group on New TB Drugs, 2022). Given that resistance to bedaquiline, the first drug to be approved for treating TB since the 1960s, is emerging globally, these new drugs are critical for future TB regimens (Chesov et al., 2022; Nair et al., 2022).

A key missing feature for TB control is a comprehensive program for prophylaxis for contacts of index cases with drug resistant TB. The toxicity and high costs of currently available Group B agents (Box 1), which would comprise the first choice for prophylaxis, are major stumbling blocks to this. Currently, the National Tuberculosis Controllers Association and CDC recommend short-course treatments (3-4 months) using rifamycin-based regimens for contacts of drug sensitive TB index cases. These could take the form of once-weekly isoniazid and rifapentine for 3 months, 3 months of daily isoniazid and rifampicin or daily rifampicin for 4 months. Six or nine months of daily isoniazid treatment is an available alternative treatment but does come with a higher toxicity risk and the longer course period can result in lower completion rates (Sterling et al., 2020). There are on-going clinical trials looking at preventative measures for household contacts of patients with MDR-TB. PHOENIx (Protecting Households On Exposure to Newly Diagnosed Index Multidrug-Resistant Tuberculosis Patients) is a phase III trial comparing the use of delamanid vs isoniazid for 26 weeks in preventing active TB in high-risk children and adult members of households with active MDR-TB cases (National Institute of Allergy and Infectious Diseases et al., 2021). The TB child multidrug-resistant preventive therapy (TB-CHAMP) phase III trial is addressing the use of levofloxacin to prevent TB disease in children under the age of 5 years, who are contacts of people with active MDR-TB (Seddon et al., 2018). More clinical trials in vulnerable populations such as healthcare workers, PLWHIV and children are needed. Furthermore, the psychosocial complexities of treating people with no (or mild) symptoms with drugs that have side-effects will need to be addressed.

Transmissibility of drug resistant strains

In the household setting, emerging evidence points to greater risk of transmission with drug resistant TB when compared to drug susceptible disease (Becerra et al., 2019). However, this effect is most likely due to delayed diagnosis or treatment of drug resistant TB rather than an increased transmissibility of resistant strains. Consistent with this a recent study from India confirmed that the high transmission of drug resistant TB, particularly in young people, was associated with delays in care including rapid diagnosis and linkage to treatment (Atre et al., 2022). As such, direct contemporaneous evidence that drug resistant strains are more transmissible in the community is lacking.

Subclinical TB and transmission

Traditionally, TB transmission has been associated with the individuals who display symptoms and are able to spread bacteria through coughing however, the role of subclinical TB in community transmission has recently garnered interest. Subclinical TB is defined by the presence of tubercle bacilli in the lung with no associated clinical TB symptoms and is radiologically and/or microbiologically detectable (Peters et al., 2019). One mechanism through which individuals with subclinical TB can contribute to transmission is through TB-unrelated chronic cough, possibly associated with air pollution or chronic lung disease (Esmail et al., 2018). Approaches to addressing chronic coughs, including treatment of viral infections may yield potential benefits (Esmail et al., 2018). Recent evidence suggests that tubercle bacteria can also be spread by tidal breathing indicating that various respiratory manoeuvres, including singing and talking, could contribute to TB transmission (Williams et al., 2020; Dinkele et al., 2022). Current estimates suggest that roughly 7-10 million individuals are living with subclinical TB, who most likely have heterogenous clinical trajectories and outcomes which impact on the ability to transmit bacteria (Kendall et al., 2021). These data illustrate that sub-clinical TB could serve as a significant driver of TB transmission. Further studies addressing this will require diagnostic tests that are accurate and easy to scale in large populations, together with appropriate bioinformatics and modelling tools to infer directionality of transmission over long periods of asymptomatic disease, sometimes in complex epidemiological settings (Kendall et al., 2021).

Another aspect to consider when looking at transmission is the mechanistic factors that dictate how the disease moves from one person to another. It has been shown that *M. tuberculosis* produces sulfolipid-1 (SL-1) which is able to activate nociceptive neurons in guinea pigs and trigger coughing (Ruhl et al., 2020). Whether a similar mechanism occurs in humans needs investigation to understand the relationship between the extent of the cough and transmission. The likelihood of transmission could be dependent on the type of *M. tuberculosis* strain present in the lungs. In mice, high transmission strains induce alveolar macrophages, in an interleukin-1 receptor-dependent manner, to migrate into the lung interstitium. This ultimately leads to granuloma formation with potential cavitary lesions, which enable the *M. tuberculosis* bacilli to enter the airways (Lovey et al., 2022). Aggregates of *M. tuberculosis*, when compared to single bacteria, face less acidification in macrophage phagosomes, are associated with an increase in bacterial replication and initiate the death of the macrophage that can lead to a macrophage-death cascade (Mahamed et al., 2017;

Rodel et al., 2021). Given that such aggregates have been identified in human granulomatous lesions (Rodel et al., 2021), together with clumps of cells detected in TB patient bioaerosols (Dinkele et al., 2021), suggests that clumping of bacterial cells could be a means of continued transmission and bacterial pathogenesis.

It is clear that extrinsic and environmental factors related to TB diagnosis, treatment and patient behaviour contribute significantly to the emergence of drug resistance in *M. tuberculosis*. Addressing these factors, together with novel health systems strengthening interventions, will significantly assist in curbing the spread of TB. The second aspect to limiting the emergence of drug resistance is developing and understanding of the bacterial factors that drive drug tolerance and the ultimate emergence of resistance. Understanding these factors is crucial to anticipate future trajectories of acquired resistance, identification of potential drug targets and development of more effective shorter regimens. Below, we highlight the contribution of some of these bacterial mechanisms and their possible roles in the development of acquired drug resistance.

Acquired drug resistance: Bacterial mechanisms for surviving antibiotic treatment

M. tuberculosis shares an extremely complex relationship with its human host. These bacteria have adapted to activate specific responses to modulate their physiology and resist stress encountered during pathogenesis. Multiple and complex stress responses induce the development of different bacterial subpopulations, which can display varying levels of tolerance to drugs, resulting in a continuous spectrum of phenotypes that contribute to disease relapse despite protracted treatment regimens. Considering this, current definitions of tolerance and persistence may be too simplistic and require revision to incorporate the associated mechanisms to describe these complex and often coexisting phenotypes.

Upon repetitive and intermittent antibiotic treatment, bacterial populations adapt and eventually become tolerant, manifesting persisters that evade death, even at very high concentrations of drug (Sulaiman and Lam, 2021). Persistent bacteria are characterised by their ability to survive high antibiotic concentrations, without genetic mutations in drug targets/in genes associated drug mechanisms of action. As they are genetically identical to their antibiotic susceptible counterparts, persistence is considered a phenotypic trait used to protect the longevity of the overall population in the face of adversity (Balaban et al., 2019; Huemer et al., 2020; Sulaiman and Lam, 2021). Persistence and the associated drug tolerance can also be defined in the context of clinical infection by lesions that do not clear because of bacterial tolerance or insufficient drug concentrations in lesions as a consequence of poor regimen adherence or inadequate drug diffusion (Sarathy et al., 2016).

The importance of tolerance as a prelude to resistance

The long-standing debate of whether antibiotic tolerance and resistance are related or two distinct phenotypes, was recently addressed through *in vitro* experiments and mathematical modelling demonstrating that tolerant *Escherichia coli* populations eventually became resistant to drugs (Levin-Reisman et al., 2017). Resistant mutants of *M. tuberculosis* were also shown to arise from antibiotic tolerant persisters explaining the ability of these bacteria to not just survive but replicate in the presence of a drug (Sebastian et al., 2017). Mechanisms contributing to antibiotic tolerance include the activity of efflux pumps, pathway redundancies, direct inactivation of drugs or modifications to the drug target, thus allowing persisters to survive in the presence of antibiotics for long periods of time, with consequent elevated MIC values (Balaban et al., 2019; Sulaiman and Lam, 2021). Combined, these mechanisms that ultimately confer tolerance should be the subject of intense study as they most likely contribute substantively to stable genetic resistance in *M. tuberculosis*. Some of these mechanisms are briefly outlined below.

Dormancy

Dormant bacteria do not replicate, displaying decreased metabolic activity thereby protecting themselves from antibiotics that require active bacterial growth to be effective. As a result, dormant bacteria display tolerance to many drugs, but through different (and sometimes overlapping) mechanisms to those that confer tolerance in replicating bacteria (Balaban et al., 2019; Tasse et al., 2021). Whilst the phenomenon of non-replicating persistence has been described for mycobacteria *in vitro*, using several different laboratory models (Gold et al., 2015; Gold and Nathan, 2017), there is insufficient evidence that a dormancy phenotype prevails in human TB. It has been reported that mycobacteria are able to sporulate, a metabolic state synonymous with dormancy, but subsequent analyses suggested that this is probably unlikely given the lack of genetic orthologues that mediate the process (Ghosh et al., 2009; Traag et al., 2010). Mycobacteria do retain the capacity to adopt non-replicative states that are associated with morphological changes however, the role of these changes in conferring antibiotic tolerance remains largely unexplored (Shleeva et al., 2011). The molecular mechanisms that have been associated with this trait may be useful to develop new TB drugs and many drug screening programs have

opted to use laboratory conditions that best mimic non-replicating persistence.

Heterogeneity in bacteria and lesions

Studies in animal models and directly in individuals with TB have shown the presence of heterogenous and divergent individual lesions within a single host, arising from differential immune potential of host cells and the varied virulence of TB bacilli (Lenaerts et al., 2015; Dhar et al., 2016). The outcome of individual lesions appears to be driven by the local tissue environment rather than the general host response, suggesting a defining role for these microenvironments in *M. tuberculosis* heterogeneity. Heterogeneity in metabolism of individual bacteria has a direct impact on cell growth rates, metabolic processes, stress responses, and drug susceptibility (Coleman et al., 2014; Lin et al., 2014; Gideon et al., 2015; Prideaux et al., 2015; Marakalala et al., 2016). These differences may not confer fitness advantages to the bacterial population under optimum growth conditions but can be beneficial for survival during stress and antibiotic treatment. Whilst there is growing appreciation of bacterial heterogeneity as an important driver of persistence in TB disease, targeting the mechanisms that drive this phenomenon will be difficult without a comprehensive mechanistic understanding of key mediators of the effect. Developing new tools to study this under physiologically relevant conditions emerges as an important research priority.

Asymmetric cell division in mycobacteria is another contributing factor for establishing a heterogeneous population both in size and elongation rate, with varying drug susceptibility (Aldridge et al., 2012; Kieser and Rubin, 2014; Logsdon and Aldridge, 2018). *M. tuberculosis* cells grown *in vitro* under stress conditions, or isolated from sputum or infected macrophages, display increased heterogeneity in cell-size suggesting that heterogeneous populations can have different sensitivity to drugs resulting in variable treatment outcomes (Vijay et al., 2017). Asymmetry in division also results in disproportionate distribution of oxidized proteins between the progeny, which is associated with a fitness cost as cells with a higher content of oxidized proteins grow slower and struggle to recover after exposure to antibiotics (Vaubourgeix et al., 2015). Single-cell heterogeneity in *M. tuberculosis* has also been linked to LamA, a divisome protein that inhibits growth at nascent new poles. Deletion of *lamA* results in loss of heterogeneity and faster killing by vancomycin and rifampicin (Rego et al., 2017).

Other mechanisms contributing to heterogeneity include growth processes such as cell elongation, DNA replication and chromosome segregation. At any given time point during growth, individual cells in a population are in different phases of elongation and division in relation to each other. Mapping of cell division cycles and chromosomal replication at the single-cell level in *Mycobacterium smegmatis* has identified cell-to-cell heterogeneity in growth rates and interdivision times, together with changes in duration of various cell cycle periods, resulting in large variations in cell size, age, ploidy, and generation times (Santi et al., 2013; Santi and McKinney, 2015). Similarly, mapping of the cell cycle in *M. tuberculosis* would enhance our understanding of bacterial heterogeneity in the context of protracted treatment and/or treatment failure. Growth rate variations dependent on nutrient availability is another widely studied characteristic contributing to heterogeneity due to the strong link between proliferation and virulence, together with the relationship between growth arrest and drug tolerance (Ray et al., 2011; Cerulus et al., 2016; Hashimoto et al., 2016).

Translesion DNA synthesis and mutagenesis

Maintenance of genomic integrity by dedicated repair systems is vital to prevent blockage of DNA replication associated with replication fork collapse. In addition to a host of DNA repair pathways, mycobacteria also have translesion DNA polymerases that are able to transiently replace the replicative polymerase to bypass lesions resulting from DNA damage. This allows for DNA damage tolerance and mutagenesis (Fuchs and Fujii, 2013). DnaE2, an alternate homologue of the replicative polymerase is associated with UV tolerance and UV-induced mutagenesis, leading to the emergence of drug resistance (Boshoff et al., 2003; Warner et al., 2010). More recently, the overexpression of DinB1, a Y-family DNA polymerase was shown to promote missense mutations in the *rpoB* gene, conferring resistance to rifampicin (Dupuy et al., 2022). The mutational signature for rifampicin resistance displayed by DinB was distinct from that of DnaE2, implicating translesion synthesis/template slippage on homopolymeric runs as a strong driver of mycobacterial genome diversification with antimicrobial resistance and host adaptation implications.

Compensatory mutations

Drug resistance in *M. tuberculosis* is exclusively due to chromosomal mutations that may confer drug resistance *via* modification or overexpression of the drug target and/or prevention of prodrug activation. These modifications may impart pleiotropic effects leading to a reduction in bacterial fitness (Andersson and Hughes, 2010) which can be mitigated by compensatory mutations by interacting epistatically with the resistance mutation (Comas et al., 2011; Gygli et al., 2017). The clinical relevance of the evolution of compensatory mutations is still poorly understood. When compared to other pathogenic bacteria, the genetic diversity of *M. tuberculosis* is low suggesting

that the accumulation of compensatory mutations may have important implications for the stability of drug resistant phenotypes (Ford et al., 2013).

Transcription

Promoter specificity in *M. tuberculosis* is driven by 13 sigma factors, small proteins that bind to the RNA polymerase holoenzyme in response to specific environmental stimuli (Rodrigue et al., 2006; Paget, 2015). Sigma factor E, SigE, is expressed in response to surface stress, low pH, oxidative stress, several antibiotics and has been proposed as a major mediator for the switch to non-replicating persistence (Balazsi et al., 2008; Dona et al., 2008; Manganelli and Provvedi, 2010; Tiwari et al., 2010; Manganelli, 2014; Pisu et al., 2017). Two SigA dependent promoters upstream of the *rpoB* gene, the target for rifampicin, have been shown to be responsible for semi-heritable tolerance in mycobacteria. Rifampicin preferentially inhibits one of the two promoters, whilst the second promoter is induced in the presence of rifampicin, resulting in accumulation of RpoB in the cytoplasm, thus supporting growth in the face of bactericidal rifampicin concentrations (Zhu et al., 2018). Mycobacteria also utilize a two-step pathway involving the glutamine amidotransferase (GatCAB) enzyme to regulate translational fidelity of glutamine and asparagine codons as an adaptive survival strategy. Mistranslation of the *rpoB* gene has been shown to reduce production of RNA polymerase and increase bacterial survival in the presence of rifampicin (Su et al., 2016). The collective role of the *M. tuberculosis* stress response pathways in drug tolerance and persistence provide an opportunity to target sigma factors as alternative strategies to shorten TB treatment.

Toxin-antitoxin systems

M. tuberculosis encode 88 toxin-antitoxin (TA) modules, that play a role in regulating adaptive responses to stresses generated by the host environment and drug treatment (Slayden et al., 2018). Most TAs are classified as type II, characterized by a toxin with endoribonuclease activity and an antitoxin that binds the toxin to neutralize its activity. Induction of antitoxin degradation is specific to the environmental stimuli, allowing the toxin to exert its effect leading to reduced metabolism and cell division arrest (Barth et al., 2019; Bordes and Genevaux, 2021). *M. tuberculosis* mutants carrying deletions in one or more of these TA systems display greater susceptibility to drugs compared to the parental strain. Different TA systems have been shown to display specific, as well as diverse roles in the presence of drugs (Singh et al., 2010; Tiwari et al., 2015). Given that TA modules directly alter metabolism, future studies assessing the effects on single cells will be valuable to determine how these systems contribute to persistence development in *M. tuberculosis*. Another challenge that needs to be addressed is the redundancy of these systems in *M. tuberculosis*. New drugs would need to target all systems concurrently or target a master metabolic mediator.

Biofilms

Mycobacterial biofilms *in vitro* are characterized by the formation of a floating pellicle at the air-medium interface. This phenomenon is of particular interest as cells that survive drug treatment generally are enriched in biofilms due to reduced growth and/or metabolism as well as reduced accessibility to drugs. Mycobacterial biofilms show extreme tolerance to isoniazid, and contain elevated numbers of persisters that survive high concentrations of rifampicin (Ojha et al., 2008). A Tn-seq screening of *M. tuberculosis* mutants unable to form biofilms identified genes responsible for biofilm formation that were also involved in stress and drug tolerance, suggesting that the hostile environment of biofilms, characterized by nutrient and oxygen depletion, has the potential to increase the proportion of persisters (Richards et al., 2019). Whilst initial studies suggested the presence of mycolic acids in biofilms, subsequent analyses pointed to the presence of cellulose in the extracellular matrix of mycobacterial biofilms (Trivedi et al., 2016). In fact, structural components of biofilm matrices are dependent on the *in vitro* model used to generate the floating pellicles (Chakraborty and Kumar, 2019). Recently, the relevance of drug tolerant biofilms to human TB was demonstrated in non-human primates and in lung tissue sections obtained from individuals with TB (Chakraborty et al., 2021). *M. tuberculosis* strains defective in biofilm formation were attenuated for survival in mice, suggesting that biofilms protect these bacilli against host immunity assaults. Moreover, mice infected with *M. tuberculosis* and subsequently treated with a cellulase to degrade biofilms displayed increased susceptibility to isoniazid and rifampicin, suggesting a role for biofilms in phenotypic drug tolerance (Chakraborty et al., 2021).

Metabolism

Many aspects of mycobacterial metabolism contribute to drug tolerance, we will focus here on a select few. Exposure of *M. tuberculosis* to stresses such as low iron, pH, or oxygen, results in the accumulation of large amounts of intracellular triacylglycerol (TAG) droplets. *M. tuberculosis* cultures from growth-limiting conditions accumulate TAG and display increased tolerance to several drugs, an effect dependent on a functional *tgs1* gene (Deb et al., 2009; Baek et al., 2011). TAG accumulation has also been implicated in carbon storage for rapid restoration of metabolic activity during resumption of growth (Daniel et al., 2004), and in the maintenance of redox homeostasis under conditions of low

respiration (Leistikow et al., 2010). In addition, the increased content of intracellular lipid inclusions, including TAGs, in caseum bacilli is linked to the development of mycobacterial caseum-induced tolerance to several first and second line drugs (Sarathy et al., 2018). Persistent bacteria have intracellular lipid bodies and monitoring sputum smears from patients on treatment, with Nile-Red for staining lipid bodies, revealed the presence of persister bacilli that did not clear at the rates as those that formed colonies using conventional culture (Kayigire et al., 2015; Sloan et al., 2015). In this context, combining different staining approaches with conventional culture may yield new clinical endpoints that enable rapid triage of new treatments. However, research in this area has been largely focused on drug sensitive TB. Whether such approaches would be useful for drug resistant TB is unclear and future work should focus on this.

Metabolomic analysis of *M. tuberculosis* treated with sublethal concentrations of isoniazid, rifampicin or streptomycin highlighted the induction of a common subset of metabolites linked to the tricarboxylic acid (TCA) cycle, glyoxylate metabolism and amino acid biosynthetic pathways (Nandakumar et al., 2014). Interrogation of the TCA cycle showed activation of the bifunctional isocitrate lyases (ICL) and methylisocitrate lyases under the oxidative stress conditions generated during antibiotic treatment, with increased activity of the glyoxylate shunt and decreased activity of the reductive arm of the TCA cycle (Kohanski et al., 2007). These data pointed to reduction in respiration to counteract the increased reactive oxygen intermediates (ROI) induced by antibiotic treatment. Consistent with this, a mutant deficient in ICL was associated with induction of several ROI responsive genes, implying that ICL has a role in counteracting endogenous oxidative stress (Nandakumar et al., 2014). These observations point to an urgent need to study the fundamental metabolism of drug resistant isolates of *M. tuberculosis* as these mechanisms may differ significantly, thus requiring a different strategy.

Phase variation

GlpK, an enzyme required for glycerol catabolism *via* the glycolytic pathway is essential for growth of *M. tuberculosis* in glycerol containing media. Interestingly, *glpK* is subjected to phase variation due to frequent and reversible frameshift mutations in its open reading frame, resulting in some clinical strains producing subpopulations of small colonies with a smooth surface phenotype with heritable tolerance to several drugs. Accumulation of these variants during drug treatment with a rapidly reversible genetic mechanism of drug tolerance has important consequences for treatment failure and relapse (Bellerose et al., 2019; Safi et al., 2019). The frameshift mutations associated with this observation are likely driven by previously mentioned translesion synthesis DNA polymerases, with a reduced fidelity for DNA replication. How these polymerases contribute to phase variation requires further investigation.

Macrophage activation

The role of efflux pumps in intracellular drug tolerance was validated through treatment of infected macrophages with drug efflux inhibitors, which resulted in reduced drug tolerance (Adams et al., 2011). *M. tuberculosis* can grow without any constraint in resting macrophages but replication of the bacilli is severely reduced in activated macrophages where the bacteria encounter a more hostile environment. Single cell studies showed that under nutrient starvation conditions, bacterial phenotypic heterogeneity increased, consisting of non-growing metabolically active and drug tolerant bacterial subpopulations. However, mice lacking interferon-γ did not display this phenotypic heterogeneity, suggesting that the level of macrophage activation may play a role in inducing drug tolerance (Manina et al., 2015). Indeed, sensitivity comparisons of bacteria residing in resting or activated macrophages with the four first line TB drugs showed differential transcriptional responses of *M. tuberculosis* to isoniazid treatment. The genes induced by isoniazid treatment were associated with regulons responsive to low pH, nutrient starvation, nitrosative or oxidative stress, and surface-damage. Exposure of intracellular bacteria to individual frontline TB drugs yielded a common set of gene expression responses, despite different modes of drug action, suggesting that macrophage activation imposes a stronger stress to the intracellular bacteria (Liu et al., 2016). As such, modulating macrophage function could directly affect drug tolerance mechanisms in tubercle bacilli, a premise of host directed therapies (HDTs), which has emerged as a promising area of research. Current drugs for diabetes, for use as HDTs, promote ROS production leading to mycobacterial killing whilst inhibition of cholesterol synthesis decreased lipid accumulation in animal models when treated together with antitubercular drugs, suggesting a potentially beneficial effect in human TB (Parihar et al., 2014; Singhal et al., 2014; Skerry et al., 2014; Turgeon et al., 2016). However, analysis of data from a national medical claim database showed no beneficial effect of these drugs during *M. tuberculosis* infection (Kang et al., 2014) suggesting the need for more controlled clinical trials to help understand the relevance of HDTs in TB infection. It is also important to assess and understand the possible drug-drug interactions between HDT and currently administered TB drug regimens for successful outcomes.

Immunometabolism

Immuno-metabolism during TB infection has important implications for pathogenesis and disease outcomes, with recent studies pointing to notable differences between drug resistant and drug sensitive TB. Following TB treatment, there are differences between the regulatory T cells of the immune system in patients with either drug susceptible or drug resistant TB, with these changes still being evident in drug resistant patients after 6 months of treatment (Tellez-Navarrete et al., 2021). Rifampicin resistant TB results in the down regulation of 35 tripartite motif (TRIM) proteins in blood leukocytes when compared to drug sensitive TB (Liu et al., 2021). A coinfection of diabetes and TB results in systemic inflammation, leading to poor outcomes (Kumar et al., 2019). Additionally, drug resistant TB strains, including RR- and MDR-TB, have been shown to be associated with an increased risk of diabetes, treatment failure and death (Kang et al., 2013; Ruesen et al., 2020). While infection with TB results in significantly higher levels of nitric oxide (NO) indicators when compared to healthy controls, these levels are not as high when the infection is with MDR-TB. Following two months of treatment, the NO levels in those individuals with drug sensitive TB returned to normal in a manner that was not seen in individuals with MDR-TB, perhaps indicating a lower level of immunological response (Butov et al., 2014). MDR and XDR-TB infected macrophages induce high levels of oxidative stress, which, as described previously, can have direct implications for drug efficacy (Tyagi et al., 2020).

MDR-TB affects various characteristics of host leukocytes, such as prolonging the circulation of a range of monocytes but decreasing the frequency of HLA-II expressing monocytes, resulting in a proinflammatory status with high IFN-γ and TNF levels months after TB treatment (Ocana-Guzman et al., 2021a; Ocana-Guzman et al., 2021b). This effect is not seen with drug susceptible TB. While inflammatory signalling and TCA cycle remodelling occurs during drug susceptible TB infection, 2 months of ineffective treatment of MDR-TB resulted in a marked upregulation of this inflammatory cascade, an effect that took a year to reverse (Collins et al., 2021). These, and related effects, should be carefully investigated to develop appropriate health systems strategies for monitoring outcomes of drug resistant TB.

Although the field of antibiotic persistence and tolerance has made great advances, much still needs to be uncovered to fully understand how these multiple mechanisms interact to generate beneficial phenotypic heterogeneity for the survival of the bacterial population during pathogenesis. Novel therapeutics aiming to eradicate persisters are desperately needed to shorten TB treatment. In addition, novel cutting-edge technologies for detecting persister bacteria in clinical specimens are urgently needed.

Covid and TB

Globally, the Covid-19 pandemic has set back the fight against TB by a decade, which is estimated to result in an additional 6.3 million people developing TB and an additional 1.4 million deaths between 2020 and 2025 (Dheda et al., 2022). In South Africa, there was a 50% reduction in the number of TB tests conducted during the initial stages of the Covid-19 pandemic. Added to this was the 12% reduction in the targeted TB treatment success rate of 90%. In response, several provinces in South Africa have developed aggressive TB plans to help monitor the TB response (Jeranji, 2021). An example is the launch of a first-of-a kind public facing TB dashboard in the Western Cape Province. The TB dashboard which includes data on TB cases, deaths, test positivity and drug resistance, from 2015 onwards was modelled based on lessons learnt from the global response in the fight against Covid-19. This interactive program aims to get the TB response in the province back on track (Jeranji, 2021). Similarly, researchers in India and across the world have called for real-time TB dashboards for recording cases and deaths (Mascarenhas, 2022).

Another effective intervention for the management of Covid-19 are mobile phone screening/tracing apps (Ruhwald et al., 2021). Based on this success, the Department of Health in South Africa has recently introduced a WhatsApp and SMS-based TB screening app called the TB Health Check app that links individuals to TB testing stations. A 12.8% TB positivity was recorded from 30 000 screens (Dheda et al., 2022). Integration of the Covid-19 and TB screening apps is currently underway with possible expansion to include HIV self-testing apps, which have already shown great promise (DiAndreth et al., 2020; Pai et al., 2021).

The Covid-19 experience has sent out a strong message that developing health interventions such as vaccines in a short space of time is achievable. Development of new tools to control the pandemic was only possible because of adequate funding and political support. TB receives only a fraction of the funding compared to Covid-19, clearly raising important questions regarding the financial disparity between these two infectious diseases. Arguably, Covid-19 may have attracted significantly greater financing compared to TB since the health, social, and economic impacts have affected not only poor countries but also wealthy ones. This was evident by the unequal rollout of Covid-19 vaccines, which prioritized vaccinating populations in wealthy countries over poorer nations (Treatment Action Group, 2021).

Future perspectives

The continued spread of resistant pathogens will undermine any gains from modern medicine, plunging health care back to the pre-antibiotic era and creating the ideal environment for

emergence of new pandemics at a global scale. Health systems need to urgently strengthen those processes that interrupt TB transmission in communities, hospitals and other congregate settings to rapidly eliminate new infections. Developing scalable and affordable genomic surveillance mechanisms for drug resistant TB in all countries will be central to limiting the spread of new infections. In addition, to prevent full blown drug resistant TB, prophylaxis of vulnerable contacts should be an urgent priority, with the outlook to achieving full coverage with reduced side effects. Given the rapid emergence of drug resistance, new research that uncovers vulnerable drug targets and highlights those processes that are fundamental to mediating drug tolerance, persistence and ultimately resistance must become urgent global priorities. Mycobacterial metabolism represents a significant departure from other well-studied bacterial systems and in this context, developing a broad knowledge based on fundamental metabolism that can be used to facilitate new drug development will require long-term investment from funding bodies. Public-Academic-Private partnerships will also serve as strong enablers to progress new compounds through the drug development pipeline. Meaningful gains in TB programs can only be achieved with this coordinated approach.

Author contributions

DL, BG, and BK collectively conceived the overall concept of the review and wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was funded by the Department of Science and Innovation (grant number DCE015) and the South African Medical Research Council (grant number KABV017).

Acknowledgments

We would like to thank Dr. Christopher Ealand and Dr. Edith Machowski for critical appraisal of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Abdulgader, S. M., Okunola, A. O., Ndlangalavu, G., Reeve, B. W. P., Allwood, B. W., Koegelenberg, C. F. N., et al. (2022). Diagnosing tuberculosis: What do new technologies allow us to (Not) do? *Respiration* 101, 979–813. doi: 10.1159/000525142

Adams, K. N., Takaki, K., Connolly, L. E., Wiedenhoft, H., Winglee, K., Humbert, O., et al. (2011). Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell* 145, 39–53. doi: 10.1016/j.cell.2011.02.022

Aldridge, B. B., Fernandez-Suarez, M., Heller, D., Ambravaneswaran, V., Irimia, D., Toner, M., et al. (2012). Asymmetry and aging of mycobacterial cells lead to variable growth and antibiotic susceptibility. *Science* 335, 100–104. doi: 10.1126/science.1216166

Andersson, D. I., and Hughes, D. (2010). Antibiotic resistance and its cost: Is it possible to reverse resistance? *Nat. Rev. Microbiol.* 8, 260–271. doi: 10.1038/nrmicro2319

Andersson, D. I., Nicoloff, H., and Hjort, K. (2019). Mechanisms and clinical relevance of bacterial heteroresistance. *Nat. Rev. Microbiol.* 17, 479–496. doi: 10.1038/s41579-019-0218-1

Atre, S. R., Jagtap, J. D., Faqih, M. I., Dumbare, Y. K., Sawant, T. U., Ambike, S. L., et al. (2022). Tuberculosis pathways to care and transmission of multidrug resistance in India. *Am. J. Respir. Crit. Care Med.* 205, 233–241. doi: 10.1164/rccm.202012-4333OC

Baek, S. H., Li, A. H., and Sassetti, C. M. (2011). Metabolic regulation of mycobacterial growth and antibiotic sensitivity. *PloS Biol.* 9, e1001065. doi: 10.1371/journal.pbio.1001065

Balaban, N. Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D. I., et al. (2019). Definitions and guidelines for research on antibiotic persistence. *Nat. Rev. Microbiol.* 17, 441–448. doi: 10.1038/s41579-019-0196-3

Balazsi, G., Heath, A. P., Shi, L., and Gennaro, M. L. (2008). The temporal response of the *Mycobacterium tuberculosis* gene regulatory network during growth arrest. *Mol. Syst. Biol.* 4, 225. doi: 10.1038/msb.2008.63

Barth, V. C., Zeng, J. M., Vvedenskaya, I. O., Ouyang, M., Husson, R. N. , and Woychik, N. A. (2019). Toxin-mediated ribosome stalling reprograms the *Mycobacterium tuberculosis* proteome. *Nat. Commun.* 10, 3035. doi: 10.1038/s41467-019-10869-8

Becerra, M. C., Huang, C. C., Lecca, L., Bayona, J., Contreras, C., Calderon, R., et al. (2019). Transmissibility and potential for disease progression of drug resistant *Mycobacterium tuberculosis*: Prospective cohort study. *BMJ* 367, l5894. doi: 10.1136/bmj.l5894

Bellerose, M. M., Baek, S. H., Huang, C. C., Moss, C. E., Koh, E. I., Proulx, M. K., et al. (2019). Common variants in the glycerol kinase gene reduce tuberculosis drug efficacy. *mBio* 10, e00663-00619. doi: 10.1128/mBio.00663-19

Beltran, C. G. G., Heunis, T., Gallant, J., Venter, R., du Plessis, N., Loxton, A. G., et al. (2020). Investigating non-sterilizing cure in TB patients at the end of successful anti-TB therapy. *Front. Cell Infect. Microbiol.* 10, 443. doi: 10.3389/fcimb.2020.00443

Bordes, P., and Genevaux, P. (2021). Control of toxin-antitoxin systems by proteases in *Mycobacterium tuberculosis*. *Front. Mol. Biosci.* 8, 691399. doi: 10.3389/fmolb.2021.691399

Boshoff, H. I., Reed, M. B., Barry, C. E. 3rd, and Mizrahi, V. (2003). DnaE2 polymerase contributes to *in vivo* survival and the emergence of drug resistance in *Mycobacterium tuberculosis*. *Cell* 113, 183–193. doi: 10.1016/s0092-8674(03)00270-8

Butov, D. O., Kuzhko, M. M., Kalmykovsmall, I. M., Kuznetsova, I. M., Butova, T. S., Grinishina, O. O., et al. (2014). Changes in nitric oxide synthase and nitrite and nitrate serum levels in patients with or without MDR-TB undergoing the intensive phase of anti-tuberculosis therapy. *Int. J. Mycobacteriol.* 3, 139–143. doi: 10.1016/j.ijmyco.2014.02.003

Carr, W., Kurbatova, E., Starks, A., Goswami, N., Allen, L., and Winston, C. (2022). Interim guidance: 4-month rifapentine-moxifloxacin regimen for the treatment of drug-susceptible pulmonary tuberculosis - United State. *MMWR Morb. Mortal Wkly Rep.* 71, 285–289. doi: 10.15585/mmwr.mm7108a1

Cerulus, B., New, A. M., Pougach, K., and Verstrepen, K. J. (2016). Noise and epigenetic inheritance of single-cell division times influence population fitness. *Curr. Biol.* 26, 1138–1147. doi: 10.1016/j.cub.2016.03.010

Chakraborty, P., Bajeli, S., Kaushal, D., Radotra, B. D. , and Kumar, A. (2021). Biofilm formation in the lung contributes to virulence and drug tolerance of *Mycobacterium tuberculosis*. *Nat. Commun.* 12, 1606. doi: 10.1038/s41467-021-217486

Chakraborty, P., and Kumar, A. (2019). The extracellular matrix of mycobacterial biofilms: Could we shorten the treatment of mycobacterial infections? *Microb. Cell* 6, 105–122. doi: 10.15698/mic2019.02.667

Chengalroyen, M. D., Beukes, G. M., Gordhan, B. G., Streicher, E. M., Churchyard, G., Hafner, R., et al. (2016). Detection and quantification of differentially culturable tubercle bacteria in sputum from patients with tuberculosis. *Am. J. Respir. Crit. Care Med.* 194, 1532–1540. doi: 10.1164/rccm.201604-0769OC

Chen, Y., Jiang, Q., Zou, J., Yang, T., Liu, Q., Luo, G., et al. (2021). Deep whole-genome sequencing reveals no evidence for heteroresistance influencing treatment outcomes among drug-susceptible tuberculosis patients. *Tuberc. (Edinb)* 130, 102120. doi: 10.1016/j.tube.2021.102120

Chesov, E., Chesov, D., Maurer, F. P., Andres, S., Utpatel, C., Barilar, I., et al. (2022). Emergence of bedaquiline resistance in a high tuberculosis burden country. *Eur. Respir. J.* 59, 2100621. doi: 10.1183/13993003.00621-2021

Coleman, M. T., Chen, R. Y., Lee, M., Lin, P. L., Dodd, L. E., Maiello, P., et al. (2014). PET/CT imaging reveals a therapeutic response to oxazolidinones in macaques and humans with tuberculosis. *Sci. Transl. Med.* 6, 265ra167. doi: 10.1126/scitranslmed.3009500

Collins, J. M., Jones, D. P., Sharma, A., Khadka, M., Liu, K. H., Kempker, R. R., et al. (2021). TCA cycle remodeling drives proinflammatory signaling in humans with pulmonary tuberculosis. *PloS Pathog.* 17, e1009941. doi: 10.1371/journal.ppat.1009941

Colwell, R. R., Brayton, P. R., Grimes, D. J., Roszak, D. B., Huq, S. A., and Palmer, L. M. (1985). Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: Implications for release of genetically engineered microorganisms. *Nat. Biotechnol.* 3, 817–820. doi: 10.1038/nbt0985-817

Comas, I., Borrell, S., Roetzer, A., Rose, G., Malla, B., Kato-Maeda, M., et al. (2011). Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. *Nat. Genet.* 44, 106–110. doi: 10.1038/ng.1038

Connell, T. G., Zar, H. J., and Nicol, M. P. (2011). Advances in the diagnosis of pulmonary tuberculosis in HIV-infected and HIV-uninfected children. *J. Infect. Dis.* 204 Suppl 4, S1151–S1158. doi: 10.1093/infdis/jir413

Daniel, J., Deb, C., Dubey, V. S., Sirakova, T. D., Abomoelak, B., Morbidoni, H. R., et al. (2004). Induction of a novel class of diacylglycerol acyltransferases and triacylglycerol accumulation in *Mycobacterium tuberculosis* as it goes into a dormancy-like state in culture. *J. Bacteriol.* 186, 5017–5030. doi: 10.1128/JB.186.15.5017-5030.2004

Dartois, V. A., and Rubin, E. J. (2022). Anti-tuberculosis treatment strategies and drug development: Challenges and priorities. *Nat. Rev. Microbiol*, 1–17. doi: 10.1038/s41579-022-00731-y

Deb, C., Lee, C. M., Dubey, V. S., Daniel, J., Abomoelak, B., Sirakova, T. D., et al. (2009). A novel *in vitro* multiple-stress dormancy model for *Mycobacterium tuberculosis* generates a lipid-loaded, drug-tolerant, dormant pathogen. *PloS One* 4, e6077. doi: 10.1371/journal.pone.0006077

Dhar, N., McKinney, J., and Manina, G. (2016). Phenotypic heterogeneity in *Mycobacterium tuberculosis*. *Microbiol. Spectr.* 4 (6), 10. doi: 10.1128/microbiolspec.TBTB2-0021-2016

Dheda, K., Gumbo, T., Maartens, G., Dooley, K. E., Murray, M., Furin, J., et al. (2019). The lancet respiratory medicine commission: 2019 update: Epidemiology, pathogenesis, transmission, diagnosis, and management of multidrug-resistant and incurable tuberculosis. *Lancet Respir. Med.* 7, 820–826. doi: 10.1016/S2213-2600(19)30263-2

Dheda, K., Limberis, J. D., Pietersen, E., Phelan, J., Esmail, A., Lesosky, M., et al. (2017). Outcomes, infectiousness, and transmission dynamics of patients with extensively drug-resistant tuberculosis and home-discharged patients with programmatically incurable tuberculosis: A prospective cohort study. *Lancet Respir. Med.* 5, 269–281. doi: 10.1016/S2213-2600(16)30433-7

Dheda, K., Perumal, T., Moultrie, H., Perumal, R., Esmail, A., Scott, A. J., et al. (2022). The intersecting pandemics of tuberculosis and COVID-19: Population-level and patient-level impact, clinical presentation, and corrective interventions. *Lancet Respir. Med* 10, 603–622. doi: 10.1016/S2213-2600(22)00092-3

Dhillon, J., Fourie, P. B., and Mitchison, D. A. (2014). Persister populations of *Mycobacterium tuberculosis* in sputum that grow in liquid but not on solid culture media. *J. Antimicrob. Chemother.* 69, 437–440. doi: 10.1093/jac/dkt357

DiAndreth, L., Jarrett, B. A., Elf, J. L., Nishath, T., Donville, B., Heidari, O., et al. (2020). Secure delivery of HIV-related and tuberculosis laboratory results to patient cell phones: A pilot comparative study. *AIDS Behav.* 24, 3511–3521. doi: 10.1007/s10461-020-02912-3

Dinkele, R., Gessner, S., McKerry, A., Leonard, B., Leukes, J., Seldon, R., et al. (2022). Aerosolization of *Mycobacterium tuberculosis* by tidal breathing. *Am. J. Respir. Crit. Care Med.* 206, 206–216. doi: 10.1164/rccm.202110-2378OC

Dinkele, R., Gessner, S., McKerry, A., Leonard, B., Seldon, R., Koch, A. S., et al. (2021). Capture and visualization of live *Mycobacterium tuberculosis* bacilli from tuberculosis patient bioaerosols. *PloS Pathog.* 17, e1009262. doi: 10.1371/journal.ppat.1009262

Dona, V., Rodrigue, S., Dainese, E., Palu, G., Gaudreau, L., Manganelli, R., et al. (2008). Evidence of complex transcriptional, translational, and posttranslational regulation of the extracytoplasmic function sigma factor σ^E in *Mycobacterium tuberculosis*. *J. Bacteriol.* 190, 5963–5971. doi: 10.1128/JB.00622-08

Dookie, N., Khan, A., Padayatchi, N., and Naidoo, K. (2022). Application of next generation sequencing for diagnosis and clinical management of drug-resistant tuberculosis: Updates on recent developments in the field. *Front. Microbiol.* 13, 775030. doi: 10.3389/fmicb.2022.775030

Dowdy, D. W., Grant, A. D., Dheda, K., Nardell, E., Fielding, K., and Moore, J. A. J. (2017). Designing and evaluating interventions to halt the transmission of tuberculosis. *J. Infect. Dis.* 216, S654–S661. doi: 10.1093/infdis/jix320

Dupuy, P., Ghosh, S., Adefisayo, O., Buglino, J., Shuman, S. , and Glickman, M. S. (2022). Distinctive roles of translesion polymerases DinB1 and DnaE2 in diversification of the mycobacterial genome through substitution and frameshift mutagenesis. *Nat. Commun.* 13, 4493. doi: 10.1038/s41467-022-32022-8

Dusthackeer, A., Balasubramanian, M., Shanmugam, G., Priya, S., Nirmal, C. R., Sam Ebenezer, R., et al. (2019). Differential culturability of *Mycobacterium tuberculosis* in culture-negative sputum of patients with pulmonary tuberculosis and in a simulated model of dormancy. *Front. Microbiol.* 10,2381. doi: 10.3389/fmicb.2019.02381

Ealand, C., Peters, J., Jacobs, O., Sewcharran, A., Ghoor, A., Golub, J., et al. (2021). Detection of mycobacterium tuberculosis complex bacilli and nucleic acids from tongue swabs in young, hospitalized children. *Front. Cell Infect. Microbiol.* 11, 696379. doi: 10.3389/fcimb.2021.696379

Esmail, H., Dodd, P. J., and Houben, R. M. G. J. (2018). Tuberculosis transmission during the subclinical period: Could unrelated cough play a part? *Lancet Respir. Med.* 6, 244–246. doi: 10.1016/S2213-2600(18)30105-X

Faksri, K., Kaewprasert, O., Ong, R. T., Suriyaphol, P., Prammananan, T., Teo, Y. Y., et al. (2019). Comparisons of whole-genome sequencing and phenotypic drug susceptibility testing for *Mycobacterium tuberculosis* causing MDR-TB and XDR-TB in Thailand. *Int. J. Antimicrob. Agents* 54, 109–116. doi: 10.1016/j.ijantimicag.2019.04.004

Feuerriegel, S., Kohl, T. A., Utpatel, C., Andres, S., Maurer, F. P., Heyckendorf, J., et al. (2021). Rapid genomic first- and second-line drug resistance prediction from clinical *Mycobacterium tuberculosis* specimens using deeplex-MycTB. *Eur. Respir. J.* 57, 2001796. doi: 10.1183/13993003.01796-2020

Ford, C. B., Shah, R. R., Maeda, M. K., Gagneux, S., Murray, M. B., Cohen, T., et al. (2013). *Mycobacterium tuberculosis* mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nat. Genet.* 45, 784–790. doi: 10.1038/ng.2656

Fuchs, R. P., and Fujii, S. (2013). Translesion DNA synthesis and mutagenesis in prokaryotes. *Cold Spring Harb. Perspect. Biol.* 5, a012682. doi: 10.1101/cshperspect.a012682

Gabaldon-Figueira, J. C., Brew, J., Dore, D. H., Umashankar, N., Chaccour, J., Orrillo, V., et al. (2021). Digital acoustic surveillance for early detection of respiratory disease outbreaks in Spain: A protocol for an observational study. *BMJ Open* 11, e051278. doi: 10.1136/bmjopen-2021-051278

Ghosh, J., Larsson, P., Singh, B., Pettersson, B. M., Islam, N. M., Sarkar, S. N., et al. (2009). Sporulation in mycobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 106, 10781–10786. doi: 10.1073/pnas.0904104106

Gideon, H. P., Phuah, J., Myers, A. J., Bryson, B. D., Rodgers, M. A., Coleman, M. T., et al. (2015). Variability in tuberculosis granuloma T cell responses exists, but a balance of pro- and anti-inflammatory cytokines is associated with sterilization. *PloS Pathog.* 11, e1004603. doi: 10.1371/journal.ppat.1004603

Gold, B., and Nathan, C. (2017). Targeting phenotypically tolerant *Mycobacterium tuberculosis*. *Microbiol. Spectr.* 5 (1), 27. doi: 10.1128/microbiolspec.TBTB2-0031-2016

Gold, B., Warrier, T., and Nathan, C. (2015). A multi-stress model for high throughput screening against non-replicating *Mycobacterium tuberculosis*. *Methods Mol. Biol.* 1285, 293–315. doi: 10.1007/978-1-4939-2450-9_18

Gordhan, B. G., Peters, J. S., McIvor, A., Machowski, E. E., Ealand, C., Waja, Z., et al. (2021). Detection of differentially culturable tubercle bacteria in sputum using mycobacterial culture filtrates. *Sci. Rep.* 11, 6493. doi: 10.1038/s41598-021-86054-z

Gygli, S. M., Borrell, S., Trauner, A., and Gagneux, S. (2017). Antimicrobial resistance in mycobacterium tuberculosis: mechanistic and evolutionary perspectives. *FEMS Microbiol. Rev.* 41, 354–373. doi: 10.1093/femsre/fux011

Hashimoto, M., Nozoe, T., Nakaoka, H., Okura, R., Akiyoshi, S., Kaneko, K., et al. (2016). Noise-driven growth rate gain in clonal cellular populations. *Proc. Natl. Acad. Sci. U.S.A.* 113, 3251–3256. doi: 10.1073/pnas.1519412113

Hossain, A. D., Jarolimova, J., Elnaiem, A., Huang, C. X., Richterman, A., and Ivers, L. C. (2022). Effectiveness of contact tracing in the control of infectious diseases: a systematic review. *Lancet Public Health* 7, e259–e273. doi: 10.1016/S2468-2667(22)00001-9

Huemer, M., Mairpady Shambat, S., Brugger, S. D., and Zinkernagel, A. S. (2020). Antibiotic resistance and persistence-implications for human health and treatment perspectives. *EMBO Rep.* 21, e51034. doi: 10.15252/embr.202051034

Jeranji, T. (2021) *Western Cape to launch COVID-style tuberculosis dashboard* (SpotlightNSP). Available at: https://www.spotlightnsp.co.za/2021/05/20/western-cape-to-launch-covid-style-tuberculosis-dashboard/ (Accessed 2022-04-25).

Kana, B. D., Gordhan, B. G., Downing, K. J., Sung, N., Vostroktunova, G., Machowski, E. E., et al. (2008). The resuscitation-promoting factors of *Mycobacterium tuberculosis* are required for virulence and resuscitation from dormancy but are collectively dispensable for growth *in vitro*. *Mol. Microbiol.* 67, 672–684. doi: 10.1111/j.1365-2958.2007.06078.x

Kang, Y. A., Choi, N. K., Seong, J. M., Heo, E. Y., Koo, B. K., Hwang, S. S., et al. (2014). The effects of statin use on the development of tuberculosis among patients with diabetes mellitus. *Int. J. Tuberc. Lung Dis.* 18, 717–724. doi: 10.5588/ijtld.13.0854

Kang, Y. A., Kim, S. Y., Jo, K. W., Kim, H. J., Park, S. K., Kim, T. H., et al. (2013). Impact of diabetes on treatment outcomes and long-term survival in multidrug-resistant tuberculosis. *Respiration* 86, 472–478. doi: 10.1159/000348374

Kargarpour Kamakoli, M., Farmanfarmaei, G., Masoumi, M., Khanipour, S., Gharibzadeh, S., Sola, C., et al. (2020). Prediction of the hidden genotype of mixed infection strains in Iranian tuberculosis patients. *Int. J. Infect. Dis.* 95, 22–27. doi: 10.1016/j.ijid.2020.03.056

Kargarpour Kamakoli, M., Sadegh, H. R., Farmanfarmaei, G., Masoumi, M., Fateh, A., Javadi, G., et al. (2017). Evaluation of the impact of polyclonal infection and heteroresistance on treatment of tuberculosis patients. *Sci. Rep.* 7, 41410. doi: 10.1038/srep41410

Katale, B. Z., Mbelele, P. M., Lema, N. A., Campino, S., Mshana, S. E., Rweyemamu, M. M., et al. (2020). Whole genome sequencing of *Mycobacterium tuberculosis* isolates and clinical outcomes of patients treated for multidrug-resistant tuberculosis in Tanzania. *BMC Genomics* 21, 174. doi: 10.1186/s12864-020-6577-1

Kayigire, X. A., Friedrich, S. O., van der Merwe, L., Donald, P. R., and Diacon, A. H. (2015). Simultaneous staining of sputum smears for acid-fast and lipid-containing *Mycobacterium tuberculosis* can enhance the clinical evaluation of antituberculosis treatments. *Tuberc. (Edinb)* 95, 770–779. doi: 10.1016/j.tube.2015.08.001

Kendall, E. A., Shrestha, S., and Dowdy, D. W. (2021). The epidemiological importance of subclinical tuberculosis. a critical reappraisal. *Am. J. Respir. Crit. Care Med.* 203, 168–174. doi: 10.1164/rccm.202006-2394PP

Kieser, K. J., and Rubin, E. J. (2014). How sisters grow apart: Mycobacterial growth and division. *Nat. Rev. Microbiol.* 12, 550–562. doi: 10.1038/nrmicro3299

Kocagoz, T., Altin, S., Turkyilmaz, O., Tas, I., Karaduman, P., Bolaban, D., et al. (2012). Efficiency of the TK culture system in the diagnosis of tuberculosis. *Diagn. Microbiol. Infect. Dis.* 72, 350–357. doi: 10.1016/j.diagmicrobio.2011.12.004

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., and Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 130, 797–810. doi: 10.1016/j.cell.2007.06.049

Kumar, N. P., Fukutani, K. F., Shruthi, B. S., Alves, T., Silveira-Mattos, P. S., Rocha, M. S., et al. (2019). Persistent inflammation during anti-tuberculosis treatment with diabetes comorbidity. *eLife* 8, e46477. doi: 10.7554/eLife.46477

Kumar, M. K., Kumar, P., and Singh, A. (2015). Recent advances in the diagnosis and treatment of childhood tuberculosis. *J. Nat. Sci. Biol. Med.* 6, 314–320. doi: 10.4103/0976-9668.159988

LaCourse, S. M., Seko, E., Wood, R., Bundi, W., Ouma, G. S., Agaya, J., et al. (2022). Diagnostic performance of oral swabs for non-sputum based TB diagnosis in a TB/HIV endemic setting. *PloS One* 17, e0262123. doi: 10.1371/journal.pone.0262123

Lalli, M., Hamilton, M., Pretorius, C., Pedrazzoli, D., White, R. G., and Houben, R. (2018). Investigating the impact of TB case-detection strategies and the consequences of false positive diagnosis through mathematical modelling. *BMC Infect. Dis.* 18, 340. doi: 10.1186/s12879-018-3239-x

Leistikow, R. L., Morton, R. A., Bartek, I. L., Frimpong, I., Wagner, K., and Voskuil, M. I. (2010). The *Mycobacterium tuberculosis* DosR regulon assists in metabolic homeostasis and enables rapid recovery from nonrespiring dormancy. *J. Bacteriol.* 192, 1662–1670. doi: 10.1128/JB.00926-09

Lenaerts, A., Barry, C. E. 3rd, and Dartois, V. (2015). Heterogeneity in tuberculosis pathology, microenvironments and therapeutic responses. *Immunol. Rev.* 264, 288–307. doi: 10.1111/imr.12252

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., and Balaban, N. Q. (2017). Antibiotic tolerance facilitates the evolution of resistance. *Science* 355, 826–830. doi: 10.1126/science.aaj2191

Lin, P. L., Ford, C. B., Coleman, M. T., Myers, A. J., Gawande, R., Ioerger, T., et al. (2014). Sterilization of granulomas is common in active and latent tuberculosis despite within-host variability in bacterial killing. *Nat. Med.* 20, 75–79. doi: 10.1038/nm.3412

Liu, S., Sun, Y., Yang, R., Ren, W., Li, C., and Tang, S. (2021). Expression profiling of TRIM gene family reveals potential diagnostic biomarkers for rifampicin-resistant tuberculosis. *Microb. Pathog.* 157, 104916. doi: 10.1016/j.micpath.2021.104916

Liu, Y., Tan, S., Huang, L., Abramovitch, R. B., Rohde, K. H., Zimmerman, M. D., et al. (2016). Immune activation of the host cell induces drug tolerance in *Mycobacterium tuberculosis* both *in vitro* and *in vivo*. *J. Exp. Med.* 213, 809–825. doi: 10.1084/jem.20151248

Logsdon, M. M., and Aldridge, B. B. (2018). Stable regulation of cell cycle events in mycobacteria: Insights from inherently heterogeneous bacterial populations. *Front. Microbiol.* 9, 514. doi: 10.3389/fmicb.2018.00514

Lovey, A., Verma, S., Kaipilyawar, V., Ribeiro-Rodrigues, R., Husain, S., Palaci, M., et al. (2022). Early alveolar macrophage response and IL-1R-dependent T cell priming determine transmissibility of *Mycobacterium tuberculosis* strains. *Nat. Commun.* 13, 884. doi: 10.1038/s41467-022-28506-2

MacLean, E., Kohli, M., Weber, S. F., Suresh, A., Schumacher, S. G., Denkinger, C. M., et al. (2020). Advances in molecular diagnosis of tuberculosis. *J. Clin. Microbiol.* 58, e01582–01519. doi: 10.1128/JCM.01582-19

MacPherson, P., Webb, E. L., Variava, E., Lala, S. G., Milovanovic, M., Ratsela, A., et al. (2019). Intensified household contact tracing, prevention and treatment support versus enhanced standard of care for contacts of tuberculosis cases in South Africa: Study protocol for a household cluster-randomised trial. *BMC Infect. Dis.* 19, 839. doi: 10.1186/s12879-019-4502-5

Mahamed, D., Boulle, M., Ganga, Y., Mc Arthur, C., Skroch, S., Oom, L., et al. (2017). Intracellular growth of mycobacterium tuberculosis after macrophage cell death leads to serial killing of host cells. *eLife* 6, e22028. doi: 10.7554/eLife.22028

Manganelli, R. (2014). Sigma factors: Key molecules in *Mycobacterium tuberculosis* physiology and virulence. *Microbiol. Spectr.* 2, MGM2–0007-2013. doi: 10.1128/microbiolspec.MGM2-0007-2013

Manganelli, R., and Provvedi, R. (2010). An integrated regulatory network including two positive feedback loops to modulate the activity of σ^E in mycobacteria. *Mol. Microbiol.* 75, 538–542. doi: 10.1111/j.1365-2958.2009.07009.x

Manina, G., Dhar, N., and McKinney, J. D. (2015). Stress and host immunity amplify *Mycobacterium tuberculosis* phenotypic heterogeneity and induce nongrowing metabolically active forms. *Cell Host Microbe* 17, 32–46. doi: 10.1016/j.chom.2014.11.016

Marakalala, M. J., Raju, R. M., Sharma, K., Zhang, Y. J., Eugenin, E. A., Prideaux, B., et al. (2016). Inflammatory signaling in human tuberculosis granulomas is spatially organized. *Nat. Med.* 22, 531–538. doi: 10.1038/nm.4073

Mascarenhas, A. (2022) *Researchers call for real-time tuberculosis data like covid-19 dashboards*. Available at: https://indianexpress.com/article/cities/pune/researchers-call-for-real-time-tuberculosis-data-like-covid-19-dashboards-7852581/ (Accessed 2022-04-25).

McAulay, K., Saito, K., Warrier, T., Walsh, K. F., Mathurin, L. D., Royal-Mardi, G., et al. (2018). Differentially detectable *Mycobacterium tuberculosis* cells in sputum from treatment-naive subjects in Haiti and their proportionate increase after initiation of treatment. *mBio* 9, e02192–02118. doi: 10.1128/mBio.02192-18

McIvor, A., Gordhan, B. G., Waja, Z., Otwombe, K., Martinson, N. A., and Kana, B. D. (2021). Supplementation of sputum cultures with culture filtrate to detect tuberculosis in a cross-sectional study of HIV-infected individuals. *Tuberc. (Edinb)* 129, 102103. doi: 10.1016/j.tube.2021.102103

Mesman, A. W., Baek, S. H., Huang, C. C., Kim, Y. M., Cho, S. N., Ioerger, T. R., et al. (2021). Characterization of drug-resistant lipid-dependent differentially

detectable *Mycobacterium tuberculosis*. *J. Clin. Med.* 10, 3249. doi: 10.3390/jcm10153249

Moore, D. A., Mendoza, D., Gilman, R. H., Evans, C. A., Hollm Delgado, M. G., Guerra, J., et al. (2004). Microscopic observation drug susceptibility assay, a rapid, reliable diagnostic test for multidrug-resistant tuberculosis suitable for use in resource-poor settings. *J. Clin. Microbiol.* 42, 4432–4437. doi: 10.1128/JCM.42.10.4432-4437.2004

Moschovis, P. P., Sampayo, E. M., Cook, A., Doros, G., Parry, B. A., Lombay, J., et al. (2021). The diagnosis of respiratory disease in children using a phone-based cough and symptom analysis algorithm: The smartphone recordings of cough sounds 2 (SMARTCOUGH-c 2) trial design. *Contemp. Clin. Trials* 101, 106278. doi: 10.1016/j.cct.2021.106278

Mukamolova, G. V., Kaprelyants, A. S., Young, D. I., Young, M., and Kell, D. B. (1998). A bacterial cytokine. *Proc. Natl. Acad. Sci. U.S.A.* 95, 8916–8921. doi: 10.1073/pnas.95.15.8916

Mukamolova, G. V., Turapov, O., Malkin, J., Woltmann, G., and Barer, M. R. (2010). Resuscitation-promoting factors reveal an occult population of tubercle bacilli in sputum. *Am. J. Respir. Crit. Care Med.* 181, 174–180. doi: 10.1164/rccm.200905-0661OC

Mukamolova, G. V., Turapov, O. A., Young, D. I., Kaprelyants, A. S., Kell, D. B., and Young, M. (2002). A family of autocrine growth factors in *Mycobacterium tuberculosis*. *Mol. Microbiol.* 46, 623–635. doi: 10.1046/j.1365-2958.2002.03184.x

Nair, P., Hasan, T., Zaw, K. K., Allamuratova, S., Ismailov, A., Mendonca, P., et al. (2022). Acquired bedaquiline resistance in Karakalpakstan, Uzbekistan. *Int. J. Tuberc. Lung Dis.* 26, 658–663. doi: 10.5588/ijtld.21.0631

Nandakumar, M., Nathan, C. , and Rhee, K. Y. (2014). Isocitrate lyase mediates broad antibiotic tolerance in *Mycobacterium tuberculosis*. *Nat. Commun.* 5, 4306. doi: 10.1038/ncomms5306

Nathan, C. (2020). Resisting antimicrobial resistance. *Nat. Rev. Microbiol.* 18, 259–260. doi: 10.1038/s41579-020-0348-5

National Institute of Allergy and Infectious Diseases, Eunice Kennedy Shriver National Institute of Child Health and Human Development and Otsuka Pharmaceutical Development and Commercialization Inc (2021). *Protecting households on exposure to newly diagnosed index multidrug-resistant tuberculosis patients (PHOENIx MDR-TB). identification no. NCT03568383*. Bethesda:National Library of Medicine.

Ocana-Guzman, R., Tellez-Navarrete, N. A., Preciado-Garcia, M., Ponce-Gallegos, M. A., Buendia-Roldan, I. , Falfan-Valencia, R., et al. (2021a). Multidrug-resistant tuberculosis patients expressing the HLA-DRB1*04 allele, and after treatment they show a low frequency of HLA-II+ monocytes and a chronic systemic inflammation. *Microb. Pathog.* 153, 104793. doi: 10.1016/j.micpath.2021.104793

Ocana-Guzman, R., Tellez-Navarrete, N. A., Ramon-Luing, L. A., Herrera, I., De Ita, M., Carrillo-Alduenda, J. L, et al (2021b). Leukocytes from patients with drug-sensitive and multidrug-resistant tuberculosis exhibit distinctive profiles of chemokine receptor expression and migration capacity*J. Immunol. Res.* 2021, 6654220. doi: 10.1155/2021/6654220

Ojha, A. K., Baughn, A. D., Sambandan, D., Hsu, T., Trivelli, X., Guerardel, Y., et al. (2008). Growth of *Mycobacterium tuberculosis* biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. *Mol. Microbiol.* 69, 164–174. doi: 10.1111/j.1365-2958.2008.06274.x

Paget, M. S. (2015). Bacterial sigma factors and anti-sigma factors: Structure, function and distribution. *Biomolecules* 5, 1245–1265. doi: 10.3390/biom5031245

Pahar, M., Klopper, M., Reeve, B., Warren, R., Theron, G., and Niesler, T. (2021). Automatic cough classification for tuberculosis screening in a real-world environment. *Physiol. Meas* 42, 105014. doi: 10.1088/1361-6579/ac2fb8

Pai, N., Esmail, A., Saha Chaudhuri, P., Oelofse, S., Pretorius, M., Marathe, G., et al. (2021). Impact of a personalised, digital, HIV self-testing app-based program on linkages and new infections in the township populations of south Africa. *BMJ Glob. Health* 6, e006032. doi: 10.1136/bmjgh-2021-006032

Pai, M., Kalantri, S., Pascopella, L., Riley, L. W., and Reingold, A. L. (2005). Bacteriophage-based assays for the rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*: A meta-analysis. *J. Infect.* 51, 175–187. doi: 10.1016/j.jinf.2005.05.017

Parihar, S. P., Guler, R., Khutlang, R., Lang, D. M., Hurdayal, R., Mhlanga, M. M., et al. (2014). Statin therapy reduces the *Mycobacterium tuberculosis* burden in human macrophages and in mice by enhancing autophagy and phagosome maturation. *J. Infect. Dis.* 209, 754–763. doi: 10.1093/infdis/jit550

Peters, J. S., Andrews, J. R., Hatherill, M., Hermans, S., Martinez, L., Schurr, E., et al. (2019). Advances in the understanding of *Mycobacterium tuberculosis* transmission in HIV-endemic settings. *Lancet Infect. Dis.* 19, e65–e76. doi: 10.1016/S1473-3099(18)30477-8

Pisu, D., Provvedi, R., Espinosa, D. M., Payan, J. B., Boldrin, F., Palu, G., et al. (2017). The alternative sigma factors SigE and SigB are involved in tolerance and persistence to antitubercular drugs. *Antimicrob. Agents Chemother.* 61, e01596–01517. doi: 10.1128/AAC.01596-17

Prideaux, B., Via, L. E., Zimmerman, M. D., Eum, S., Sarathy, J., O'Brien, P., et al. (2015). The association between sterilizing activity and drug distribution into tuberculosis lesions. *Nat. Med.* 21, 1223–1227. doi: 10.1038/nm.3937

Ray, J. C., Tabor, J. J., and Igoshin, O. A. (2011). Non-transcriptional regulatory processes shape transcriptional network dynamics. *Nat. Rev. Microbiol.* 9, 817–828. doi: 10.1038/nrmicro2667

Rego, E. H., Audette, R. E., and Rubin, E. J. (2017). Deletion of a mycobacterial divisome factor collapses single-cell phenotypic heterogeneity. *Nature* 546, 153–157. doi: 10.1038/nature22361

Richards, J. P., Cai, W., Zill, N. A., Zhang, W., and Ojha, A. K. (2019). Adaptation of *Mycobacterium tuberculosis* to biofilm growth is genetically linked to drug tolerance. *Antimicrob. Agents Chemother.* 63, e01213–01219. doi: 10.1128/AAC.01213-19

Rodel, H. E., Ferreira, I., Ziegler, C. G. K., Ganga, Y., Bernstein, M., Hwa, S. H., et al. (2021). Aggregated *Mycobacterium tuberculosis* enhances the inflammatory response. *Front. Microbiol.* 12. doi: 10.3389/fmicb.2021.757134

Rodrigue, S., Provvedi, R., Jacques, P. E., Gaudreau, L., and Manganelli, R. (2006). The σfactors of *Mycobacterium tuberculosis*. *FEMS Microbiol. Rev.* 30, 926–941. doi: 10.1111/j.1574-6976.2006.00040.x

Ruesen, C., Chaidir, L., Ugarte-Gil, C., van Ingen, J., Critchley, J. A., Hill, P. C., et al. (2020). Diabetes is associated with genotypically drug-resistant tuberculosis. *Eur. Respir. J.* 55, 1901891. doi: 10.1183/13993003.01891-2019

Ruhl, C. R., Pasko, B. L., Khan, H. S., Kindt, L. M., Stamm, C. E., Franco, L. H., et al. (2020). *Mycobacterium tuberculosis* sulfolipid-1 activates nociceptive neurons and induces cough. *Cell* 181, 293-305.e211. doi: 10.1016/j.cell.2020.02.026

Ruhwald, M., Carmona, S., and Pai, M. (2021). Learning from COVID-19 to reimagine tuberculosis diagnosis. *Lancet Microbe* 2, e169–e170. doi: 10.1016/S2666-5247(21)00057-4

Rupani, M. P., Shah, C. J., Dave, J. D., Trivedi, A. V., and Mehta, K. G. (2021). 'We are not aware of notification of tuberculosis': A mixed-methods study among private practitioners from western India. *Int. J. Health Plann. Manage* 36, 1052–1068. doi: 10.1002/hpm.3151

Safi, H., Gopal, P., Lingaraju, S., Ma, S., Levine, C., Dartois, V., et al. (2019). Phase variation in *Mycobacterium tuberculosis glpK* produces transiently heritable drug tolerance. *Proc. Natl. Acad. Sci. U.S.A.* 116, 19665–19674. doi: 10.1073/pnas.1907631116

Saito, K., Warrier, T., Somersan-Karakaya, S., Kaminski, L., Mi, J., Jiang, X., et al. (2017). Rifamycin action on RNA polymerase in antibiotic-tolerant *Mycobacterium tuberculosis* results in differentially detectable populations. *Proc. Natl. Acad. Sci. U.S.A.* 114, E4832–E4840. doi: 10.1073/pnas.1705385114

Santi, I., Dhar, N., Bousbaine, D., Wakamoto, Y., and McKinney, J. D. (2013). Single-cell dynamics of the chromosome replication and cell division cycles in mycobacteria. *Nat. Commun.* 4,2470. doi: 10.1038/ncomms3470

Santi, I., and McKinney, J. D. (2015). Chromosome organization and replisome dynamics in *Mycobacterium smegmatis*. *mBio* 6, e01999–e01914. doi: 10.1128/mBio.01999-14

Sarathy, J. P., Via, L. E., Weiner, D., Blanc, L., Boshoff, H., Eugenin, E. A., et al. (2018). Extreme drug tolerance of *Mycobacterium tuberculosis* in caseum. *Antimicrob. Agents Chemother.* 62, e02266-02217. doi: 10.1128/AAC.02266-17

Sarathy, J. P., Zuccotto, F., Hsinpin, H., Sandberg, L., Via, L. E., Marriner, G. A., et al. (2016). Prediction of drug penetration in tuberculosis lesions. *ACS Infect. Dis.* 2, 552–563. doi: 10.1021/acsinfecdis.6b00051

Sebastian, J., Swaminath, S., Nair, R. R., Jakkala, K., Pradhan, A., and Ajitkumar, P. (2017). *De novo* emergence of genetically resistant mutants of *Mycobacterium tuberculosis* from the persistence phase cells formed against antituberculosis drugs *In vitro*. *Antimicrob. Agents Chemother.* 61, e01343–01316. doi: 10.1128/AAC.01343-16

Seddon, J. A., Garcia-Prats, A. J., Purchase, S. E., Osman, M., Demers, A. M., Hoddinott, G., et al. (2018). Levofloxacin versus placebo for the prevention of tuberculosis disease in child contacts of multidrug-resistant tuberculosis: study protocol for a phase III cluster randomised controlled trial (TB-CHAMP). *Trials* 19, 693. doi: 10.1186/s13063-018-3070-0

Shah, N. S., Auld, S. C., Brust, J. C., Mathema, B., Ismail, N., Moodley, P., et al. (2017). Transmission of extensively drug-resistant tuberculosis in South Africa. *N Engl. J. Med.* 376, 243–253. doi: 10.1056/NEJMoa1604544

Sheen, P., Rodriguez, J., Alcantara, R., Vargas, J., Grandjean, L., Moore, D. A. J., et al. (2022). Alternative cost-effective media to facilitate MODS culture for diagnostics of tuberculosis. *Tuberc. (Edinb)* 135, 102225. doi: 10.1016/j.tube.2022.102225

Shin, S. S., Modongo, C., Baik, Y., Allender, C., Lemmer, D., Colman, R. E., et al. (2018). Mixed *Mycobacterium tuberculosis*-strain infections are associated with poor treatment outcomes among patients with newly diagnosed tuberculosis,

independent of pretreatment heteroresistance. *J. Infect. Dis.* 218, 1974–1982. doi: 10.1093/infdis/jiy480

Shleeva, M., Goncharenko, A., Kudykina, Y., Young, D., Young, M., and Kaprelyants, A. (2013). Cyclic AMP-dependent resuscitation of dormant mycobacteria by exogenous free fatty acids. *PloS One* 8, e82914. doi: 10.1371/journal.pone.0082914

Shleeva, M. O., Kudykina, Y. K., Vostroknutova, G. N., Suzina, N. E., Mulyukin, A. L., and Kaprelyants, A. S. (2011). Dormant ovoid cells of *Mycobacterium tuberculosis* are formed in response to gradual external acidification. *Tuberc. (Edinb)* 91, 146–154. doi: 10.1016/j.tube.2010.12.006

Singhal, A., Jie, L., Kumar, P., Hong, G. S., Leow, M. K., Paleja, B., et al. (2014). Metformin as adjunct antituberculosis therapy. *Sci. Transl. Med.* 6, 263ra159. doi: 10.1126/scitranslmed.3009885

Singh, R., Barry, C. E.3rd, and Boshoff, H. I. (2010). The three RelE homologs of mycobacterium tuberculosis have individual, drug-specific effects on bacterial antibiotic tolerance. *J. Bacteriol.* 192, 1279–1291. doi: 10.1128/JB.01285-09

Skerry, C., Pinn, M. L., Bruiners, N., Pine, R., Gennaro, M. L., and Karakousis, P. C. (2014). Simvastatin increases the *in vivo* activity of the first-line tuberculosis regimen. *J. Antimicrob. Chemother.* 69, 2453–2457. doi: 10.1093/jac/dku166

Slayden, R. A., Dawson, C. C., and Cummings, J. E. (2018). Toxin-antitoxin systems and regulatory mechanisms in *Mycobacterium tuberculosis*. *Pathog. Dis.* 76. doi: 10.1093/femspd/fty039

Sloan, D. J., Mwandumba, H. C., Garton, N. J., Khoo, S. H., Butterworth, A. E., Allain, T. J., et al. (2015). Pharmacodynamic modeling of bacillary elimination rates and detection of bacterial lipid bodies in sputum to predict and understand outcomes in treatment of pulmonary tuberculosis. *Clin. Infect. Dis.* 61, 1–8. doi: 10.1093/cid/civ195

Sterling, T. R., Njie, G., Zenner, D., Cohn, D. L., Reves, R., Ahmed, A., et al. (2020). Guidelines for the treatment of latent tuberculosis infection: Recommendations from the national tuberculosis controllers association and CD. *MMWR Recomm. Rep.* 69, 1–11. doi: 10.15585/mmwr.rr6901a1

Sulaiman, J. E., and Lam, H. (2021). Evolution of bacterial tolerance under antibiotic treatment and its implications on the development of resistance. *Front. Microbiol.* 12, 617412. doi: 10.3389/fmicb.2021.617412

Su, H. W., Zhu, J. H., Li, H., Cai, R. J., Ealand, C., Wang, X., et al. (2016). The essential mycobacterial amidotransferase GatCAB is a modulator of specific translational fidelity. *Nat. Microbiol.* 1, 16147. doi: 10.1038/nmicrobiol.2016.147

Tasse, J., Dieppois, G., Peyrane, F., and Tesse, N. (2021). Improving the ability of antimicrobial susceptibility tests to predict clinical outcome accurately: Adding metabolic evasion to the equation. *Drug Discovery Today* 26, 2182–2189. doi: 10.1016/j.drudis.2021.05.018

Tellez-Navarrete, N. A., Ramon-Luing, L. A., Munoz-Torrico, M., Preciado-Garcia, M., Medina-Quero, K., Hernandez-Pando, R., et al. (2021). Anti-tuberculosis chemotherapy alters TNFR2 expression on CD4+ lymphocytes in both drug-sensitive and -resistant tuberculosis: however, only drug-resistant tuberculosis maintains a pro-inflammatory profile after a long time. *Mol. Med.* 27, 76. doi: 10.1186/s10020-021-00320-4

Tiwari, P., Arora, G., Singh, M., Kidwai, S., Narayan, O. P., and Singh, R. (2015). MazF ribonucleases promote *Mycobacterium tuberculosis* drug tolerance and virulence in guinea pigs. *Nat. Commun.* 6, 6059. doi: 10.1038/ncomms7059

Tiwari, A., Balazsi, G., Gennaro, M. L., and Igoshin, O. A. (2010). The interplay of multiple feedback loops with post-translational kinetics results in bistability of mycobacterial stress response. *Phys. Biol.* 7, 36005. doi: 10.1088/1478-3975/7/3/036005

Traag, B. A., Driks, A., Stragier, P., Bitter, W., Broussard, G., Hatfull, G., et al. (2010). Do mycobacteria produce endospores? *Proc. Natl. Acad. Sci. U.S.A.* 107, 878–881. doi: 10.1073/pnas.0911299107

Treatment Action Group (2021). *Tuberculosis research funding trends 2005 - 2020* (New York: Treatment Action Group).

Trivedi, A., Mavi, P. S., Bhatt, D., and Kumar, A. (2016). Thiol reductive stress induces cellulose-anchored biofilm formation in *Mycobacterium tuberculosis*. *Nat. Commun.* 7, 11392. doi: 10.1038/ncomms11392

Turapov, O., O'Connor, B. D., Sarybaeva, A. A., Williams, C., Patel, H., Kadyrov, A. S., et al. (2016). Phenotypically adapted *Mycobacterium tuberculosis* populations from sputum are tolerant to first-line drugs. *Antimicrob. Agents Chemother.* 60, 2476–2483. doi: 10.1128/AAC.01380-15

Turgeon, R. D., Barry, A. R., and Pearson, G. J. (2016). Familial hypercholesterolemia: Review of diagnosis, screening, and treatment. *Can. Fam Physician* 62, 32–37.

Tyagi, P., Pal, V. K., Agrawal, R., Singh, S., Srinivasan, S., and Singh, A. (2020). *Mycobacterium tuberculosis* reactivates HIV-1 *via* exosome-mediated resetting of cellular redox potential and bioenergetics. *mBio* 11, e03293–03219. doi: 10.1128/mBio.03293-19

Variava, E., and Martinson, N. (2018). Drug-resistant tuberculosis: the rise of the monos. *Lancet Infect. Dis.* 18, 705–706. doi: 10.1016/S1473-3099(18)30247-0

Vaubourgeix, J., Lin, G., Dhar, N., Chenouard, N., Jiang, X., Botella, H., et al. (2015). Stressed mycobacteria use the chaperone ClpB to sequester irreversibly oxidized proteins asymmetrically within and between cells. *Cell Host Microbe* 17, 178–190. doi: 10.1016/j.chom.2014.12.008

Vijay, S., Vinh, D. N., Hai, H. T., Ha, V. T. N., Dung, V. T. M., Dinh, T. D., et al. (2017). Influence of stress and antibiotic resistance on cell-length distribution in *Mycobacterium tuberculosis* clinical isolates. *Front. Microbiol.* 8, 2296. doi: 10.3389/fmicb.2017.02296

Warner, D. F., Ndwandwe, D. E., Abrahams, G. L., Kana, B. D., Machowski, E. E., Venclovas, C., et al. (2010). Essential roles for imuA'- and imuB-encoded accessory factors in DnaE2-dependent mutagenesis in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13093–13098. doi: 10.1073/pnas.1002614107

Williams, C. M., Abdulwhhab, M., Birring, S. S., De Kock, E., Garton, N. J., Townsend, E., et al. (2020). Exhaled *Mycobacterium tuberculosis* output and detection of subclinical disease by face-mask sampling: prospective observational studies. *Lancet Infect. Dis.* 20, 607–617. doi: 10.1016/S1473-3099(19)30707-8

Working Group on New TB Drugs (2022). Available at: https://www.newtbdrugs.org/pipeline/clinical (Accessed 2022-07-18).

World Health Organization (2020). *WHO consolidated guidelines on tuberculosis, module 4: Treatment - drug-resistant tuberculosis treatment* (Geneva: World Health Organization).

World Health Organization (2021a). *Global tuberculosis report 2021* (Geneva: World Health Organization).

World Health Organization (2021b). *WHO consolidated guidelines on tuberculosis. module 3: Diagnosis - rapid diagnostics for tuberculosis detection* (Geneva: World Health Organization).

Yuen, C. M., Amanullah, F., Dharmadhikari, A., Nardell, E. A., Seddon, J. A., Vasilyeva, I., et al. (2015). Turning off the tap: stopping tuberculosis transmission through active case-finding and prompt effective treatment. *Lancet* 386, 2334–2343. doi: 10.1016/S0140-6736(15)00322-0

Zainabadi, K., Walsh, K. F., Vilbrun, S. C., Mathurin, L. D., Lee, M. H., Saito, K., et al. (2021). Characterization of differentially detectable *Mycobacterium tuberculosis* in the sputum of subjects with drug-sensitive or drug-resistant tuberculosis before and after two months of therapy. *Antimicrob. Agents Chemother.* 65, e0060821. doi: 10.1128/AAC.00608-21

Zhu, J. H., Wang, B. W., Pan, M., Zeng, Y. N., Rego, H., and Javid, B. (2018). Rifampicin can induce antibiotic tolerance in mycobacteria *via* paradoxical changes in *rpoB* transcription. *Nat. Commun.* 9, 4218. doi: 10.1038/s41467-018-06667-3

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 29 September 2022
DOI 10.3389/fcimb.2022.938032

OPEN ACCESS

EDITED BY
Ben Gold,
Weill Cornell Medicine, United States

REVIEWED BY
Anil Ojha,
Wadsworth Center, United States
Ahmed Gaballah,
U1052 Centre de Recherche en Cancerologie de Lyon (INSERM), France

*CORRESPONDENCE
Amit Singh
asingh@iisc.ac.in
Karl Drlica
drlicaka@njms.rutgers.edu

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 06 May 2022
ACCEPTED 21 July 2022
PUBLISHED 29 September 2022

CITATION
Singh A, Zhao X and Drlica K (2022) Fluoroquinolone heteroresistance, antimicrobial tolerance, and lethality enhancement.
Front. Cell. Infect. Microbiol. 12:938032.
doi: 10.3389/fcimb.2022.938032

Fluoroquinolone heteroresistance, antimicrobial tolerance, and lethality enhancement

Amit Singh[1,2]*, Xilin Zhao[3,4] and Karl Drlica[3]*

[1]Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India, [2]Centre for Infectious Disease Research, Indian Institute of Science, Bangalore, India, [3]Public Health Research Institute and Department of Microbiology, Biochemistry and Molecular Genetics, New Jersey Medical School, Rutgers Biomedical and Health Sciences, Rutgers University, Newark, NJ, United States, [4]State Key Laboratory of Molecular Vaccinology and Molecular Diagnostics, School of Public Health, Xiamen University, Xiamen, China

With tuberculosis, the emergence of fluoroquinolone resistance erodes the ability of treatment to interrupt the progression of MDR-TB to XDR-TB. One way to reduce the emergence of resistance is to identify heteroresistant infections in which subpopulations of resistant mutants are likely to expand and make the infections fully resistant: treatment modification can be instituted to suppress mutant enrichment. Rapid DNA-based detection methods exploit the finding that fluoroquinolone-resistant substitutions occur largely in a few codons of DNA gyrase. A second approach for restricting the emergence of resistance involves understanding fluoroquinolone lethality through studies of antimicrobial tolerance, a condition in which bacteria fail to be killed even though their growth is blocked by lethal agents. Studies with *Escherichia coli* guide work with *Mycobacterium tuberculosis*. Lethal action, which is mechanistically distinct from blocking growth, is associated with a surge in respiration and reactive oxygen species (ROS). Mutations in carbohydrate metabolism that attenuate ROS accumulation create pan-tolerance to antimicrobials, disinfectants, and environmental stressors. These observations indicate the existence of a general death pathway with respect to stressors. *M. tuberculosis* displays a variation on the death pathway idea, as stress-induced ROS is generated by NADH-mediated reductive stress rather than by respiration. A third approach, which emerges from lethality studies, uses a small molecule, N-acetyl cysteine, to artificially increase respiration and additional ROS accumulation. That enhances moxifloxacin lethality with *M. tuberculosis* in culture, during infection of cultured macrophages, and with infection of mice. Addition of ROS stimulators to fluoroquinolone treatment of tuberculosis constitutes a new direction for suppressing the transition of MDR-TB to XDR-TB.

KEYWORDS

antimycobacterial, oxidative stress, fluoroquinolone, respiration, N-acetyl cysteine, redox biosensor, reductive stress, resistance

1 Introduction

1.1 Overview

Antimicrobial treatment of tuberculosis has led to widespread emergence of resistance, particularly to the two most effective first-line agents, rifampicin and isoniazid. The resulting disease, called multidrug-resistant tuberculosis (MDR-TB), increased in prevalence by over 20% annually between 2008 and 2016 (Lange et al., 2018). Treatment of MDR-TB requires many months with second-line agents (a fluoroquinolone and one of three injectable drugs such as kanamycin, amikacin, or capreomycin). MDR-TB that acquires resistance to second-line agents, including a fluoroquinolone, is termed extensively drug-resistant tuberculosis (XDR-TB), a disease that is exceptionally difficult to cure. Results from resistance surveys are concerning: by 2018 XDR-TB accounted for about 6% of MDR-TB cases (WHO, 2013; WHO, 2019), and the increase in XDR-TB cases was almost 10-fold between 2011 and 2018 (WHO, 2013; WHO, 2019). Finding ways to halt the progression from MDR-TB to XDR-TB is a major healthcare priority.

We are focusing on improving the effectiveness of fluoroquinolones, the most potent of the second-line drugs used against MDR-TB. Addition of a C-8 methoxy group to ciprofloxacin-like agents, as found with moxifloxacin and gatifloxacin, improves lethal action, especially against resistant mutants (Dong et al., 1998; Zhao et al., 1999). However, this structural change my be insufficient, since older, less potent quinolone derivatives are still widely used. Moreover, the fluoroquinolones are used extensively for many other infections – inadvertent pre-treatment of TB may contribute to the emergence of fluoroquinolone-resistant tuberculosis (Bernardo and Yew, 2009), especially since the quinolones generate resistant mutants (Malik et al., 2010; Malik et al., 2012a). Even when treatment is brief, pretreatment with fluoroquinolone is associated with the emergence of resistance (Ginsburg et al., 2003). Thus, the emergence of fluoroquinolone-resistant *M. tuberculosis* is likely to remain a problem until ways are developed to suppress it.

In the present review we consider three fluoroquinolone issues: heteroresistance, tolerance, and enhancement. Heteroresistant cultures contain significant subpopulations of resistant mutants but score as susceptible when tested phenotypically. Maintaining selective pressure can lead to fully resistant infections. Tolerant bacteria are not killed by antimicrobials. Studies of tolerance lead to the formulation of a stress-mediated death pathway that may be exploited. Finally, unique features of *M. tuberculosis* have led to a way to enhance moxifloxacin-mediated killing. These studies of fluoroquinolones are likely to be of broad interest, because some of the principles appear to apply to antimicrobials in general. For example, heteroresistance is a general property of bacteria, and the death pathway appears to be common to antibiotics, disinfectants, and environmental stress (Zeng et al., 2022). We begin by discussing two key characteristics of bacteria, resistance and tolerance.

1.2 Resistance and tolerance

Resistance occurs when an isolate has an MIC above an empirically determined breakpoint. Mechanistically, resistance is the inability of the drug to form an initial bacterial lesion and thereby the inability to block bacterial growth. In general, resistance can be caused by reduced drug uptake, drug degradation, increased efflux, or the inability of the antimicrobial to interact with its molecular target. For the quinolones, resistance arises from the failure to form drug-gyrase-DNA complexes that would otherwise rapidly block DNA replication (Drlica et al., 2019). Resistance forces the patient to rely on the immune system to clear infection. In the case of tuberculosis, immune-based clearance can be ineffective (Pawlowski et al., 2012), which makes resistance particularly problematic.

Antimicrobial tolerance is elevated bacterial survival during treatment with a lethal antibiotic in the absence of a decrease in bacteriostatic susceptibility (no increase in MIC) (Tuomanen et al., 1986). Many lines of evidence support the idea that resistance and tolerance are mechanistically distinct (reviewed in (Drlica and Zhao, 2021)). For fluoroquinolones, and likely most lethal stressors, killing arises in part from macromolecular destruction by reactive oxygen species (ROS). Tolerance appears to be specific interference with ROS-mediated effects. For example, an iron chelator and a radical scavenger reduce killing by fluoroquinolones with little or no effect on MIC (reviewed in (Drlica and Zhao, 2021)). In principle, tolerance is expected to make clearing infection difficult and contribute to tuberculosis relapse. Tolerance also contributes to elevated frequency of resistance (Levin-Reisman et al., 2017; Shee et al., 2022), probably by allowing bacterial numbers to remain high and by reducing the killing of resistant mutant subpopulations. We note that quinolones also kill bacteria by chromosome fragmentation – examples exist in which interference with ROS accumulation fails to block killing completely (Malik et al., 2007; Keren et al., 2013).

1.3 Mutant selection window

Detection of resistant mutants at various fluoroquinolone concentrations reveals that mutants are most readily recovered when concentration exceeds wild-type MIC, which exerts selective pressure, but below the MIC of the least susceptible mutant subpopulation, a value that suppresses the outgrowth of resistant mutants. The latter value is termed the mutant prevention concentration (MPC; see Figure 1); The concentration range between MIC and MPC is called the

mutant selection window, since mutant subpopulations are selectively amplified in that range. Validation of the selection window idea has focused on pathogens other than *M. tuberculosis* (Cui et al., 2006; Drlica and Zhao, 2007; Zhu et al., 2012; Ni et al., 2014; Zhang et al., 2014; Xiong et al., 2016), but MPC has been measured with *M. tuberculosis* cultures (Dong et al., 2000; Rodriguez et al., 2004) and in an animal model of tuberculosis (Almeida et al., 2007).

In principle, amplification of mutant subpopulations can be restricted by keeping relevant tissue concentrations above the MPC. However, that is difficult in practice due to the high drug concentrations required: they may have adverse effects on patients. Consequently, doses designed to cure disease tend to place drug concentrations inside the selection window, thereby selectively enriching resistant subpopulations with every treatment. Thus, designing dosing strategies to simply cure disease (Wald-Dickler et al., 2018) has a fundamental flaw with respect to the emergence of resistance.

When resistant subpopulations are detectable during infection, the overall pathogen population is heterogeneous; the infection is said to be heteroresistant. Below we discuss heteroresistance, which we consider to be an early stage in antimicrobial-mediated evolution to bacterial resistance.

2 Heteroresistance

2.1 Overview

For many bacterial pathogens, heteroresistant infections often respond favorably to antimicrobial treatment, largely because the dominant, susceptible portion of the population is controlled well enough for host defense systems to clear infection. In such situations, heteroresistance is a problem mainly for immunocompromised patients. However, a heteroresistant infection can evolve to full resistance. An example is seen with colistin resistance of *Enterobacter* (reviewed in (Band and Weiss, 2019)). An isolate was examined in which 1 to 10% of the population grew in the presence of 1000-times the colistin concentration normally used to block growth of susceptible cells. In the presence of the drug, the resistant subpopulation rapidly expanded. In this case, expansion was transient, probably reflecting induction of colistin-resistance genes. When mice were infected with the heteroresistant strain ($1/10^5$ bacterial cells tested resistant), the bacteria failed to respond to a colistin treatment that protected mice infected by a fully susceptible strain. Thus, massive enrichment can occur during drug exposure.

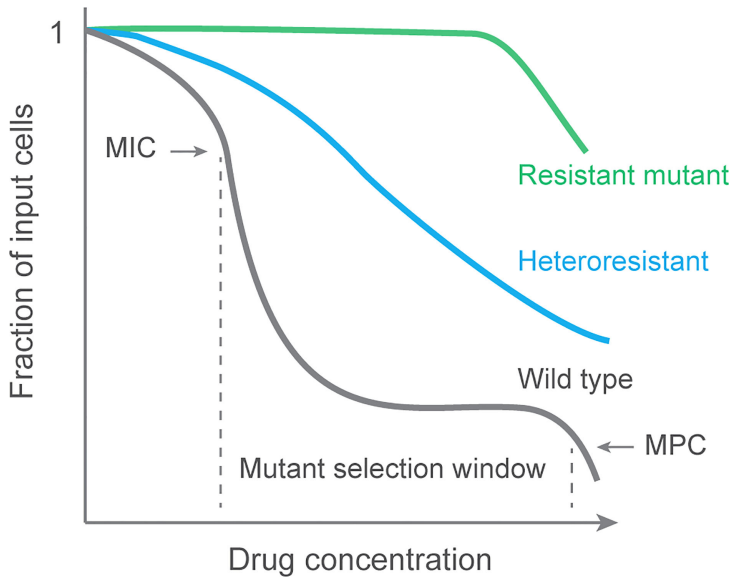


FIGURE 1
Population analysis profile and mutant selection window. Data are generated by applying a bacterial culture to a series of agar plates containing various concentrations of antimicrobial. After incubation to allow colony formation, colonies are counted, and the number is plotted for each drug concentration as a fraction of the input. Resistant cultures are unaffected by the drug until concentrations are very high. A fully susceptible culture (wild type) exhibits a sharp drop in colony recovery at MIC. A second sharp drop occurs at the MIC of the least susceptible mutant subpopulation (MPC). Selective enrichment of resistant mutants occurs at concentrations between MIC and MPC, a range called the mutant selection window (Zhao and Drlica, 2001). A population containing a mixture of susceptible and resistant subpopulations is called heteroresistant. Data for wild-type *M. tuberculosis* can be found in reference (Zhou et al., 2000).

Heteroresistance is observed with *M. tuberculosis* in both HIV-positive and HIV-negative patients (Zetola et al., 2014). Moreover, it is detected for many antimicrobials, including ethambutol, isoniazid, rifampicin, fluoroquinolones, streptomycin, pyrazinamide, and amikacin. Thus, heteroresistance is a general phenomenon with *M. tuberculosis*. It is also a general problem, because the disease is usually treated intensely for many months. During long incubations, resistant bacterial subpopulations tend to be enriched. Indeed, a quarter of MDR *M. tuberculosis* isolates can be heteroresistant to fluoroquinolones (Zhang et al., 2012; Eilertson et al., 2014). If phenotypic heteroresistance is greater than 1% by drug susceptibility testing, the infection is considered resistant (Canetti et al., 1963). Thus, with *M. tuberculosis*, heteroresistance is taken as a strong warning of future resistance. That makes rapid detection methods important.

2.2 Detection of heteroresistance: General considerations

For rapidly growing bacteria, heteroresistance is easily detected by observing colonies in the zone of inhibition created by a spot of antimicrobial on agar where a lawn of bacteria form (see example with E-test strips in (Pournaras et al., 2005)). If colonies inside the inhibition zone are positive for resistance using MIC-based tests, the overall population is considered heteroresistant. When those colonies continue to appear resistant after multiple rounds of growth using drug-free medium, heteroresistance is considered stable. However, many examples have been reported in which heteroresistance is lost during subculturing in drug-free medium. Such situations are termed unstable heteroresistance. The "colonies-within-the-inhibition-zone" test can be used by diagnostic laboratories to detect heteroresistance with samples that would otherwise be considered susceptible. Unfortunately, slow pathogen growth renders this method of little utility with *M. tuberculosis*.

The gold standard for demonstrating heteroresistance is finding "resistant" subpopulations in a population analysis profile (PAP (deLencastre et al., 1991); see Figure 1). A fully susceptible isolate will show a sharp decrease in colony number when drug concentration in agar reaches the MIC. Such is seen with laboratory isolates of *M. tuberculosis*: resistant colonies can be recovered, but they are rare (Zhou et al., 2000). A heteroresistant population is seen as a more gradual drop in colony recovery (Figure 1). Integration of heteroresistance data and normalization to a reference strain lacking detectible heteroresistance generates a single number to compare heteroresistance among pathogen samples.

Since performing a full population analysis is labor intensive, a variation is applied to *M. tuberculosis*. An infection is deemed resistant if the proportion of colonies that are resistant exceeds 1%. This phenotypic method can be very sensitive for mutant detection, but it has two drawbacks. First, incubation times are long due to the slow growth of *M. tuberculosis*. For example, detection of resistance to fluoroquinolones and second-line injectables by conventional methods (a two-step process) takes approximately 15–30 days. Second, subculturing from sputum samples can alter the size of the mutant subpopulation due to selective advantage or disadvantage (Metcalfe et al., 2017). Moreover, long incubation times can make the induction of resistance an important factor. For quinolones, colony number increases dramatically on agar plates over the course of days with rapidly growing bacteria (Malik et al., 2010) and over the course of several weeks with *M. tuberculosis* (Malik et al., 2012a). DNA-based detection methods have been developed to overcome these problems. For example, the time required to detect resistance by a commercial line-probe test (MTBDR*sl*) is 1–2 days (Ajbani et al., 2012). The key for detecting DNA-based fluoroquinolone resistance is knowing which nucleotide sequence changes cause resistance.

2.3 Detecting heteroresistance: Fluoroquinolone-resistance alleles

Most clinically relevant fluoroquinolone resistance derives from amino acid substitutions in the target protein, DNA gyrase (*M. tuberculosis* lacks the related enzyme, topoisomerase IV, which would otherwise contribute to resistance). The resistance alterations map in narrow regions of the two subunits of gyrase, GyrA and GyrB (these short regions have been termed quinolone-resistance-determining regions; QRDRs).

For GyrA, the QRDR, initially found with *E. coli* (Yoshida et al., 1990), comprises codons 90, 91, and 94 in *M. tuberculosis*. Changes at these positions probably interfere with the interaction of the carboxy end of the quinolone with the QRDR of GyrA (Aldred et al., 2014). Since the structure of this end of quinolones is common to the class, the GyrA substitutions are likely to confer resistance to all quinolones. However, different substitutions at a given codon confer different levels of protection, as indicated by different proportions of mutant recovery at different fluoroquinolone concentrations on agar plates (Zhou et al., 2000), from infected mice (Bernard et al., 2016), and among clinical isolates (Table 1).

Studies with *E. coli* reveal that an A67S substitution also reduces susceptibility (Malik et al., 2006), although the main effect of this allele is on lethal action. We speculated that this substitution weakens the GyrA-GyrA interface, thereby stimulating gyrase subunit dissociation and chromosome fragmentation (Malik et al., 2006). Since the corresponding allele (A74S in *M. tuberculosis*) can be detected with *M. smegmatis* and *M. tuberculosis* in "low-level resistant" mutants, the A to S substitution also likely affects bacteriostatic activity (Zhou et al., 2000). Indeed, introduction of the allele into a

TABLE 1 Examples of GyrA alleles associated with resistance *in vivo*.

G88C/A	D89N/G	A90V	S91P	D94H	D94A/Y/N	D94G	Ref
		24	6		11	42	(Ajbani et al., 2012)
		9			12	41	(Hillemann et al., 2009)
3		40	16	3	6	30	(Chakravorty et al., 2011)
		30			9	44	(Zhang et al., 2012)
	13	5	5	7	11	15	(Bernard et al., 2016)[a]

[a]Murine infection.
Percent of single alleles recovered from infections.

laboratory strain of *M. tuberculosis* increased MIC by 2- to 4-fold (Malik et al., 2012b). Moreover, examination of purified, recombinant A74S gyrase shows a decrease in sensitivity to ofloxacin and moxifloxacin (about 8- and 14-fold reduction, respectively (Lau et al., 2011)). Thus, including the A74S allele in DNA-based tests for heteroresistance may be appropriate.

Studies with *E. coli* also associate GyrB substitutions with resistance to some quinolones (Yoshida et al., 1990). With *M. tuberculosis*, 15% of resistant isolates contain point mutations in *gyrB* (85% map in *gyrA*). These *gyrB* alleles are likely responsible for resistance, since they reduce fluoroquinolone sensitivity when present in purified, recombinant gyrases (Aubry et al., 2006; Kim et al., 2011). To define the GyrB QRDR, 19 *gyrB* alleles were transduced into a laboratory strain of *M. tuberculosis* that was then examined for susceptibility to fluoroquinolones using the phenotypic proportion method to define resistance (Malik et al., 2012b). By this test, the QRDR is almost 90 codons long.

The protective activity of gyrase mutations observed *in vitro* does not always carry over to clinical resistance. For example, the *E. coli* GyrA G81C substitution is very protective (Mustaev et al., 2014), and the equivalent substitution in *M. tuberculosis* (G88C) is readily selected on drug-containing agar (Zhou et al., 2000). But the G88C allele is rarely recovered among clinical isolates (Chakravorty et al., 2011). Conversely, not every amino acid change seen in resistant cells reduces susceptibility. Indeed, some substitutions increase susceptibility (Aubry et al., 2006). Thus, fitness is likely to play a role in determining which alleles are relevant for DNA-based assays. Nevertheless, the correlation between gyrase alleles and resistance has been good enough to encourage the development of DNA-based assays that shorten assay time for MDR-TB to XDR-TB conversion from weeks to a day. These assays tend to focus on GyrA substitutions.

2.4 Assays for heteroresistance

2.4.1 Line probe assay

Knowledge of the GyrA QRDR allows specific amplification methods to produce DNA fragments that are characteristic of particular alleles. The fragments can then be separated by gel electrophoresis; heteroresistance is observed by the presence of both wild-type and mutant fragments (Rinder et al., 2001). Sensitivity is improved by reverse hybridization. Paper strips are prepared in which regions of wild-type DNA and mutant DNA are placed at specific spots. Regions of sample DNA are amplified by PCR, labeled, and hybridized to DNA on the strip. Resistance is scored by hybridization to mutant fragments and by the absence of the equivalent wild-type allele; heteroresistance produces a mixed result.

Commercial assay kits are available for performing line probe assays. One called MTBDR*sl* is designed to detect resistance to fluoroquinolones (GyrA alleles) and second-line injectable drugs in samples from MDR-TB cases (Hillemann et al., 2009). For fluoroquinolone resistance using *gyrA* alleles, the concordance between the phenotypic test and the positive MTBDR*sl* assay is 90% (Hillemann et al., 2009; Ajbani et al., 2012). Thus, a rapid test for fully resistant and fully susceptible cultures is in place even without including GyrB-mediated resistance.

Occasionally discordance is observed between drug-susceptibility and DNA-based tests: DNA assays indicate resistance, but only susceptibility is seen following bacterial outgrowth. This result is explained by heteroresistance, which is clear when specimens display both mutant and wild-type bands in line-probe assays (Hillemann et al., 2009). A fitness advantage would allow susceptible bacteria to dominate during the outgrowth needed for phenotypic drug susceptibility testing. That would make DNA methods more efficient at detecting resistant mutant subpopulations when applied to primary specimens. This increased efficiency might then allow the standard for resistance *via* drug susceptibility testing to be relaxed from 1% heteroresistance to perhaps 19% with DNA-based methods (Vargas et al., 2021).

Heteroresistance below the threshold, whether 1% or 19%, may not assure the emergence of resistance, but it would serve as an early warning and could affect treatment decisions. In this scenario, the reliability of a particular assay at low levels of heteroresistance is important. For example, PCR-based diagnostic tests have a specificity problem when mutant subpopulations are small, because templates from the dominant bacterial population can create false-positive signals due to mis-priming, mis-incorporation, and mis-hybridization (DNA polymerase error frequency limits sensitivity to 0.1 to 0.2%). Another challenge for PCR-based methods arises from

laboratory contamination by amplicons from previous assays. Cross-contamination using open-tube assays is estimated to be almost 4% (Warren et al., 2004; vanRie et al., 2005). Closed-tube methods would reduce laboratory contamination (Huang et al., 2011; Rice et al., 2012; Hu et al., 2014), but they require improvement in sensitivity for heteroresistant infections. Thus, interpretation of DNA-based test results is likely to depend on the method employed.

2.4.2 DNA sequence determination

When DNA samples are amplified by PCR and nucleotide sequences are determined for the regions of interest, results are obtained rapidly. Many laboratories have access to the Sanger sequencing method, making it a popular assay. However, sensitivity is a problem, since in some cases the mutant frequency needs to be above 50% for detection (Folkvardsen et al., 2013). In one example, Sanger sequencing reported only 3 samples as resistant of 9 scoring resistant by the proportion drug susceptibility method (Bernard et al., 2016).

Sensitivity is improved by performing the sequencing with very large numbers (millions) of parallel determinations (deep or next-generation sequencing). The general strategy uses reversible-terminator sequencing-by-synthesis technology to provide end-to-end sequencing and many short reads. In this method, genomic DNA is extracted from bacterial cells, enzymatically sheared into small fragments, and tagged with Illumina-specific DNA identifiers. These unique identifiers allow multiple DNA fragments to be sequenced at the same time. The short, tagged fragments of DNA are purified, samples are normalized to specific concentrations, pooled, and loaded into the sequencer. The data are then computationally analyzed. When the method is extended to the whole genome, sensitivity is below 5% for resistant mutants, perhaps as low as 0.2% (Nimmo et al., 2020). A disadvantage of deep sequencing is that data handling is cumbersome: bioinformatic improvements are needed for general utility (Operario et al., 2017).

2.4.3 Sloppy molecular beacons

Molecular beacons are oligonucleotides in which a probe sequence is situated between ends that are complementary and form base pairs. One end contains a fluorophore and the other a quencher. Hybridization of the probe with its target sequence destabilizes the base pairing of the ends, separating the quencher from the fluorophore. The probe-target interaction is seen as fluorescence. Sloppy molecular beacons have unusually long probe sequences that allow hybridization to long target regions that can have considerable mismatch. The mismatches lower the melting temperature of the probe-target interaction as an indicator of different gyrase alleles (Chakravorty et al., 2011).

In one iteration, the sloppy molecular beacon assay amplified the *M. tuberculosis gyrA* QRDR using asymmetrical PCR. Then probing was with two sloppy molecular beacons that spanned the entire QRDR. By testing DNA targets corresponding to all known QRDR mutations, the Alland laboratory (Chakravorty et al., 2011) found that one or both sloppy beacons produced a melting temperature shift of at least 3.6°C for each mutation. That shift is readily detectable. The assay also identifies mixtures of wild-type and mutant DNA, with QRDR mutants identified in heteroresistant samples containing as little as 10 to 20% mutant DNA. Since fluorophores emitting different wave lengths are available, a single assay tube can report the presence of specific mutations associated with distinct changes in melting temperature for each fluorophore.

2.4.4 Digital PCR

Studies in cancer biology are driving the development of DNA-based assays for heteroresistance. With digital PCR (Vogelstein and Kinsler, 1999), the sample is diluted into a set of wells in a multi-well plate so a given well has only a single molecule of DNA (only wild-type DNA is present in most wells). Amplification of DNA in the wells reveals either the presence or absence of mutant DNA. The fraction of wells scoring positive for mutant estimates the percent of the sample containing mutant DNA. The sensitivity of digital PCR is limited only by the number of wells tested.

Digital PCR has been used with *M. tuberculosis* by combining wild-type DNA with DNA carrying resistance alleles in *gyrA*, *katG, rpoB*, and *rrs*. This assay can reveal heteroresistance of 1 mutant to 1,000 wild-type cells (Pholwat et al., 2013). For such sensitivity with sputum, the samples must have more than 1,000 bacilli per ml (*M. tuberculosis* content, which varies among sputum samples, can exceed one million CFU (Yajko et al., 1995; Brindle et al., 2001; Diacon et al., 2007)).

2.4.5 SuperSelective primers

Another strategy, also from cancer diagnosis, employs SuperSelective primers for real-time PCR assays (Vargas et al., 2016). In this test, a DNA primer is synthesized in which one region, the anchor, hybridizes strongly to a portion of the target DNA being probed. The anchor is separated from a detector region, called the "foot", by a long stretch of nucleotides expected to mispair with the target, thereby forming a loop. The foot is designed to hybridize only with the mutant nucleotide sequence in the target. The resulting hybrid is then used to prime real-time PCR. The SuperSelective primer method detects multiple mutations in the same reaction tube by using fluorophores having different colors to discriminate among amplification products.

2.4.6 CRISPR

This bacterial process recognizes and destroys foreign nucleic acids. The recognition aspect is applied to mutant detection by transcribing DNA samples from the pathogen

and then incubating the transcripts with the Cas13a protein system plus a quenched, fluorescently labeled reporter RNA. When the target RNA is recognized by Cas13a, which is designed to occur only if the resistance mutation is present, collateral damage in the reporter RNA will occur, thereby eliminating quenching and generating a fluorescent signal. This method, called SHERLOCK (Gootenberg et al., 2017), has single-molecule sensitivity, similar to droplet digital PCR and quantitative PCR (qPCR). It also has point-of-care diagnostic features. The CRISPR system functions with *M. tuberculosis* (Rock et al., 2017).

2.4.7 iPLEX gold

In this method, single-nucleotide primer extension incorporates a nucleotide having a distinctive mass modification for identification by mass spectroscopy (Bouakaze et al., 2011). The method can detect multiple resistance alleles in the same reaction mixture. In one application, a reconstruction experiment reported one amikacin-resistant cell per 200 wild-type cells (Zhang et al., 2013).

2.4.8 Conclusions

Detection methods vary significantly in their ability to detect heteroresistance when the resistant allele is rare. They also differ in the ease of use: commercial kits are available for the line probe assays, while deep sequencing requires bioinformatics expertise. Still unknown is the clinical significance of low-level heteroresistance: not every mutant amplifies to full resistance in patients. One of the results of DNA-based assays is the realization that two general types of heteroresistance occur in tuberculosis.

2.5 Two forms of heteroresistance

2.5.1 Mixed infections

Heterogeneity can arise from co-infection with multiple, dissimilar infecting strains of *M. tuberculosis*. These mixed infections may be common when the spread of disease leads to super-infection. High levels of mixed infection indicate poor infection control (failure to isolate patients, control of hospital air flow, etc.). They tend to occur where tuberculosis and resistant disease are common.

Mixed infections have been identified using methods that reveal very different DNA fingerprints (IS*6110* RFLP or VNTR patterns) (Shamputa et al., 2004; Kargarpour Kamakoli et al., 2017). In a report from Tashkent, Uzbekistan (Hofmann-Thiel et al., 2009), sputum samples subjected to DNA analysis showed that five of seven heteroresistant isolates were composed of different strains. Three of these mixed infections were newly diagnosed in untreated patients; consequently, continuous antimicrobial pressure is not required to create mixed infections.

2.5.2 Clonal heteroresistance

Heteroresistance can also evolve along clonal lines (within-host heteroresistance). This phenomenon is common when super-infection is rare and treatment of individual patients is poor. In this scenario, intermittent drug exposure, due to suboptimal dosing and/or factors that affect compliance, allows cycles of bacterial population expansion followed by selective reduction. Spontaneous heterogeneity is expected, because the bacterial burden can be high: some tuberculosis patients harbor on the order of 10^9 bacilli (Canetti, 1965; Mitchison, 1984). Bacterial load is probably an important factor in the emergence of resistance, since an abnormally high mutation rate does not seem to be the cause (for cultured *M. tuberculosis*, mutation rate is similar to that of other bacteria (McGrath et al., 2014)).

The complex dynamics of clonal heteroresistance are illustrated by a South African study (Post et al., 2004). The study subjects suffered from MDR-TB that persisted despite treatment for more than a year. Since the community prevalence of MDR-TB was low (0.3% in new patients, 1.7% in previously treated patients), clonal heterogeneity was more likely than mixed infection. Indeed, examination of sputum samples from 13 HIV-negative MDR-TB patients, taken at two-week intervals, showed that all contained *M. tuberculosis* having a single IS*6110* RFLP type and spoligotype pattern: superinfection was not observed.

Nucleotide sequence analysis for several genes showed that resistance patterns for infections changed during the course of sampling. For example, one patient was tested for mutations in *gyrA, embB*, and *katG* over 56 weeks of therapy. At the start of sampling, the three genes were wild type, while at weeks 4 and 6, the *katG* marker was resistant. It later returned to wild type. The *embB* marker became resistant by week 6 and remained resistant throughout the observation period. The *gyrA* gene showed a mixture of alleles at week 6; in later samples, transient changes occurred among several *gyrA* resistance forms, often mixed with wild-type alleles. After 48 weeks, *gyrA* was a mixture of resistant and wild-type alleles. By week 52, a different *gyrA* allele (D94G) became dominant. Isolates from two other patients also contained different alleles of drug-resistance genes. These marker fluctuations illustrate the dynamic and varied nature of clonal heteroresistance with *M. tuberculosis*.

The heteroresistance detected in sputum samples arises in part from independent clonal evolution in distinct regions of the lung. When surgical samples of lung were examined from 3 patients following long-term therapy, DNA IS*6110* fingerprints were identical for *M. tuberculosis* from different lung regions: the isolates within individual patients appeared to be clonally related (Kaplan et al., 2003). In one patient, a streptomycin-resistant strain was found in an open lesion, but wild-type cells were seen in a closed granuloma. Wild-type cells were also recovered from sputum. A second patient carried bacteria with two different *gyrA* resistance alleles when obtained from open lesions, while wild-type *gyrA* was recovered from two closed lesions. A third

patient harbored three types of *M. tuberculosis*: 1) bacteria from apparently normal lung tissue had wild-type genes *for embB, katG*, and *rrs*, 2) cells from sputum and four pathological sites had *embB* and *katG* resistance markers but wild-type *rrs*, and 3) bacteria from another pathological site exhibited resistance for all three genes. These findings, plus similar observations in another study (Vadwai et al., 2011) and in autopsies (Lieberman et al., 2016), lead to the idea that resistance evolution occurs independently in different lung compartments and that wild-type cells can survive treatment (they may be tolerant; see discussion of tolerance below). The results of sputum analyses probably reflect granulomas from different regions opening and releasing bacteria at different times.

The complex evolution of resistance alleles arising in different lung compartments suggests that analysis of multiple sputum samples may be necessary to accurately assess the diversity of bacterial populations in an infection. Survival of wild-type cells is particularly worrisome if those cells are genetically tolerant. As indicated by *E. coli* studies, such cells would not be killed by any antimicrobial.

3 Antimicrobial tolerance

Antimicrobial tolerance is the ability of a bacterium to survive lethal treatment without exhibiting an increase in MIC, a measure of susceptibility to antimicrobial-mediated growth inhibition. Knowledge of how antimicrobials kill bacteria is expected to lead to methods for measuring the prevalence of tolerance (MIC-based assays are uninformative). That knowledge should also lead to strategies for restricting the selection of tolerant mutants. A key idea, based in part on quinolone studies, is that lethal stress elicits a general stress response in which ROS accumulates and damages macromolecules (reviewed in (Drlica and Zhao, 2021)). Below we outline studies with *E. coli* to provide a framework, and then we address work with *M. tuberculosis* that expands the framework.

3.1 The *E. coli* ROS paradigm

In 2007 the Collins laboratory reported that three diverse antimicrobials stimulate the accumulation of ROS in *E. coli* (Kohanski et al., 2007). ROS are thought to be byproducts of respiration, and indeed lethal doses of fluoroquinolone do stimulate a burst of respiration (Dwyer et al., 2014). Subsequent work solidified the conclusion that severe stress elicits a cellular response that is self-destructive: genes that are protective at low stress levels can become destructive at high ones (Wu et al., 2011; Dorsey-Oresto et al., 2013). We have suggested that repair of topoisomerase-DNA lesions (e.g. double-stranded DNA breaks), which is a large energy-consuming process when observed in eukaryotic cells (Hoeijmakers, 2009), stimulates increased respiration (Dahan-Grobgeld et al., 1998; Lobritz et al., 2015; Brace et al., 2016; Hong et al., 2019; Drlica and Zhao, 2021). Elevated respiration generates superoxide and subsequently hydrogen peroxide. In the presence of iron, Fenton chemistry converts hydrogen peroxide to hydroxyl radical, which damages many molecule types and oxidizes deoxynucleotides that subsequently lead to lethal, incomplete base-excision repair (Takahashi et al., 2017; Gruber and Walker, 2018; Gruber et al., 2022). That ROS cause death rather than being caused by death is indicated by the observation that ROS-mediated death continues even after removal of the primary stressor (Hong et al., 2019). Additional support for causality comes from mutations in protective genes, such as *katG* (catalase), increasing ROS-mediated death (Wang and Zhao, 2009; Dwyer et al., 2014; Luan et al., 2018).

Many aspects of Collins' early work were challenged (Keren et al., 2013; Liu and Imlay, 2013; Imlay, 2015) as summarized in (Drlica and Zhao, 2021), which led us to seek a clear demonstration of a lethal stress response without using an experimental approach that relies on perturbing levels of ROS. We expected that the existence of a general lethal stress response would be revealed by the enrichment and characterization of anti-death mutants that were concurrently tolerant to many stressor types. Since by definition tolerance has no effect on MIC (Tuomanen et al., 1986), obtaining tolerant mutants required that we challenge bacterial cultures with an agent for which resistance (increased MIC) is selected rarely, if at all (recovery of resistant mutants would obscure the presence of tolerant mutants). After multiple rounds of screening with phenol, tolerant mutants were recovered (Zeng et al., 2022). These spontaneous, anti-death mutants of *E. coli* survived treatment by bactericidal agents that included antibiotics, disinfectants, and environmental stressors. As required, these mutants retained their bacteriostatic susceptibility (unchanged MIC) to the agents. The pan-tolerance (anti-death) phenotype demonstrated the existence of a death pathway common to many, if not all lethal stressors.

Characterization of the mutants revealed genes involved in carbohydrate metabolism (Zeng et al., 2022). In particular, mutations were found in *ptsI* (phosphotransferase) and *cyaA* (cAMP), thereby defining a novel activity of these genes as upstream regulators of the stress-mediated death pathway. The anti-death effect was reversed by genetic complementation, exogenous cAMP, or a Crp variant that bypasses cAMP binding for activation. Moreover, mutations in the same genes were obtained when screening was performed using multiple challenges with antimicrobials rather than with phenol. Downstream events that were blocked by the mutations included a metabolic shift from the TCA cycle to glycolysis and the pentose phosphate pathway, suppression of stress-mediated ATP surges, and reduced accumulation of ROS.

Thus, the tolerance genes showed that upstream signals from diverse stress-mediated lesions stimulate shared, late-stage, ROS-mediated events that damage macromolecules. Cultures of these stable, pan-tolerant mutants grew normally and were therefore distinct from tolerance derived from growth defects (described below).

Phenol, alcohol, and chlorhexidine are commonly used disinfectants. Thus, pan-tolerance leads to the idea that massive, unrestricted disinfectant use could contribute to antibiotic tolerance and eventually resistance. The recent surge in disinfectant use due to the COVID-19 pandemic may provide retrospective evidence that disinfectant consumption contributes to tolerance and ultimately resistance. Particularly insidious is the possibility that pan-tolerance weakens host defenses – the lethal activity of three agents used by the immune system (hypochlorite, hydrogen peroxide, and low pH) is reduced by pan-tolerance. Since tolerance can arise as single-gene mutations, it may be more common than we realize: tolerance could threaten the widespread use of disinfectants.

A second type of tolerance derives from decreased metabolism as described in (Brauner et al., 2016). For example, it is well known that metabolic downshift, such as entering stationary phase of culture growth, interferes with quinolone lethality (Gutierrez et al., 2017). A variety of genes whose products interfere with growth, such as toxin-antitoxin pairs (HipBA, VapBC), tRNA synthetases (MetG), metabolic enzymes (PrsA, GlpD), and many other gene products that extend the lag before exponential growth following release from stationary phase (Fridman et al., 2014), have been associated with this type of tolerance. These genes are said to be part of a tolerome (Brauner et al., 2016). This down-shift tolerance is important, as it has been associated with serious clinical consequences for treatment of blood infections involving *S. aureus* (Liu et al., 2020). A unifying idea is that the basis of growth-defect tolerance is suppression of ROS accumulation by the associated metabolic downshift.

3.2 The *M. tuberculosis* ROS paradigm

As a first approximation, fluoroquinolones kill mycobacteria much as seen with *E. coli* and other bacteria: killing is rapid, it is partially blocked by the protein synthesis inhibitor chloramphenicol, and it is affected by the C-7 fluoroquinolone substituent (Dong et al., 1998; Malik and Drlica, 2006). As with *E. coli*, we expect ROS to play a central role in fluoroquinolone lethality with *M. tuberculosis*. Indeed, moxifloxacin increases ROS with cultured *M. tuberculosis*, as detected by an oxidation-sensitive fluorescent dye and by a redox-sensitive biosensor (Shee et al., 2022). Involvement of hydrogen peroxide is supported by a moxifloxacin-mediated increase in peroxide and the suppression of lethality by adding catalase to the growth medium (hydrogen peroxide diffuses between the cell interior and exterior, making it vulnerable to exogenous catalase-mediated degradation). Moreover, agents that interfere with ROS accumulation (bipyridyl and thiourea) lower ROS and increase survival (Shee et al., 2022). Moxifloxacin also increases the expression of genes involved in the oxidative stress response, iron-sulfur cluster biogenesis, and DNA repair (Shee et al., 2022). In addition, the idea that fluoroquinolones have two ways to kill *M. tuberculosis* is supported by ROS appearing to act at low drug concentration but killing continuing at high concentration independent of ROS (Shee et al., 2022), as observed with *E. coli* (Keren et al., 2013). Thus, in many ways fluoroquinolone action in *M. tuberculosis* is similar to that reported for *E. coli*.

Surprisingly, and in contrast with *E. coli* work, moxifloxacin suppresses oxygen consumption in *M. tuberculosis* and decreases expression of *M. tuberculosis* genes involved in respiration and carbon catabolism. Thus, the two bacterial species differ in the source of ROS and therefore in the early steps of the death pathway.

In *M. tuberculosis*, ROS accumulation derives from reductive stress, a phenomenon in which diminished respiration leads to the accumulation of NADH (Mavi et al., 2020). Dissipation of the NADH overload by overexpression of *Lactobacillus brevis* NADH oxidase reduces the ROS surge, diminishes free iron accumulation, and protects *M. tuberculosis* from moxifloxacin-mediated killing (Shee et al., 2022). These data fit with the known ability of NADH to mobilize bound iron and maintain iron in a reduced state (Jaeschke et al., 1992), both of which can drive the generation of hydroxyl radical *via* Fenton chemistry (Vilcheze et al., 2013). Thus, moxifloxacin-induced, ROS-mediated killing of *M. tuberculosis* appears to depend on elevated levels of NADH and iron rather than elevated respiration (Figure 2). Nevertheless, if respiration could be artificially increased during moxifloxacin treatment, that increase might raise ROS levels even higher and increase moxifloxacin lethality.

4 Lethality enhancement

Fluoroquinolones kill cells by two processes: stimulation of ROS accumulation and chromosome fragmentation. The relative contribution of the two processes to killing likely depends on quinolone structure (Malik et al., 2007), DNA repair, and quinolone concentration (Malik et al., 2007). Structural considerations favor the use of moxifloxacin (Malik and Drlica, 2006) and are not discussed further. Since ROS-mediated killing is more pronounced at low fluoroquinolone concentration, which is generally kept low to minimize adverse effects, ROS-based strategies are important. Most of our discussion of lethality enhancement with *M. tuberculosis* focuses on increasing respiration when the normal response to fluoroquinolone (moxifloxacin) exposure is to decrease it (Shee

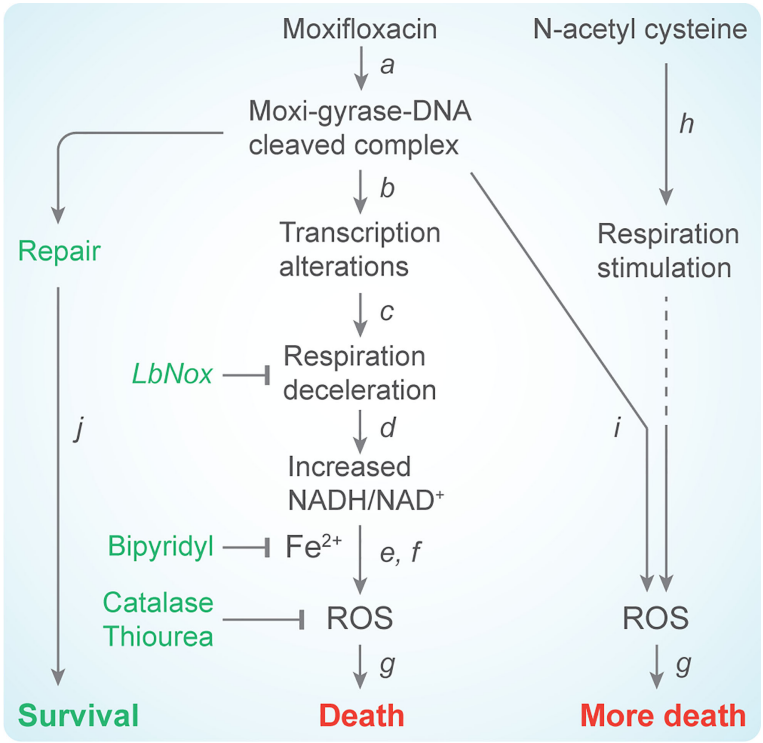


FIGURE 2
Scheme describing moxifloxacin-mediated killing of *M. tuberculosis* enhanced by NAC. **(a)** Moxifloxacin enters *M. tuberculosis* and traps gyrase on DNA as bacteriostatic drug-enzyme-DNA complexes in which the DNA is broken. This step is reversible. **(b)** The bacterium responds by down-regulating expression of genes involved in respiration. **(c)** The transcriptional changes result in reduced rate of respiration. **(d)** NADH levels and the ratio of NADH to NAD^+ increase; over-expression of *LbNox*, an NADH oxidase, interferes with downstream events. **(e)** NADH increases the free Fe^{2+} pool by releasing Fe from ferritin-bound forms and keeps it in a reduced state. Bipyridyl, an Fe chelator, blocks downstream events. **(f)** Elevated Fe^{2+} promotes the Fenton reaction and production of hydroxyl radical. Thiourea, a radical scavenger, blocks downstream events. **(g)** ROS damage macromolecules and cause death in a self-amplifying process, as indicated by exogenous catalase blocking the killing when added after removal of moxifloxacin. **(h)** Addition of N-acetyl cysteine to cells stimulates respiration and **(i)** provides more ROS from moxifloxacin-mediated lesions. NAC alone does not induce ROS or trigger death. The additional ROS increase killing by moxifloxacin. **(j)** Repair of moxifloxacin-mediated lesions, NADH dissipation, Fe sequestration, and ROS detoxification mechanisms contribute to survival.

et al., 2022). Then we briefly mention suppression of repair as a way to enhance killing of *M. smegmatis* and potentially *M. tuberculosis*.

4.1 Cysteine reduces drug tolerance in *M. tuberculosis*

A small-molecule enhancer emerged from studies of cysteine (Vilchèze and Jacobs, 2021). When this amino acid is administered to cultured *E. coli*, its oxidation to cystine by transition metals, such as copper and iron, can mediate the production of ROS (Park and Imlay, 2003). Cysteine is also rapidly converted to cystine in *M. tuberculosis* (Vilchèze et al., 2017). As expected, the combination of cysteine and isoniazid plus rifampicin leads to cation-dependent oxidative stress and DNA damage (Vilchèze et al., 2017). The result, using cysteine at 4 mM, was a drop in *M. tuberculosis* culture density from 10^7 CFU/ml to 0-10 CFU/ml (Vilchèze et al., 2017). In the absence of cysteine, isoniazid plus rifampicin lowered culture density by only 3 logs, and resistant bacteria emerged 7 days after treatment initiation. The effect of cysteine was not observed under anaerobic conditions or upon treatment with the iron chelator deferoxamine (Vilchèze et al., 2017), which together suggest an ROS-based phenomenon. Although cysteine fails to perturb the $NADH/NAD^+$ balance expected from elevated H_2O_2 concentrations (Vilchèze et al., 2017), it does raise respiration, apparently by transiently shifting the ratio of bacterial menaquinol-9 (MKH2) to menaquinone-9 (MK) towards MKH2 (Vilchèze et al., 2017). That shift would prevent the entry of the bacillus into a stress-mediated, quasi dormant state that would otherwise reduce the effects of lethal stressors.

In support of the MKH2:MK hypothesis, we recently found that drug tolerance exhibited by intra-phagosomal *M.*

tuberculosis depends partly on cysteine disposal mechanisms, such as Fe-S cluster biogenesis, the trans-sulfuration pathway, and mycothiol biosynthesis (Mishra et al., 2019; Mishra et al., 2021). Disruption of these processes reduces survival by ~ 9-fold upon treatment with a combination of isoniazid and rifampicin at 3X MIC (Mishra et al., 2019; Mishra et al., 2021). Cysteine alone only modestly reduces dissolved O_2 in culture media containing *M. tuberculosis* (from ~ 75% to 60% in 300 seconds) while its combination with isoniazid more dramatically lowers dissolved O_2 (from 80% to 40% in 300 seconds) (Vilchèze et al., 2017). Isoniazid alone does not affect dissolved O_2 concentration in culture media (Vilchèze et al., 2017). These observations suggest that an endogenous increase in cysteine, either from inefficient fluxing or from exogenous supplementation, accelerates respiration and induces redox imbalance, thereby increasing the lethality of anti-TB drugs that act by elevating ROS. Unfortunately, cysteine, even at low, micromolar concentrations, is cytotoxic for macrophages (Vilchèze et al., 2017). However a related thiol, N-acetyl cysteine (NAC), is well tolerated by macrophages (Vilchèze et al., 2017) and patients (Nagrale et al., 2013).

4.2 N-acetyl cysteine during tuberculosis

Although the stimulation of lethal activity by NAC parallels that observed with cysteine, the effect on respiration is quantitatively distinct. Addition of NAC results in only a small, 0.95- to 1.25-fold increase in oxygen consumption as compared to a 4- to 5-fold increase with cysteine (Vilchèze and Jacobs, 2021). Moreover, an *in vivo* labelling study with mice indicates that NAC uptake and deacetylation may not be adequate to maintain the cellular pool of cysteine and the downstream production of glutathione, an antioxidant that would protect from stress-mediated lethality. Instead of being an active source of cysteine, NAC is readily desulfurated to produce hydrogen sulfide (H_2S) (Ezeriņa et al., 2018). H_2S is a signaling molecule known to increase oxygen consumption in *M. tuberculosis* by activating the energy-inefficient cytochrome BD oxidase mode of respiration (Kunota et al., 2021). It is possible that differences in the way by which cysteine (increased MKH2/MKH ratio) and NAC (cytochrome BD oxidase) stimulate respiration have distinct effects on the kinetics of oxygen consumption and thereby on the stimulation of killing associated with antimicrobial treatment of *M. tuberculosis*.

NAC also exerts host anti-mycobacterial properties by 1) increasing the production of the cytokines interleukin-2 (IL-2), interleukin-12 (IL-12), and interferon-gamma (IFN-γ), 2) decreasing other interleukins (IL-10, IL-1, IL-6) and tumor necrosis factor alpha (TNF-α), and 3) elevating host glutathione and S-nitrosoglutathione levels (Venketaraman et al., 2006; Guerra et al., 2011; Teskey et al., 2018; Cao et al., 2018). The net effect is improved immunological activities of natural killer cells and macrophages (Morris et al., 2013; Allen et al., 2015). Thus, it is not surprising that NAC displays beneficial effects in animal models of experimental tuberculosis (Palanisamy et al., 2011; Amaral et al., 2016), although significant host-species differences are seen. For example, with infected mice, NAC alone significantly reduces the bacterial load in lungs after seven days of treatment (Amaral et al., 2016), but in a guinea pig model, even 60 days of NAC treatment fails to reduce the lung bacillary load despite a decrease in lesion burden and extent of necrosis (Palanisamy et al., 2011). However, NAC does reduce the bacterial load in guinea pig spleens after 30 days of treatment (Palanisamy et al., 2011). NAC appears to delay the dissemination of *M. tuberculosis* to the spleen, perhaps due to a protective effect of NAC on lung vasculature and reduction of lesion necrosis (both lesion necrosis and loss of vascular integrity are important for extra-pulmonary dissemination of *M. tuberculosis*) (Palanisamy et al., 2011).

Clinical effects of NAC have been encouraging. For example, in a double-blind, randomized trial with 67 therapy-naïve TB patients, NAC, when combined with first-line anti-TB therapy (ATT), increased smear conversion from 58% to almost 96% after three weeks of treatment (Mahakalkar et al., 2017). Radiological improvement of the infected lung was evident in the NAC + ATT group (Mahakalkar et al., 2017). In another example, NAC reduced hepatotoxicity, which occurs in ~25% of patients with uncomplicated TB (hepatotoxicity can affect therapy adherence (Possuelo et al., 2008; Baniasadi et al., 2010)). Since HIV infection is one of the predisposing factors for hepatotoxicity, NAC is being tested for safety when combined with first-line ATT in patients coinfected with HIV and *M. tuberculosis*. In one study, the safety profile of the combination was similar to that of ATT alone (Safe et al., 2020). These results have encouraged an ongoing cohort study (TB-SEQUEL; ClinicalTrials.gov Identifier: NCT03702738) using a higher dose of NAC (1200 mg rather than 600 mg) to evaluate safety and smear conversion in patients with TB and TB-HIV. Since NAC appears to be useful during treatment of TB, we examined the effects of NAC on moxifloxacin-mediated killing of *M. tuberculosis*.

4.3 NAC stimulates moxifloxacin-mediated killing with cultured *M. tuberculosis*

NAC alone induces a rapid increase in oxygen consumption rate (Vilchèze and Jacobs, 2021; Shee et al., 2022) that completely exhausts the reserve respiratory capacity of *M. tuberculosis* (Shee et al., 2022). Adding NAC to moxifloxacin treatment reverses the respiratory slowdown seen for moxifloxacin alone, as indicated

by increased oxygen consumption rate (Shee et al., 2022). Moreover, NAC-stimulated respiration enhances ROS accumulation more than seen with moxifloxacin alone. Indeed, supplementation of moxifloxacin at 1X and 5X MIC with 1 mM of NAC reduced bacterial survival by 21- and 11-fold, respectively (Shee et al., 2022). These observations are summarized schematically in Figure 2.

We emphasize that NAC concentrations that increase lethality have no effect on moxifloxacin MIC (Shee et al., 2022). Thus, the effect of NAC on moxifloxacin lethality is largely due to accelerated respiration and the associated ROS surge rather than a modification of the primary interaction between quinolone and DNA gyrase (cleaved-complex formation). This result strongly supports our contention that blocking growth and killing cells are mechanistically distinct. Nevertheless, NAC reduced MPC, a bacteriostatic parameter, by two-fold. Apparently killing mutant subpopulations is important in MPC determination (Cui et al., 2006).

4.4 NAC potentiates moxifloxacin efficacy in infected macrophages and mice

Since NAC augments host-cell glutathione biosynthesis and reduces host-generated ROS, it was unclear how NAC would affect killing of *M. tuberculosis* by moxifloxacin inside macrophages. Using an *M. tuberculosis* H37Rv strain that expresses the redox biosensor Mrx1-roGFP2 (strain *Mtb*-roGFP2), we found that moxifloxacin treatment of THP-1 macrophages, infected with *Mtb*-roGFP2, oxidizes the biosensor. Supplementation with non-toxic concentrations of NAC (1 mM to 2 mM) increased biosensor oxidation more than moxifloxacin alone, and a combination of moxifloxacin + NAC increased the level of oxidative stress by 2-fold beyond that observed for moxifloxacin alone (Shee et al., 2022). Most important, the moxifloxacin + NAC combination decreased the bacillary burden in macrophages 5-10 times more than moxifloxacin alone.

When we performed experiments with infected mice using a short moxifloxacin treatment (10 days), the moxifloxacin + NAC combination reduced bacterial burden by 4- and 12-fold more than moxifloxacin alone for lung and spleen, respectively. NAC alone had no effect on lung and spleen bacillary load (Shee et al., 2022).

Since fluoroquinolone-containing therapies are important for halting the transition of MDR-TB to XDR, we also examined the effect of NAC on the selection of moxifloxacin-resistant mutants in mice (Shee et al., 2022). We discovered that treatment with moxifloxacin alone increased the emergence of resistant strains of *M. tuberculosis*, as expected for induction of resistance by the quinolones (Malik et al., 2010; Malik et al., 2012a). NAC supplementation reduced the recovery of moxifloxacin-resistant mutants by 8-fold (Shee et al., 2022). Thus, NAC stimulates the lethal action of moxifloxacin and reduces the emergence of resistance *in vivo*.

4.5 NAC-mediated potentiation of lethal action with drug combinations

Several studies solidify the potential utility of NAC by showing that the compound, when added to first-line and several second-line anti-TB drug combinations, increases killing (Vilchèze et al., 2017; Vilchèze and Jacobs, 2021). For example, co-administration of NAC with inhibitors of the electron transport chain, such as bedaquiline, clofazimine, and Q203, kills cultured *M. tuberculosis* by 2 $\log_{10}$ more than bedaquiline or clofazimine or Q203 alone (Lamprecht et al., 2016). Since many antibiotics, such as isoniazid, rifampicin, and clofazimine, induce ROS in *M. tuberculosis* as part of their lethal action (Yano et al., 2011; Bhaskar et al., 2014; Piccaro et al., 2014; Tyagi et al., 2015; Nair et al., 2019), NAC likely increases respiration and the lethal action of drug combinations (Vilchèze et al., 2017; Vilchèze and Jacobs, 2021). These observations were counter-intuitive in the case of isoniazid, a prodrug that is oxidatively activated by catalase (KatG) and shows elevated activity when in combination with superoxide generators (Tyagi et al., 2015). As an antioxidant, NAC is expected to reduce the levels of free radicals such as superoxide and H_2O_2; thus, the mycobactericidal activity of isoniazid is anticipated to diminish when co-administered with NAC. Here, the explanation is that NAC is a poor scavenger of oxidants, such as H_2O_2 and superoxide, for which it has an extremely low rate constant (0.16 $M^{-1}s^{-1}$ [H_2O_2] and 68 $M^{-1}s^{-1}$ [superoxide], at pH 7.4 and 37°C) (Ezeriņa et al., 2018). Therefore, it is likely that enhancement of respiration and an associated increase in ROS upon treatment with NAC potentiate the antimycobacterial activity of anti-TB drugs.

NAC reduces treatment time: when combined with two first-line (isoniazid + rifampicin) or three second-line anti-TB drugs (ofloxacin + kanamycin + ethionamide or with moxifloxacin + amikacin + clofazimine), NAC reduced the time necessary to sterilize *M. tuberculosis* cultures treated with each of the combinations from 5-10 days to only 3-7 days (Vilchèze and Jacobs, 2021). Thus, NAC appears to be useful with combination therapies, as required for control of tuberculosis. Whether the contribution of the drugs in the combination therapies is additive has not been reported.

We noticed that the influence of NAC on moxifloxacin lethality differs from its effect on isoniazid and rifampicin when cells are cultured in synthetic medium. For example, with moxifloxacin the killing effect of NAC was evident at days 1 to 2 post-treatment (Shee et al., 2022): with isoniazid and rifampicin, lethality was seen only after 6 to 7 days post-treatment (Vilchèze et al., 2017).

4.6 ROS-mediated lethality as a kinetic phenomenon

Several lines of evidence indicate that ROS-mediated effects accelerate death without increasing the extent of killing. This phenomenon was first noticed with *S. aureus* where interference of ROS accumulation (treatment with bipyridyl plus thiourea) delayed killing by moxifloxacin (15 x MIC) for about 60 min and elevated survival by 20-fold after 120 min without an effect on minimal bactericidal concentration (MBC), a measurement involving a long incubation time (Liu et al., 2012). With *E. coli* and *M. tuberculosis*, perturbations of ROS affect the rate of killing after removal of the stressor but not the extent (Hong et al., 2019; Shee et al., 2022). The kinetic effects of ROS also fit with the increase in hydrogen peroxide being transient (Vilchèze et al., 2017) and with NAC stimulating killing of *M. tuberculosis* in mice at short incubation times (Shee et al., 2022) but not at a longer one (Vilchèze and Jacobs, 2021).

Acceleration of killing without an increase in extent has important implications for clinical application: the optimal dosing interval must be determined. If it is very short, *i.e.* frequent dosing is required, NAC might be of limited utility in resource-poor environments where patients cannot be repeatedly treated at short intervals.

4.7 Suppression of repair

A different form of enhancement is seen with the DNA repair pathway termed homologous repair-recombination. This system involves formation of Holliday junctions and the Ruv resolvase (Singh, 2017). We found that the absence of the *M. smegatis* Ruv resolvase increases the bacteriostatic and bactericidal activities of moxifloxacin. Treatment of *ruvAB*-deficient cells with thiourea and 2,2-bipyridyl lowers moxifloxacin killing to wild-type levels. Thus, the absence of *ruvAB* may stimulate a lethal pathway involving ROS. The hexapeptide WRWCR, which traps the Holliday junction substrate of RuvAB, potentiates moxifloxacin-mediated lethality by ten-fold (Long et al., 2015). This observation has yet to be exploited.

5 Concluding remarks

The fluoroquinolones are important agents for impeding the conversion of MDR-TB to XDR-TB. Human clinical studies indicate that the early bactericidal activity of moxifloxacin is similar to that of first-line anti-TB-drugs, such as isoniazid and rifampicin (Nuermberger et al., 2004; Pletz et al., 2004; Dorman et al., 2021). Moreover, a recent human clinical trial suggests that the efficacy of a four-month treatment with a combination of rifapentine and moxifloxacin was comparable to the standard six-month regimen of isoniazid, rifampicin, ethambutol, and pyramidazide (Dorman et al., 2021). However, the clinical situation is likely complex: moxifloxacin shows poor penetration into caseous regions of tubercular granulomas in a rabbit model of experimental tuberculosis (Prideaux et al., 2015; Sarathy et al., 2019). Low, local moxifloxacin concentrations may promote the emergence of fluoroquinolone resistance (Forsman et al., 2021).

General strategies have emerged for slowing the transition from MDR-TB to XDR-TB. Implementing rapid, DNA-based tests for fluoroquinolone heteroresistance will reveal the emergence of fluoroquinolone resistance before full resistance has been reached. That would enable introduction of treatment options. The most straightforward action is to advise patients of the danger, the importance of not missing doses. Another is to discontinue use of fluoroquinolone types that are only marginally effective anti-tuberculosis agents. A third is to alter the treatment protocol so that other anti-tuberculosis agents, such as rifapentine in combination with moxifloxacin, are introduced.

A second, general approach is to increase fluoroquinolone lethality to suppress the emergence of tolerance and the probability of relapse. Addition of NAC to moxifloxacin treatment is the most promising avenue, as it makes the drug more lethal and less likely to select fluoroquinolone-resistant mutants. The current problem with NAC is the kinetic nature of ROS-mediated killing, because an appropriate dosing interval is unknown. Whether that problem can be solved with derivatives of NAC is also unknown. One approach led to testing of N-acetylcysteine amide (NACA), a derivative of NAC having higher bioavailability (Vilchèze and Jacobs, 2021). This agent failed to improve the activity of drugs in *M. tuberculosis*-infected mice beyond that observed with NAC, and a more severe lung pathology was observed with isoniazid + rifampicin + NACA treatment when compared with isoniazid + rifampicin or isoniazid + rifampicin + NAC combinations (Vilchèze and Jacobs, 2021). Nevertheless, assays are now in place to explore other derivatives of NAC.

Many fluoroquinolone-related questions remain unanswered. For example, what is the prevalence of tolerance and is it a major cause of relapse? Measuring tolerance on a large scale is labor intensive (MIC plus kill curves); thus, it is not readily implemented by clinical laboratories. A bigger question that extends beyond tuberculosis is whether our massive use of disinfectants is applying sufficient selective pressure for widespread emergence of tolerance and subsequently even more resistance.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and each approved it for publication.

Funding

The authors' work has been supported by the Wellcome Trust/DBT India Alliance Grants, IA/S/16/2/502700 (AS); Department of Biotechnology (DBT) Grants (BT/PR13522/COE/34/27/2015, BT/PR29098/Med/29/1324/2018, BT/HRD/NBA/39/07/2018-19, and BT/PR39308/DRUG/134/86/2021 (AS); the Revati and Satya Nadham Atluri Chair Professorship (AS), and the National Natural Science Foundation of China (82172316, XZ).

Acknowledgments

We thank Bo Shopsin for critical comments on the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Ajbani, K., Nikam, C., Kazi, M., Gray, C., Boehme, C., Balan, K., et al. (2012). Evaluation of genotype MTBDRsl assay to detect drug resistance associated with fluoroquinolones, aminoglycosides and ethambutol on clinical sediments. *PloS One* 7 (11), e49433. doi: 10.1371/journal.pone.0049433

Aldred, K. J., Kerns, R. J., and Osheroff, N. (2014). Mechanism of quinolone action and resistance. *Biochemistry* 53 (10), 1565–1574. doi: 10.1021/bi5000564

Allen, M., Bailey, C., Cahatol, I., Dodge, L., Yim, J., Kassissa, C., et al. (2015). Mechanisms of control of *Mycobacterium tuberculosis* by NK cells: Role of glutathione. *Front. Immunol.* 6, 508. doi: 10.3389/fimmu.2015.00508

Almeida, D., Nuermberger, E., Tyagi, S., Bishai, W. R., and Grosset, J. (2007). *In vivo* validation of the mutant selection window hypothesis with moxifloxacin in a murine model of tuberculosis. *Antimicrob. Agents Chemother.* 51 (12), 4261–4266. doi: 10.1128/AAC.01123-07

Amaral, E. P., Conceição, E. L., Costa, D. L., Rocha, M. S., Marinho, J. M., Cordeiro-Santos, M., et al. (2016). N-acetyl-cysteine exhibits potent anti-mycobacterial activity in addition to its known anti-oxidative functions. *BMC Microbiol.* 16 (1), 251. doi: 10.1186/s12866-016-0872-7

Aubry, A., Veziris, N., Cambau, E., Truffot-Pernot, C., Jarlier, V., and Fisher, L. M. (2006). Novel gyrase mutations in quinolone-resistant and -hypersusceptible clinical isolates of *Mycobacterium tuberculosis*: functional analysis of mutant enzymes. *Antimicrob. Agents Chemother.* 50 (1), 104–112. doi: 10.1128/AAC.50.1.104-112.2006

Band, V. I., and Weiss, D. S. (2019). Heteroresistance: A cause of unexplained antibiotic treatment failure? *PloS Pathog.* 15 (6), e1007726. doi: 10.1371/journal.ppat.1007726

Baniasadi, S., Eftekhari, P., Tabarsi, P., Fahimi, F., Raoufy, M. R., Masjedi, M. R., et al. (2010). Protective effect of n-acetylcysteine on antituberculosis drug-induced hepatotoxicity. *Eur. J. Gastroenterol. Hepatol.* 22 (10), 1235–1238. doi: 10.1097/MEG.0b013e32833aa11b

Bernard, C., Aubry, A., Chauffour, A., Brossier, F., Robert, J., and Veziris, N. (2016). *In vivo mycobacterium tuberculosis* fluoroquinolone resistance emergence: a complex phenomenon poorly detected by current diagnostic tests. *J. Antimicrob. Chemother.* 71 (12), 3465–3472. doi: 10.1093/jac/dkw344

Bernardo, J., and Yew, W. W. (2009). How are we creating fluoroquinolone-resistant tuberculosis? *Am. J. Respir. Crit. Care Med.* 180 (4), 288–289. doi: 10.1164/rccm.200906-0863ED

Bhaskar, A., Chawla, M., Mehta, M., Parikh, P., Chandra, P., Bhave, D., et al. (2014). Reengineering redox sensitive GFP to measure mycothiol redox potential of *Mycobacterium tuberculosis* during infection. *PloS Pathog.* 10 (1), e1003902. doi: 10.1371/journal.ppat.1003902

Bouakaze, C., Keyser, C., Gonzalez, A., Sougakoff, W., Veziris, N., Dabernat, H., et al. (2011). Matrix-assisted laser desorption ionization-time of flight mass spectrometry-based single nucleotide polymorphism genotyping assay using iPLEX gold technology for identification of *Mycobacterium tuberculosis* complex species and lineages. *J. Clin. Microbiol.* 49, 3292–3299. doi: 10.1128/JCM.00744-11

Brace, L. E., Vose, S. C., Stanya, K., Gathungu, R. M., Marur, V. R., Longchamp, A., et al. (2016). Increased oxidative phosphorylation in response to acute and chronic DNA damage. *NPJ Aging Mech. Dis.* 2, 16022. doi: 10.1038/npjamd.2016.22

Brauner, A., Fridman, O., Gefen, O., and Balaban, N. Q. (2016). Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat. Rev. Microbiol.* 14 (5), 320–330. doi: 10.1038/nrmicro.2016.34

Brindle, R., Odhiambo, J., and Mitchison, D. (2001). Serial counts of *Mycobacterium tuberculosis* in sputum as surrogate markers of the sterilising activity of rifampicin and pyrazinamide in treating pulmonary tuberculosis. *BMC Pulm. Med.* 1, 2. doi: 10.1186/1471-2466-1-2

Canetti, G. (1965). The j. burns amberson lecture: present aspects of bacterial resistance in tuberculosis. *Am. Rev. Resp. Dis.* 92, 687–703.

Canetti, G., Froman, S., Grosset, J., Hauduroy, P., Langerová, M., Mahler, H., et al. (1963). Mycobacteria: laboratory methods for testing drug sensitivity and resistance. *Bull. World Health Organ* 29, 564–578.

Cao, R., Teskey, G., Islamoglu, H., Abrahem, R., Munjal, S., Gyurjian, K., et al. (2018). Characterizing the effects of glutathione as an immunoadjuvant in the treatment of tuberculosis. *Antimicrob. Agents Chemother.* 62 (11), e01132–18. doi: 10.1128/AAC.01132-18

Chakravorty, S., Aladegbami, B., Thoms, K., Lee, J., Lee, E., Rajan, V., et al. (2011). Rapid detection of fluoroquinolone-resistant and heteroresistant *Mycobacterium tuberculosis* by use of sloppy molecular beacons and dual melting-temperature codes in a real-time PCR assay. *J. Clin. Microbiol.* 49, 932–940. doi: 10.1128/JCM.02271-10

Cui, J., Liu, Y., Wang, R., Tong, W., Drlica, K., and Zhao, X. (2006). The mutant selection window in rabbits infected with staphylococcus aureus. *J. Infect. Dis.* 194 (11), 1601–1608. doi: 10.1086/508752

Dahan-Grobgeld, E., Livneh, Z., Maretzek, A. F., Polak-Charcon, S., Eichenbaum, Z., and Degani, H. (1998). Reversible induction of ATP synthesis by DNA damage and repair in escherichia coli. *In vivo* NMR studies. *J. Biol. Chem.* 273 (46), 30232–30238. doi: 10.1074/jbc.273.46.30232

deLencastre, H., SáFigueiredo, A., Urban, C., Rahal, J., and Tomasz, A. (1991). Multiple mechanisms of methicillin resistance and improved methods for detection in clinical isolates of staphylococcus aureus. *Antimicrob. Agents Chemother.* 35, 632–639. doi: 10.1128/AAC.35.4.632

Diacon, A., Patientia, R., Venter, A., vanHelden, P., Smith, P., McIlleron, H., et al. (2007). Early bactericidal activity of high-dose rifampin in patients with pulmonary tuberculosis evidenced by positive sputum smears. *Antimicrob. Agents Chemother.* 51, 2994–2996. doi: 10.1128/AAC.01474-06

Dong, Y., Xu, C., Zhao, X., Domagala, J., and Drlica, K. (1998). Fluoroquinolone action against mycobacteria: effects of c-8 substituents on growth, survival, and resistance. *Antimicrob. Agents Chemother.* 42 (11), 2978–2984. doi: 10.1128/AAC.42.11.2978

Dong, Y., Zhao, X., Kreiswirth, B. N., and Drlica, K. (2000). Mutant prevention concentration as a measure of antibiotic potency: studies with clinical isolates of

mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 44 (9), 2581–2584. doi: 10.1128/AAC.44.9.2581-2584.2000

Dorman, S. E., Nahid, P., Kurbatova, E. V., Phillips, P. P. J., Bryant, K., Dooley, K. E., et al. (2021). Four-month rifapentine regimens with or without moxifloxacin for tuberculosis. *New Engl. J. Med.* 384 (18), 1705–1718. doi: 10.1056/NEJMoa2033400

Dorsey-Oresto, A., Lu, T., Mosel, M., Wang, X., Salz, T., Drlica, K., et al. (2013). YihE kinase is a central regulator of programmed cell death in bacteria. *Cell Rep.* 3 (2), 528–537. doi: 10.1016/j.celrep.2013.01.026

Drlica, K., and Zhao, X. (2007). Mutant selection window hypothesis updated. *Clin. Infect. Dis.* 44 (5), 681–688. doi: 10.1086/511642

Drlica, K., and Zhao, X. (2021). Bacterial death from treatment with fluoroquinolones and other lethal stressors. *Expert Rev. Anti Infect. Ther.* 19 (5), 601–618. doi: 10.1080/14787210.2021.1840353

Drlica, K., Zhao, X., Malik, M., Hiasa, H., Mustaev, A., and Kerns, R. J. (2019). "Fluoroquinolone resistance," in *Bacterial resistance to antibiotics: From molecules to man*. Eds. B. Bonev and E. W. B. N. Brown (Wiley Blackwell, United Kingdom) 125–161.

Dwyer, D. J., Belenky, P., Yang, J. H., MacDonald, I. C., Martell, J. D., Takahashi, N., et al. (2014). Antibiotics induce redox-related physiological alterations as part of their lethality. *Proc. Natl. Acad. Sci. U. S. A.* 111 (20), E2100–E2109. doi: 10.1073/pnas.1401876111

Eilertson, B., Maruri, F., Blackman, A., Herrera, M., Samuels, D. C., and Sterling, T. R. (2014). High proportion of heteroresistance in gyrA and gyrB in fluoroquinolone-resistant *Mycobacterium tuberculosis* clinical isolates. *Antimicrob. Agents Chemother.* 58 (6), 3270–3275. doi: 10.1128/AAC.02066-13

Ezeriņa, D., Takano, Y., Hanaoka, K., Urano, Y., and Dick, T. P. (2018). N-acetyl cysteine functions as a fast-acting antioxidant by triggering intracellular H(2)S and sulfane sulfur production. *Cell Chem. Biol.* 25 (4), 447–59.e4. doi: 10.1016/j.chembiol.2018.01.011

Folkvardsen, D., Thomsen, V., Rigouts, L., Rasmussen, E., Bang, D., Bernaerts, G., et al. (2013). Rifampin heteroresistance in *Mycobacterium tuberculosis* cultures as detected by phenotypic and genotypic drug susceptibility test methods. *J. Clin. Microbiol.* 51, 4220–4222. doi: 10.1128/JCM.01602-13

Forsman, L., Niward, K., Kuhlin, J., Zheng, X., Zheng, R., Ke, R., et al (2021). Suboptimal moxifloxacin and levofloxacin drug exposure during treatment of patients with multidrug-resistant tuberculosis: results from a prospective study in China. *Eur. Respir. J.* 57 (3), 2003463. doi: 10.1183/13993003.03463-2020

Fridman, O., Goldberg, A., Ronin, I., Shoresh, N., and Balaban, N. Q. (2014). Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature* 513 (7518), 418–421. doi: 10.1038/nature13469

Ginsburg, A. S., Hooper, N., Parrish, N., Dooley, K. E., Dorman, S. E., Booth, J., et al. (2003). Fluoroquinolone resistance in patients with newly diagnosed tuberculosis. *Clin. Infect. Dis.* 37 (11), 1448–1452. doi: 10.1086/379328

Gootenberg, J., Abudayyeh, O., Lee, J., Essletzbichler, P., Dy, A., Joung, J., et al. (2017). Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science* 356, eaam9321. doi: 10.1126/science.aam9321

Gruber, C. C., Babu, V. M. P., Livingston, K., Joisher, H., and Walker, G. C. (2022). Degradation of the *Escherichia coli* essential proteins DapB and dxr results in oxidative stress, which contributes to lethality through incomplete base excision repair. *mBio* 29, e0375621. doi: 10.1128/mbio.03756-21

Gruber, C. C., and Walker, G. C. (2018). Incomplete base excision repair contributes to cell death from antibiotics and other stresses. *DNA Repair (Amst)* 71, 108–117. doi: 10.1016/j.dnarep.2018.08.014

Guerra, C., Morris, D., Sipin, A., Kung, S., Franklin, M., Gray, D., et al. (2011). Glutathione and adaptive immune responses against *Mycobacterium tuberculosis* infection in healthy and HIV infected individuals. *PloS One* 6 (12), e28378. doi: 10.1371/journal.pone.0028378

Gutierrez, A., Jain, S., Bhargava, P., Hamblin, M., Lobritz, M. A., and Collins, J. J. (2017). Understanding and sensitizing density-dependent persistence to quinolone antibiotics. *Mol. Cell.* 68 (6), 1147–54 e3. doi: 10.1016/j.molcel.2017.11.012

Hillemann, D., Rusch-Gerdes, S., and Richter, E. (2009). Feasibility of the GenoType MTBDRsl assay for fluoroquinolone, amikacin-capreomycin, and ethambutol resistance testing of *Mycobacterium tuberculosis* strains and clinical specimens. *J. Clin. Microbiol.* 47 (6), 1767–1772. doi: 10.1128/JCM.00081-09

Hoeijmakers, J. H. (2009). DNA Damage, aging, and cancer. *N Engl. J. Med.* 361 (15), 1475–1485. doi: 10.1056/NEJMra0804615

Hofmann-Thiel, S., vanIngen, J., Feldmann, K., Turaev, L., Uzakova, G., Murmusaeva, G., et al. (2009). Mechanisms of heteroresistance to isoniazid and rifampin of *Mycobacterium tuberculosis* in Tashkent, Uzbekistan. *Eur. Respir. J.* 33, 368–374. doi: 10.1183/09031936.00089808

Hong, Y., Zeng, J., Wang, X., Drlica, K., and Zhao, X. (2019). Post-stress bacterial cell death mediated by reactive oxygen species. *Proc. Natl. Acad. Sci. U. S. A.* 116 (20), 10064–10071. doi: 10.1073/pnas.1901730116

Huang, Q., Liu, Z., Liao, Y., Chen, X., Zhang, Y., and Li, Q. (2011). Multiplex fluorescence melting curve analysis for mutation detection with dual-labeled, self-quenched probes. *PloS One* 6, e19206. doi: 10.1371/journal.pone.0019206

Hu, S., Li, G., Li, H., Liu, X., Niu, J., Quan, S., et al. (2014). Rapid detection of isoniazid resistance in *Mycobacterium tuberculosis* isolates by use of real-time-PCR-based melting curve analysis. *J. Clin. Microbiol.* 52, 1644–1652. doi: 10.1128/JCM.03395-13

Imlay, J. A. (2015). Diagnosing oxidative stress in bacteria: not as easy as you might think. *Curr. Opin. Microbiol.* 24, 124–131. doi: 10.1016/j.mib.2015.01.004

Jaeschke, H., Kleinwaechter, C., and Wendel, A. (1992). NADH-dependent reductive stress and ferritin-bound iron in allyl alcohol-induced lipid peroxidation *in vivo*: the protective effect of vitamin e. *Chem. Biol. Interact.* 81 (1-2), 57–68. doi: 10.1016/0009-2797(92)90026-H

Kaplan, G., Post, F., Moreira, A., Wainwright, H., Kreiswirth, B., Tanverdi, M., et al. (2003). *Mycobacterium tuberculosis* growth at the cavity surface: a microenvironment with failed immunity. *Infect. Immun.* 71, 7099–7108. doi: 10.1128/IAI.71.12.7099-7108.2003

Kargarpour Kamakoli, M., Sadegh, H. R., Farmanfarmaei, G., Masoumi, M., Fateh, A., Javadi, G., et al. (2017). Evaluation of the impact of polyclonal infection and heteroresistance on treatment of tuberculosis patients. *Sci. Rep.* 7, 41410. doi: 10.1038/srep41410

Keren, I., Wu, Y., Inocencio, J., Mulcahy, L. R., and Lewis, K. (2013). Killing by bactericidal antibiotics does not depend on reactive oxygen species. *Science* 339 (6124), 1213–1216. doi: 10.1126/science.1232688

Kim, H., Nakajima, C., Yokoyama, K., Rahim, Z., Kim, Y. U., Oguri, H., et al. (2011). Impact of the E540V amino acid substitution in GyrB of *Mycobacterium tuberculosis* on quinolone resistance. *Antimicrob. Agents Chemother.* 55 (8), 3661–3667. doi: 10.1128/AAC.00042-11

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., and Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 130 (5), 797–810. doi: 10.1016/j.cell.2007.06.049

Kunota, T. T. R., Rahman, M. A., Truebody, B. E., Mackenzie, J. S., Saini, V., Lamprecht, D. A., et al. (2021). Mycobacterium tuberculosis H(2)S functions as a sink to modulate central metabolism, bioenergetics, and drug susceptibility. *Antioxid. (Basel)* 10 (8), 1285. doi: 10.3390/antiox10081285

Lamprecht, D. A., Finin, P. M., Rahman, M. A., Cumming, B. M., Russell, S. L., Jonnala, S. R., et al. (2016). Turning the respiratory flexibility of *Mycobacterium tuberculosis* against itself. *Nat. Commun.* 7, 12393. doi: 10.1038/ncomms12393

Lange, C., Chesov, D., Heyckendorf, J., Leung, C. C., Udwadia, Z., and Dheda, K. (2018). Drug-resistant tuberculosis: An update on disease burden, diagnosis and treatment. *Respirology* 23 (7), 656–673. doi: 10.1111/resp.13304

Lau, R. W., Ho, P. L., Kao, R. Y., Yew, W. W., Lau, T. C., Cheng, V. C., et al. (2011). Molecular characterization of fluoroquinolone resistance in *Mycobacterium tuberculosis*: functional analysis of *gyrA* mutation at position 74. *Antimicrob. Agents Chemother.* 55 (2), 608–614. doi: 10.1128/AAC.00920-10

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., and Balaban, N. Q. (2017). Antibiotic tolerance facilitates the evolution of resistance. *Science* 355 (6327), 826–830. doi: 10.1126/science.aaj2191

Lieberman, T., Wilson, D., Misra, R., Xiong, L., Moodley, P., Cohen, T., et al. (2016). Genomic diversity in autopsy samples reveals within-host dissemination of HIV-associated mycobacterium tuberculosis. *Nat. Med.* 22, 1470–1474. doi: 10.1038/nm.4205

Liu, J., Gefen, O., Ronin, I., Bar-Meir, M., and Balaban, N. Q. (2020). Effect of tolerance on the evolution of antibiotic resistance under drug combinations. *Science* 367 (6474), 200–204. doi: 10.1126/science.aay3041

Liu, Y., and Imlay, J. A. (2013). Cell death from antibiotics without the involvement of reactive oxygen species. *Science* 339 (6124), 1210–1213. doi: 10.1126/science.1232751

Liu, Y., Liu, X., Qu, Y., Wang, X., Li, L., and Zhao, X. (2012). Inhibitors of reactive oxygen species accumulation delay and/or reduce the lethality of several antistaphylococcal agents. *Antimicrob. Agents Chemother.* 56 (11), 6048–6050. doi: 10.1128/AAC.00754-12

Lobritz, M. A., Belenky, P., Porter, C. B. M., Gutierrez, A., Yang, J. H., Schwarz, E. G., et al. (2015). Antibiotic efficacy is linked to bacterial cellular respiration. *P Natl. Acad. Sci. U.S.A.* 112 (27), 8173–8180. doi: 10.1073/pnas.1509743112

Long, Q., Du, Q., Fu, T., Drlica, K., Zhao, X., and Xie, J. (2015). Involvement of holliday junction resolvase in fluoroquinolone-mediated killing of mycobacterium smegmatis. *Antimicrob. Agents Chemother.* 59 (3), 1782–1785. doi: 10.1128/AAC.04434-14

Luan, G., Hong, Y., Drlica, K., and Zhao, X. (2018). Suppression of reactive oxygen species accumulation accounts for paradoxical bacterial survival at high quinolone concentration. *Antimicrob. Agents Chemother.* 62 (3), 01622–17. doi: 10.1128/AAC.01622-17

Mahakalkar, S. M., Nagrale, D., Gaur, S., Urade, C., Murhar, B., and Turankar, A. (2017). N-acetylcysteine as an add-on to directly observed therapy short-I therapy in fresh pulmonary tuberculosis patients: A randomized, placebo-controlled, double-blinded study. *Perspect. Clin. Res.* 8 (3), 132–136. doi: 10.4103%2F2229-3485.210450

Malik, M., Chavda, K., Zhao, X., Shah, N., Hussain, S., Kurepina, N., et al. (2012a). Induction of mycobacterial resistance to quinolone class antimicrobials. *Antimicrob. Agents Chemother.* 56 (7), 3879–3887. doi: 10.1128/AAC.00474-12

Malik, M., and Drlica, K. (2006). Moxifloxacin lethality against *Mycobacterium tuberculosis* in the presence and absence of chloramphenicol. *Antimicrob. Agents Chemother.* 50 (8), 2842–2844. doi: 10.1128/AAC.00250-06

Malik, M., Hoatam, G., Chavda, K., Kerns, R. J., and Drlica, K. (2010). Novel approach for comparing the abilities of quinolones to restrict the emergence of resistant mutants during quinolone exposure. *Antimicrob. Agents Chemother.* 54 (1), 149–156. doi: 10.1128/AAC.01035-09

Malik, M., Hussain, S., and Drlica, K. (2007). Effect of anaerobic growth on quinolone lethality with escherichia coli. *Antimicrob. Agents Chemother.* 51 (1), 28–34. doi: 10.1128/AAC.00739-06

Malik, S., Willby, M., Sikes, D., Tsodikov, O. V., and Posey, J. E. (2012b). New insights into fluoroquinolone resistance in *Mycobacterium tuberculosis*: functional genetic analysis of gyrA and gyrB mutations. *PloS One* 7 (6), e39754. doi: 10.1371/journal.pone.0039754

Malik, M., Zhao, X., and Drlica, K. (2006). Lethal fragmentation of bacterial chromosomes mediated by DNA gyrase and quinolones. *Mol. Microbiol.* 61, 810–825. doi: 10.1111/j.1365-2958.2006.05275.x

Mavi, P. S., Singh, S., and Kumar, A. (2020). Reductive stress: New insights in physiology and drug tolerance of mycobacterium. *Antioxid. Redox Signal.* 32 (18), 1348–1366. doi: 10.1089/ars.2019.7867

McGrath, M., GeyvanPittius, N., vanHelden, P., Warren, R., and Warner, D. (2014). Mutation rate and the emergence of drug resistance in mycobacterium tuberculosis. *J. Antimicrob. Chemother.* 69, 292–302. doi: 10.1093/jac/dkt364

Metcalfe, J. Z., Streicher, E., Theron, G., Colman, R. E., Penaloza, R., Allender, C., et al. (2017). *Mycobacterium tuberculosis* subculture results in loss of potentially clinically relevant heteroresistance. *Antimicrob. Agents Chemother.* 61 (11), 00888–17. doi: 10.1128/AAC.00888-17

Mishra, R., Kohli, S., Malhotra, N., Bandyopadhyay, P., Mehta, M., Munshi, M., et al. (2019). Targeting redox heterogeneity to counteract drug tolerance in replicating mycobacterium tuberculosis. *Sci. Transl. Med.* 11 (518). doi: 10.1126/scitranslmed.aaw6635

Mishra, R., Yadav, V., Guha, M., and Singh, A. (2021). Heterogeneous host-pathogen encounters coordinate antibiotic resilience in mycobacterium tuberculosis. *Trends Microbiol.* 29 (7), 606–620. doi: 10.1016/j.tim.2020.10.013

Mitchison, D. A. (1984). Drug resistance in mycobacteria. *Brit. Med. Bull.* 40, 84–90. doi: 10.1093/oxfordjournals.bmb.a071952

Morris, D., Guerra, C., Khurasany, M., Guilford, F., Saviola, B., Huang, Y., et al. (2013). Glutathione supplementation improves macrophage functions in HIV. *J. Interferon Cytokine Res.* 33 (5), 270–279. doi: 10.1089/jir.2012.0103

Mustaev, A., Malik, M., Zhao, X., Kurepina, N., Luan, G., Oppegard, L. M., et al. (2014). Fluoroquinolone-gyrase-DNA complexes: two modes of drug binding. *J. Biol. Chem.* 289 (18), 12300–12312. doi: 10.1074/jbc.M113.529164

Nagrale, D., Mahakalkar, S., and Gaur, S. (2013). Supplementation of n-acetylcysteine as an adjuvant in treatment of newly diagnosed pulmonary tuberculosis patients: A prospective, randomized double blind, placebo controlled study. *Eur. Respir. J.* 42 (Suppl 57), P2833.

Nair, R. R., Sharan, D., Sebastian, J., Swaminath, S., and Ajitkumar, P. (2019). Heterogeneity of ROS levels in antibiotic-exposed mycobacterial subpopulations confers differential susceptibility. *Microbiol. (Reading)* 165 (6), 668–682. doi: 10.1099/mic.0.000797

Nimmo, C., Brien, K., Millard, J., Grant, A. D., Padayatchi, N., Pym, A. S., et al. (2020). Dynamics of within-host *Mycobacterium tuberculosis* diversity and heteroresistance during treatment. *EBioMedicine* 55, 102747. doi: 10.1016/j.ebiom.2020.102747

Ni, W., Song, X., and Cui, J. (2014). Testing the mutant selection window hypothesis with *Escherichia coli* exposed to levofloxacin in a rabbit tissue cage infection model. *Eur. J. Clin. Microbiol. Infect. Dis.* 33 (3), 385–389. doi: 10.1007/s10096-013-1968-8

Nuermberger, E. L., Yoshimatsu, T., Tyagi, S., O'Brien, R. J., Vernon, A. N., Chaisson, R. E., et al. (2004). Moxifloxacin-containing regimen greatly reduces time to culture conversion in murine tuberculosis. *Am. J. Respir. Crit. Care Med.* 169 (3), 421–426. doi: 10.1164/rccm.200310-1380OC

Operario, D. J., Koeppel, A. F., Turner, S. D., Bao, Y., Pholwat, S., Banu, S., et al. (2017). Prevalence and extent of heteroresistance by next generation sequencing of multidrug-resistant tuberculosis. *PloS One* 12 (5), e0176522. doi: 10.1371/journal.pone.0176522

Palanisamy, G. S., Kirk, N. M., Ackart, D. F., Shanley, C. A., Orme, I. M., and Basaraba, R. J. (2011). Evidence for oxidative stress and defective antioxidant response in guinea pigs with tuberculosis. *PloS One* 6 (10), e26254. doi: 10.1371/journal.pone.0026254

Park, S., and Imlay, J. A. (2003). High levels of intracellular cysteine promote oxidative DNA damage by driving the fenton reaction. *J. Bacteriol.* 185 (6), 1942–1950. doi: 10.1128/JB.185.6.1942-1950.2003

Pawlowski, A., Jansson, M., Skold, M., Rottenberg, M. E., and Kallenius, G. (2012). Tuberculosis and HIV co-infection. *PloS Pathog.* 8 (2), e1002464. doi: 10.1371/journal.ppat.1002464

Pholwat, S., Stroup, S., Foongladda, S., and Houpt, E. (2013). Digital PCR to detect and quantify heteroresistance in drug resistant mycobacterium tuberculosis. *PloS One* 8, e57238. doi: 10.1371/journal.pone.0057238

Piccaro, G., Pietraforte, D., Giannoni, F., Mustazzolu, A., and Fattorini, L. (2014). Rifampin induces hydroxyl radical formation in mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 58 (12), 7527–7533. doi: 10.1128/AAC.03169-14

Pletz, M. W., De Roux, A., Roth, A., Neumann, K. H., Mauch, H., and Lode, H. (2004). Early bactericidal activity of moxifloxacin in treatment of pulmonary tuberculosis: a prospective, randomized study. *Antimicrob. Agents Chemother.* 48 (3), 780–782. doi: 10.1128/AAC.48.3.780-782.2004

Possuelo, L. G., Castelan, J. A., de Brito, T. C., Ribeiro, A. W., Cafrune, P. I., Picon, P. D., et al. (2008). Association of slow n-acetyltransferase 2 profile and anti-TB drug-induced hepatotoxicity in patients from southern Brazil. *Eur. J. Clin. Pharmacol.* 64 (7), 673–681. doi: 10.1007/s00228-008-0484-8

Post, F., Willcox, P., Mathema, B., Steyn, L., Shean, K., Ramaswamy, S., et al. (2004). Genetic polymorphism in *Mycobacterium tuberculosis* isolates from patients with chronic multidrug-resistant tuberculosis. *J. Inf. Dis.* 190, 99–106. doi: 10.1086/421501

Pournaras, S., Ikonomidis, A., Markogiannakis, A., Maniatis, A., and Tsakris, A. (2005). Heteroresistance to carbapenems in acinetobacter baumannii. *J. Antimicrob. Chemother.* 55, 1055–1056. doi: 10.1093/jac/dki115

Prideaux, B., Via, L. E., Zimmerman, M. D., Eum, S., Sarathy, J., O'Brien, P., et al. (2015). The association between sterilizing activity and drug distribution into tuberculosis lesions. *Nat. Med.* 21 (10), 1223–1227. doi: 10.1038/nm.3937

Rice, J., Reis, A., Rice, L., Carver-Brown, R., and Wangh, L. (2012). Fluorescent signatures for variable DNA sequences. *Nucleic Acids Res.* 40, e164. doi: 10.1093/nar/gks731

Rinder, H., Mieskes, K., and Loscher, T. (2001). Heteroresistance in mycobacterium tuberculosis. *Int. J. Tuberc. Lung Dis.* 5, 339–345.

Rock, J., Hopkins, F., Chavez, A., Diallo, M., Chase, M., Gerrick, E., et al. (2017). Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform. *Nat. Microbiol.* 2, 16274. doi: 10.1038/nmicrobiol.2016.274

Rodriguez, J. C., Cebrian, L., Lopez, M., Ruiz, M., Jimenez, I., and Royo, G. (2004). Mutant prevention concentration: comparison of fluoroquinolones and linezolid with mycobacterium tuberculosis. *J. Antimicrob. Chemother.* 53 (3), 441–444. doi: 10.1093/jac/dkh119

Safe, I. P., Lacerda, M. V. G., Printes, V. S., Praia Marins, A. F., Rebelo Rabelo, A. L., Costa, A. A., et al. (2020). Safety and efficacy of n-acetylcysteine in hospitalized patients with HIV-associated tuberculosis: An open-label, randomized, phase II trial (RIPENACTB study). *PloS One* 15 (6), e0235381. doi: 10.1371/journal.pone.0235381

Sarathy, J., Blanc, L., Alvarez-Cabrera, N., O'Brien, P., Dias-Freedman, I., Mina, M., et al. (2019). Fluoroquinolone efficacy against tuberculosis is driven by penetration into lesions and activity against resident bacterial populations. *Antimicrob. Agents Chemother.* 63 (5), 02516–18. doi: 10.1128/AAC.02516-18

Shamputa, I., Rigouts, L., Eyongeta, L., ElAila, N., vanDeun, A., Salim, A., et al. (2004). Genotypic and phenotypic heterogeneity among *Mycobacterium tuberculosis* isolates from pulmonary tuberculosis patients. *J. Clin. Microbiol.* 42, 5528–5536. doi: 10.1128/JCM.42.12.5528-5536.2004

Shee, S., Singh, S., Tripathi, A., Thakur, C., Kumar, A. T., Das, M., et al. (2022). Moxifloxacin-mediated killing of *Mycobacterium tuberculosis* involves respiratory downshift, reductive stress, and ROS accumulation. *Antimicrob. Agents Chemother. (in press).* doi: 10.1101/2022.04.04.486929

Singh, A. (2017). Guardians of the mycobacterial genome: A review on DNA repair systems in mycobacterium tuberculosis. *Microbiology* 163 (12), 1740–1758. doi: 10.1099/mic.0.000578

Takahashi, N., Gruber, C. C., Yang, J. H., Liu, X., Braff, D., Yashaswini, C. N., et al. (2017). Lethality of MalE-LacZ hybrid protein shares mechanistic attributes with oxidative component of antibiotic lethality. *Proc. Natl. Acad. Sci. U. S. A.* 114 (34), 9164–9169. doi: 10.1073/pnas.1707466114

Teskey, G., Cao, R., Islamoglu, H., Medina, A., Prasad, C., Prasad, R., et al. (2018). The synergistic effects of the glutathione precursor, NAC and first-line

antibiotics in the granulomatous response against mycobacterium tuberculosis. *Front. Immunol.* 9, 2069. doi: 10.3389/fimmu.2018.02069

Tuomanen, E., Durack, D. T., and Tomasz, A. (1986). Antibiotic tolerance among clinical isolates of bacteria. *Antimicrob. Agents Chemother.* 30 (4), 521–527. doi: 10.1128/AAC.30.4.521

Tyagi, P., Dharmaraja, A. T., Bhaskar, A., Chakrapani, H., and Singh, A. (2015). *Mycobacterium tuberculosis* has diminished capacity to counteract redox stress induced by elevated levels of endogenous superoxide. *Free Radic. Biol. Med.* 84, 344–354. doi: 10.1016/j.freeradbiomed.2015.03.008

Vadwai, V., Daver, G., Udwadia, Z., Sadani, M., Shetty, A., and Rodrigues, C. (2011). Clonal population of *Mycobacterium tuberculosis* strains reside within multiple lung cavities. *PloS One* 6, e24770. doi: 10.1371/journal.pone.0024770

vanRie, A., Victor, T., Richardson, M., Johnson, R., vanderSpuy, G., Murray, E., et al. (2005). Reinfection and mixed infection cause changing *Mycobacterium tuberculosis* drug-resistance patterns. *Am. J. Respir. Crit. Care Med.* 172, 636–642. doi: 10.1164/rccm.200503-449OC

Vargas, R., Freschi, L., Marin, M., Epperson, L. E., Smith, M., Oussenko, I., et al. (2021). In-host population dynamics of mycobacterium tuberculosis complex during active disease. *Elife* 10, e61805. doi: 10.7554/eLife.61805

Vargas, D., Kramer, F., Tyagi, S., and Marras, S. (2016). Multiplex real-time PCR assays that measure the abundance of extremely rare mutations associated with cancer. *PloS One* 11, e0156546. doi: 10.1371/journal.pone.0156546

Venketaraman, V., Rodgers, T., Linares, R., Reilly, N., Swaminathan, S., Hom, D., et al. (2006). Glutathione and growth inhibition of mycobacterium tuberculosis in healthy and HIV infected subjects. *AIDS Res. Ther.* 3, 5. doi: 10.1186/1742-6405-3-5

Vilcheze, C., Hartman, T., Weinrick, B., and Jacobs, W. R.Jr. (2013). *Mycobacterium tuberculosis* is extraordinarily sensitive to killing by a vitamin c-induced fenton reaction. *Nat. Commun.* 4, 1881. doi: 10.1038/ncomms2898

Vilchèze, C., Hartman, T., Weinrick, B., Jain, P., Weisbrod, T. R., Leung, L. W., et al. (2017). Enhanced respiration prevents drug tolerance and drug resistance in mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. U.S.A.* 114 (17), 4495–4500. doi: 10.1073/pnas.1704376114

Vilchèze, C., and Jacobs, W. R.Jr. (2021). The promises and limitations of n-acetylcysteine as a potentiator of first-line and second-line tuberculosis drugs. *Antimicrob. Agents Chemother.* 65 (5), e01703–20. doi: 10.1128/AAC.01703-20

Vogelstein, B., and Kinsler, K. (1999). Digital PCR. *Proc. Natl. Acad. Sci. U.S.A.* 96, 9236–9241. doi: 10.1073/pnas.96.16.9236

Wald-Dickler, N., Holtom, P., and Spellberg, B. (2018). Busting the myth of "Static vs cidal": A systemic literature review. *Clin. Infect. Dis.* 66 (9), 1470–1474. doi: 10.1093/cid/cix1127

Wang, X., and Zhao, X. (2009). Contribution of oxidative damage to antimicrobial lethality. *Antimicrob. Agents Chemother.* 53, 1395–1402. doi: 10.1128/AAC.01087-08

Warren, R., Victor, T., Streicher, E., Richardson, M., Beyers, N., GeyvanPittius, N., et al. (2004). Patients with active tuberculosis often have different strains in the same sputum specimen. *Am. J. Crit. Care Med.* 169, 610–614. doi: 10.1164/rccm.200305-714OC

WHO (2013). *Global tuberculosis report 2013* Vol. 2013 (Geneva: World Health Organization).

WHO (2019). *Global tuberculosis report 2019* Vol. 2019 (Geneva: World Health Organization).

Wu, X., Wang, X., Drlica, K., and Zhao, X. (2011). A toxin-antitoxin module in *Bacillus subtilis* can both mitigate and amplify effects of lethal stress. *PloS One* 6 (8), e23909. doi: 10.1371/journal.pone.0023909

Xiong, M., Wu, X., Ye, X., Zhang, L., Zeng, S., Huang, Z., et al. (2016). Relationship between cefquinome PK/PD parameters and emergence of resistance of *Staphylococcus aureus* in rabbit tissue-cage infection model. *Front. Microbiol.* 7, 874. doi: 10.3389/fmicb.2016.00874

Yajko, D., Wagner, C., Tevere, V., Kocagöz, T., Hadley, W., and Chambers, H. (1995). Quantitative culture of *Mycobacterium tuberculosis* from clinical sputum specimens and dilution endpoint of its detection by the amplicor PCR assay. *J. Clin. Microbiol.* 33, 1944–1947. doi: 10.1128/jcm.33.7.1944-1947.1995

Yano, T., Kassovska-Bratinova, S., Teh, J. S., Winkler, J., Sullivan, K., Isaacs, A., et al. (2011). Reduction of clofazimine by mycobacterial type 2 NADH:quinone oxidoreductase: a pathway for the generation of bactericidal levels of reactive oxygen species. *J. Biol. Chem.* 286 (12), 10276–10287. doi: 10.1074/jbc.M110.200501

Yoshida, H., Bogaki, M., Nakamura, M., and Nakamura, S. (1990). Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of escherichia coli. *Antimicrob. Agents Chemother.* 34 (6), 1271–1272. doi: 10.1128/AAC.34.6.1271

Zeng, J., Hong, Y., Zhao, N., Liu, Q., Zhu, W., Xiao, L., et al. (2022). A broadly applicable, stress-mediated bacterial death pathway regulated by the phosphotransferase system (PTS) and the cAMP-crp cascade. *Proc. Natl. Acad. Sci. U. S. A.* 119 (23), e2118566119. doi: 10.1073/pnas.2118566119

Zetola, N., Modongo, C., Moonan, P., Ncube, R., Matlhagela, K., Sepako, E., et al. (2014). Clinical outcomes among persons with pulmonary tuberculosis caused by *Mycobacterium tuberculosis* isolates with phenotypic heterogeneity in results of drug-susceptibility tests. *J. Infect. Dis.* 209, 1754–1763. doi: 10.1093/infdis/jiu040

Zhang, B., Gu, X., Li, Y., Li, X., Gu, M., Zhang, N., et al. (2014). *In vivo* evaluation of mutant selection window of cefquinome against *Escherichia coli* in piglet tissue-cage model. *BMC Vet. Res.* 10, 297. doi: 10.1186/s12917-014-0297-1

Zhang, X., Zhao, B., Huang, H., Zhu, Y., Peng, J., Dai, G., et al. (2013). Co-Occurrence of amikacin-resistant and -susceptible *Mycobacterium tuberculosis* isolates in clinical samples from Beijing, China. *J. Antimicrob. Chemother.* 68, 1537–1542. doi: 10.1093/jac/dkt082

Zhang, X., Zhao, B., Liu, L., Zhu, Y., Zhao, Y., and Jin, Q. (2012). Subpopulation analysis of heteroresistance to fluoroquinolone in *Mycobacterium tuberculosis* isolates from Beijing, China. *J. Clin. Microbiol.* 50 (4), 1471–1474. doi: 10.1128/JCM.05793-11

Zhao, X., and Drlica, K. (2001). Restricting the selection of antibiotic-resistant mutants: a general strategy derived from fluoroquinolone studies. *Clin. Inf. Dis.* 33 (Suppl 3), S147–SS56. doi: 10.1086/321841

Zhao, B. Y., Pine, R., Domagala, J., and Drlica, K. (1999). Fluoroquinolone action against clinical isolates of *Mycobacterium tuberculosis*: effects of a c-8 methoxyl group on survival in liquid media and in human macrophages. *Antimicrob. Agents Chemother.* 43 (3), 661–666. doi: 10.1128/AAC.43.3.661

Zhou, J., Dong, Y., Zhao, X., Lee, S., Amin, A., Ramaswamy, S., et al. (2000). Selection of antibiotic-resistant bacterial mutants: allelic diversity among fluoroquinolone-resistant mutations. *J. Infect. Dis.* 182 (2), 517–525. doi: 10.1086/315708

Zhu, Y. L., Hu, L. F., Mei, Q., Cheng, J., Liu, Y. Y., Ye, Y., et al. (2012). Testing the mutant selection window in rabbits infected with methicillin-resistant staphylococcus aureus exposed to vancomycin. *J. Antimicrob. Chemother.* 67 (11), 2700–2706. doi: 10.1093/jac/dks280

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 03 October 2022
DOI 10.3389/fcimb.2022.1007958

OPEN ACCESS

EDITED BY
Ben Gold,
Weill Cornell Medicine, United States

REVIEWED BY
Christina Stallings,
Washington University in St. Louis, United States
Camus Nimmo,
Francis Crick Institute, United Kingdom
Greg Cook,
University of Otago, New Zealand

*CORRESPONDENCE
Sarah M. Fortune
sfortune@hsph.harvard.edu

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 31 July 2022
ACCEPTED 14 September 2022
PUBLISHED 03 October 2022

CITATION
Stanley S, Liu Q and Fortune SM (2022) *Mycobacterium tuberculosis* functional genetic diversity, altered drug sensitivity, and precision medicine.
Front. Cell. Infect. Microbiol. 12:1007958.
doi: 10.3389/fcimb.2022.1007958

Mycobacterium tuberculosis functional genetic diversity, altered drug sensitivity, and precision medicine

Sydney Stanley, Qingyun Liu and Sarah M. Fortune*

Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, Boston, MA, United States

In the face of the unrelenting global burden of tuberculosis (TB), antibiotics remain our most effective tools to save lives and control the spread of *Mycobacterium tuberculosis* (Mtb). However, we confront a dual challenge in our use of antibiotics: simplifying and shortening the TB drug regimen while also limiting the emergence and propagation of antibiotic resistance. This task is now more feasible due to the increasing availability of bacterial genomic data at or near the point of care. These resources create an opportunity to envision how integration of bacterial genetic determinants of antibiotic response into treatment algorithms might transform TB care. Historically, Mtb drug resistance studies focused on mutations in genes encoding antibiotic targets and the resulting increases in the minimal inhibitory concentrations (MICs) above a breakpoint value. But recent progress in elucidating the effects of functional genetic diversity in Mtb has revealed various genetic loci that are associated with drug phenotypes such as low-level MIC increases and tolerance which predict the development of resistance and treatment failure. As a result, we are now poised to advance precision medicine approaches in TB treatment. By incorporating information regarding Mtb genetic characteristics into the development of drug regimens, clinical care which tailors antibiotic treatment to maximize the likelihood of success has come into reach.

KEYWORDS

***mycobacterium tuberculosis*, antibiotic resistance, genetic diversity, precision medicine, bacterial genomics, molecular diagnostics, TB drug regimen, MIC**

Introduction

As the tuberculosis (TB) pandemic caused by *Mycobacterium tuberculosis* (Mtb) rages on, we must find new ways to augment the efficacy of antibiotics, our most powerful weapons in this war (The World Health Organization, 2021). Precision medicine represents a promising strategy, which would involve methods to mitigate the

occurrence of treatment failure by predicting the antibiotic sensitivities of individual strains based on their genotype and modifying the drug regimen accordingly (Lange et al., 2020). Adopting precision medicine approaches such as tailored antibiotic treatment regimens informed by the results of next-generation molecular detection of mutations known to alter drug susceptibility could facilitate shortened treatment regimens and mitigate the development of resistance (Lange et al., 2020). However, such strategies require a roadmap of the relationship between bacterial variation, antibiotic sensitivities, and treatment outcomes. In this review, we will discuss advancements in our understanding of how Mtb genetic diversity affects antibiotic susceptibility, which has expanded conventional metrics and measures of drug sensitivity. We will highlight opportunities to apply this knowledge to bolster current efforts towards precision medicine for TB.

The genesis of our current understanding of drug resistance

The challenge of drug resistance in Mtb became clear almost immediately after the discovery of streptomycin (SM) in 1943 (Schatz et al., 1944). By 1947 two randomized controlled trials had been conducted to assess the efficacy of SM for TB (Marshall et al., 1948; Fox et al., 1954; Fox et al., 1999). In these trials, there was no long-term survival benefit to SM treatment; almost all patients receiving SM initially improved but most subsequently relapsed with drug resistant Mtb. These findings led to the recognition that Mtb with reduced antibiotic susceptibility would reliably emerge after a period of antibiotic selection, at least with a single agent.

In the late 1940's and early 1950's, drug susceptibility was measured by comparing the lowest concentration of a drug that inhibited the *in vitro* growth of strains isolated from patients to that of a reference strain, H37Rv, which had been derived from a lung lesion 50 years earlier (Mitchison, 1949). The presumption was that most Mtb strains at the time were drug sensitive by definition because they had not faced drug treatment before. More formally, the diagnostic assumption was that bacterial growth distributions in the presence of drug were normally distributed, and that all strains falling above this normal distribution were equally likely to fail SM treatment. This understanding simplified the technical complexity of standardizing measurements of drug responses and led the field to solutions for establishing resistance, such as the calibrated "minimum inhibitory concentration" (MIC), which was a comparative measure response in strains deemed "probably sensitive" (from newly diagnosed patients) and "probably resistant" (from treatment failures) (Fox et al., 1999). These approaches have now matured into the so-called proportion method whereby the critical concentration is the lowest concentration of drug, established by international convention, which inhibits 99% of the growth of a population of "phenotypically wild type" Mtb (The World Health Organization, 2018). The MIC is now defined as the lowest concentration of an antibiotic that inhibits visible bacterial growth of an individual strain and is often used by bench scientists (Schön et al., 2020). The MBC or minimum bactericidal concentration is the lowest concentration that kills 99% of the initial bacterial population and is utilized mostly in clinical settings (National Committee for Clinical Laboratory Standards, 1999). Even though the field recognizes a difference between low and high resistance to some antibiotics such as isoniazid (INH) or fluoroquinolones (Malik et al., 2012; Lempens et al., 2018), proportion methods such as these - or genetic proxies of proportion methods - remain the most commonly used approach to defining drug susceptibility in Mtb, and has had the effect of enforcing a dichotomized understanding of antibiotic resistance, where the "wildtype" is assumed to be more or less homogeneously sensitive and functionally drug naïve.

We note that that the assumption that "wildtype" Mtb is drug naïve was challenged early on by the increasing frequency of primary drug resistance, that is drug resistance in treatment naïve patients. By 1964, not even 20 years after the first SM trial, 11% of primary TB cases in Hong Kong were drug resistant (and 10% resistant to INH and/or SM in Kenya by 1974), reflecting remarkable drug pressure on the extant bacterial population (Hong Kong Government Tuberculosis ServiceBritish Medical Research Council Co-operative Investigation, 1964; An East African and British Medical Research Council Co-operative Investigation, 1978) even as measured by dichotomous assays. We propose that it is likely that the population distribution of other forms of altered drug susceptibility, not captured by conventional measures such as MIC assays and therefore not countered through subsequent regimen modifications, were also under this immense selective pressure and increasingly prevalent in the population.

Nonetheless, in a pragmatic field, the dichotomous definition of resistance became accepted as adequate where there was little capacity to measure drug susceptibility or tailor therapy based on bacterial features. Thus, as the modern short course chemotherapy regimens were developed through the 1980's, length of treatment was established in part to minimize the effects of preexisting bacterial or host differences in drug responses that contributed to treatment failure. Radically simpler regimens were first tried in the 1950s - 3 months of INH alone, for example, which actually led to durable cure in nearly half of patients (46% or 62/134) (Blowers and Cooke, 1954), but the focus was on developing a universal regimen because there was little capacity to identify and act on predictors of short course therapy success or failure.

Given that drug regimens are designed around dichotomized measures of Mtb antibiotic resistance, in the genomic age the

field has focused on delineating the bacterial genetic determinants that mediate MIC shifts above the critical concentration resistance breakpoint. This resulted in studies that identified and characterized antibiotic resistance determinants by zeroing in on individual mutations in the genes encoding drug targets or drug activators, such as single nucleotide polymorphisms in *katG* and *inhA*, *rpoB*, *embB*, *pncA*, and *rpsL* and *rrs* conferring resistance to INH, RIF, pyrazinamide (PZA), ethambutol (EMB), and SM respectively (Finken et al., 1993; Scorpio et al., 1997; Sreevatsan et al., 1997; Rozwarski et al., 1998; Ramaswamy et al., 2003; Hazbón et al., 2006; Juréen et al., 2008; Palomino and Martin, 2014). Such studies informed the development of GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA), the first test designed for rapid molecular detection of Mtb and RIF resistance (Boehme et al., 2010). Later advancements include Xpert MTB/XDR, which completes rapid susceptibility testing for additional first- and second-line drugs to partially address multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB (Cao et al., 2021; Penn-Nicholson et al., 2022). These tests and other accessible rapid diagnostics represent a transformative first-step towards TB precision medicine. However, there is considerable space for improvement because drug resistance-conferring mutations and breakpoint MICs are not completely predictive of treatment outcomes (Liu et al., 2021). Therefore, the field has begun to consider the wider effect of Mtb genetic diversity on not only antibiotic resistance but other clinically-relevant antibiotic susceptibility phenotypes.

Moving beyond dichotomous measures of drug resistance

While the clinical TB field was focused on acquired, high-level drug resistance determined by MIC changes above a breakpoint value, the bench sciences have been rapidly expanding our understanding of the clinical implications of variation in MIC below the critical concentration and the associated phenotypes. This includes small increases in MIC below the resistance threshold and also tolerance and persistence phenotypes that have been reviewed previously (Lewis and Shan, 2017; Boldrin et al., 2020; Schrader et al., 2020). These also include a range of environmentally-driven alterations in drug susceptibility such as differentially culturable bacteria (Chengalroyen et al., 2016; Turapov et al., 2016), "fat and lazy" macrophage resident bacteria (Garton et al., 2008; Daniel et al., 2011), or bacterial subpopulations created by adaptive regulatory systems like the toxin-antitoxin systems (Slayden et al., 2018).

Historically, there has been little effort to incorporate these other forms of "drug conditioning" into clinical treatment frameworks because they have been somewhere between hard and impossible to measure in a clinical setting and of unclear clinical importance. However, there is data to suggest that we should pay attention to the spectrum of altered drug responses under high-level drug resistance, the mass of ice under the tip of the iceberg.

MIC measurements are accessible to clinical labs. Indeed, a range of MIC levels for Mtb clinical isolates were observed as early as 1953 when the methodology for the MIC determination of INH, SM, and para-aminosalicylic acid (PAS) was becoming standardized, but this was assumed part of the "wildtype", drug sensitive distribution (Medical Research Council, 1953). However, in a landmark study, Colangeli et al. determined that putatively drug susceptible Mtb strains with higher MICs to RIF and INH are more likely to result in treatment failure (Colangeli et al., 2018). This observation indicates that small increases in MIC are clinically-relevant even though they have been historically overlooked and suggest a potential benefit of having the tools to identify these features.

With the advent of inexpensive sequencing technology, bacterial genomic data has also become widely available. This has been leveraged by methods such as candidate gene approaches to determine if mutations in known target genes of first-line drugs predict phenotypic antibiotic susceptibilities and bacterial genome-wide association studies (GWAS) to identify bacterial genetic variations associated with high-level drug resistance (Zhang et al., 2013; Coll et al., 2018; CRyPTIC consortium and the 100,000 genomes project, 2018; Hicks et al., 2018; Farhat et al., 2019; Lai and Ioerger, 2020). While the latter were originally undertaken to identify missing genetic determinants of MICs above breakpoint values, these studies have unearthed a suite of "stepping stone" mutations (Hicks et al., 2018; Safi et al., 2019; Hicks et al., 2020; Liu et al., 2022; Martini et al., 2022). These are mutations that at a minimum facilitate the acquisition of drug resistance but presumptively this occurs by reducing treatment efficacy. There have been few treatment cohorts with good metadata sufficiently powered to identify these factors through their direct association with treatment outcomes. However, the population genomic argument is that the selection in the population is de facto evidence of their benefit to the bacterium. We would argue that these bacterial factors will become even more important as we push to shorten treatment, though relative importance of specific factors may differ by regimen (Turkova et al., 2022).

The ultimate importance of identifying and parsing the genetic basis of these other forms of drug susceptibilities depends on the implications of and ability to act on this knowledge. We would argue that critical factors include (A) ability to modify drug regimen based on this information and (B) implications for new drugs – that is, mutations that do not alter drug responses in a target-specific fashion but broadly alter responses in a way that will compromise new regimens.

Non-canonical bacterial genetical determinants and low-level MIC shifts

The genetic basis of the low-level INH and RIF MIC shifts associated with treatment failure by Colangeli et al. was not defined. However, several studies have suggested a range of candidate variants. These include mutations in both known pathway genes and novel pathways. Mutations associated with antibiotic resistance differ in how much they modulate MIC, for example the *katG* S315T mutation is associated with large, resistance-level increases in the MIC of INH, while other mutations such as the *fabG1* C-15T polymorphism upstream of the INH target *inhA* results in smaller MIC shifts and low-level resistance (Lavender et al., 2005; Palomino and Martin, 2014). As for non-canonical targets, a Lineage 1 (L1) subclade-defining mutation in *ndh* (R268H) was associated with a ~2-fold increase in INH MIC, deemed neutral by the authors but only because it did not meet a standardized definition of resistance despite being present in 9.5% of INH-resistant strains but in none of the susceptible isolates, consistent with the changes in drug susceptibility associated with treatment failure (Lee et al., 2001; Merker et al., 2020).

Additionally, non-target variants associated with drug resistance that may contribute to moderate yet clinically-relevant MIC changes were identified by bacterial population GWAS studies (Zhang et al., 2013; Coll et al., 2018; Hicks et al., 2018; Farhat et al., 2019; Lai and Ioerger, 2020). Mutations in *dnaA*, which are found in 3.2% of all clinical strains globally, have been linked to INH resistance and have ~2-fold increase in MIC (Hicks et al., 2020). Low-level INH MIC-shifts may be relevant for new and old regimens alike, where the new 4-month regimen (rifapentine, moxifloxacin) still contains an INH backbone (Dorman et al., 2021). Further, *rnaseJ* is highly mutated in drug-resistant clinical strains of Mtb (Martini et al., 2022). While deletion of the gene does not alter the MIC to RIF nor INH, it does increase multidrug tolerance (Martini et al., 2022). Mutations in *resR* (Rv1830), a gene undergoing positive selection in Mtb clinical isolates, result in a slight MIC increase to INH, but more interestingly leads to faster bacterial recovery from drug treatment (Liu et al., 2022). ResR belongs to a regulatory pathway with WhiB2 and WhiA, and mutations in these genes were associated with canonical drug resistance and relapse after antibiotic treatment (Liu et al., 2022). Around 1.5%-9.7% of drug-sensitive Mtb strains from high-burden TB countries carry mutations in these genes, but mutations are present in 22.2% of strains from patients who failed treatment in the global REMoxTB phase 3 regimen-shortening trial (Bryant et al., 2013; Jindani et al., 2014; Liu et al., 2022).

Low-level MIC shifts have also been observed for second-line drugs. Mutations in *gidB* are associated with low-level SM resistance, but patients infected with Mtb strains carrying the *rrs* mutation that confers high-level amikacin resistance may still benefit from SM treatment despite the presence of *gidB* variants (Spies et al., 2011; Wong et al., 2011; Cohen et al., 2015; Cohen et al., 2020). *gidB* mutation may also explain the 15% of SM-resistant Mtb strains that lack mutations in *rrs* and *rpsL* (Wong et al., 2011). Even though SM is no longer used in the treatment of TB, this example illustrates the diagnostic benefit of non-target genes that mediate low-level resistance (Cohen et al., 2020). Mutations that impair the monooxygenase *Rv0565c* are associated with low-level resistance to the second-line antibiotic ethionamide (ETH) (Hicks et al., 2019). Like loss-of-function (LOF) mutations in *ald* that result in D-cycloserine resistance, Rv0565c mutations are found exclusively in MDR strains of Mtb, suggesting selection by second-line drug regimens (Desjardins et al., 2016; Hicks et al., 2019). Interestingly, low-level resistance has also been described for BDQ and CFZ, mediated by mutations in non-target genes including the regulator of the MmpS5-MmpL5 efflux pump system and *pepQ* (Andries et al., 2014; Almeida et al., 2016), and is more prevalent than predicted by exposure to these drugs.

Mtb genetic variation and antibiotic susceptibility

The original framing of altered drug susceptibility in Mtb implicitly posited that there were not clinically relevant differences in antibiotic susceptibility between Mtb strains before antibiotic exposure. However, as the L1 subclade-defining mutation in *ndh* suggests, it has become clear that this is an oversimplified view (Oppong et al., 2019; Merker et al., 2020).

Numerous studies reviewed elsewhere have indicated that L2 and L4 strains are more associated with drug resistance, MDR, and XDR (Gygli et al., 2017; Shanmugam et al., 2022). Several studies have demonstrated that L2 Mtb strains acquire drug resistances more rapidly *in vitro* (Ford et al., 2013), and are also more likely to develop RIF resistance after becoming resistant to INH (Torres Ortiz et al., 2021). This may reflect factors such as different mutation rates or epistatic interactions with lineage- or sublineage-associated mutations (Borrell and Gagneux, 2011; Ford et al., 2013; Torres Ortiz et al., 2021).

Other epistatic effects have been described. Fenner et al. discovered that compared to other lineages, L1 strains are more likely to carry a specific a promoter mutation in *inhA*, a drug resistance determinant that encodes the enzymatic target of INH (Fenner et al., 2012). In addition, Mtb genetic background modulates the level of INH resistance conferred by other mutations known to mediate resistance, which suggests an epistatic interaction between drug resistance mutations and lineage genetic diversity (Fenner et al., 2012). Similarly, the essentiality of *katG*, the activator of INH, was shown to differ depending on strain genetic background (Carey et al., 2018). Further, the degree of essentiality for *katG* is associated with the

katG mutation rate, suggesting that the epistatic interaction between *katG* essentiality and strain genetic background is associated with the development of drug resistance (Carey et al., 2018).

Torres et al. identified "pre-resistance" genomic loci and polymorphisms associated with increased risk for drug resistance acquisition which could inform precision medicine efforts to predict and preempt the occurrence (Torres Ortiz et al., 2021). Following the idea of pre-resistance, a subset of L2 strains carry an ancestral polymorphism in *gidB*, which could mediate some level of intrinsic SM resistance (Spies et al., 2011). Additionally, a subgroup of MDR L4 strains share a mutation in *tlyA*, a gene that mediates resistance to the second-line drug capreomycin (Walker et al., 2018; Merker et al., 2020). And fortunately, altered susceptibility mediated by clade-defining mutations is not always bad news. For example, a LOF mutation in *whib7* shared by a subgroup of L1 strains results in increased macrolide sensitivity (Li et al., 2022). This subgroup is estimated to cause 43,000 cases of MDR-TB per year (Li et al., 2022; Edokimov et al., 2022). Further, specific clades of L1 and L4 carry LOF mutations in *mmpl5* that could render them hypersusceptible to bedaquiline (BDQ) and clofazimine (CFZ) (Andries et al., 2014; Merker et al., 2020). Given the geographical distributions of Mtb lineage subgroups, these data underscore the need for drug susceptibility diagnostics that detect variants associated with the regional context (Manson et al., 2017).

Host-pathogen interactions shaping Mtb drug responses

In the cases of lineage or sublineage defining variants in genes like *whib7* or *mmpl5*, Mtb strains have acquired altered sensitivity to drugs they have not seen before. This is often attributed to genetic drift. However, the alternative model is that there are some host environments which select for bacterial features that that are also advantageous to Mtb in the face of drug (Hicks et al., 2018; Safi et al., 2019). By identifying variants that alter Mtb antibiotic susceptibility in host-relevant contexts, the field has diversified the suite of candidate mutations that could improve molecular diagnostics.

Several studies have linked Mtb antibiotic sensitivity to features of the host environment such as carbon source availability. For example, Mtb glycerol starvation triggers a stress-resistance response that in turn promote multidrug antibiotic tolerance (Safi et al., 2019). This phenotype is mediated by transient frameshift mutations that impair the glycerol kinase-encoding gene *glpK*, mutations in which are associated with MDR and XDR Mtb strains (Bellerose et al., 2019; Safi et al., 2019). Nonsynonmoyous *glpK* mutations were identified in 6.6% of Mtb strains from a Peruvian cohort (Bellerose et al., 2019). Mutations in *glpK* accrue during *in vitro* drug treatment and human infection, so this serves as another example of how bacterial adaptation to the host may augment antibiotic susceptibility (Bellerose et al., 2019; Safi et al., 2019; Vargas and Farhat, 2020). Mutations in the transcription factor encoded by *prpR* are associated with drug resistant Mtb clinical strains (Hicks et al., 2018). *prpR* mutations mediate drug tolerance to INH, RIF, and the second-line antibiotic ofloxacin (OFL) only during macrophage infection or in liquid media supplemented with propionate (Hicks et al., 2018). *prpR* mutations are enriched in Chinese clinical isolates, with a prevalence of 8-10% (Hicks et al., 2018). The glycerol and propionate phenotypes are consistent with other studies that investigated Mtb drug tolerance and phosphoenolpyruvate starvation in the context of the nonreplicating state induced by hypoxia, a stress relevant to the lung environment that selects for resistance (Liu et al., 2016; Lim et al., 2021). Similarly, Dhar and McKinney discovered that *cydC* and the rv0096–rv0101 gene set are persistence genetic determinants that alter Mtb clearance in C57BL/6 mice treated with INH (Dhar and McKinney, 2010). The persistence phenotypes are dependent on the mouse tissue environment, suggesting an interaction between the Mtb physiological changes caused by mutations in these genes, INH, and components of the mouse tissue (Dhar and McKinney, 2010).

Bacterial variants and differences in host environments are likely to be compounded by host differences in the pharmacokinetics and pharmacodynamics of drug metabolism. It is clear that drug exposure matters. RIF concentrations 2 hours post-dosing is associated with TB treatment success (Ramachandran et al., 2017). Further, Chigutsa et al. determined that Mtb infection sterilization can be predicted by a nonlinear relationship between patient antibiotic concentrations and bacterial MICs (Chigutsa et al., 2015). Indeed, data suggest that altered susceptibility indicated by strain associated differences in MIC can be overcome with increased dosing (Ruesen et al., 2018).

Conclusion and discussion

Traditionally, Mtb antibiotic response studies focused on bacterial mutations in genes involved in the drug mechanism of action and MIC shifts above the resistance threshold. But examination of the larger role of Mtb genetic diversity and the associated clinically-relevant drug phenotypes has unearthed new avenues that can be exploited to develop the precision medicine toolkit and ultimately improve patient outcomes (Cohen et al., 2019) (Figure 1). By expanding the scope of molecular diagnostic tests, we can identify mutations in strains that allow us to predict antibiotic sensitivities and treatment failure, which direct us in optimizing the drug regimen and even dosing to maximize the likelihood of treatment success.

We recognize that there are barriers to precision medicine in TB, including the cost of developing and implementing new tools and also epistatic interactions between diagnostic Mtb mutations and bacterial and host factors that could alter the phenotype penetrance and predictive power across populations. Therefore, we propose further research into understanding the

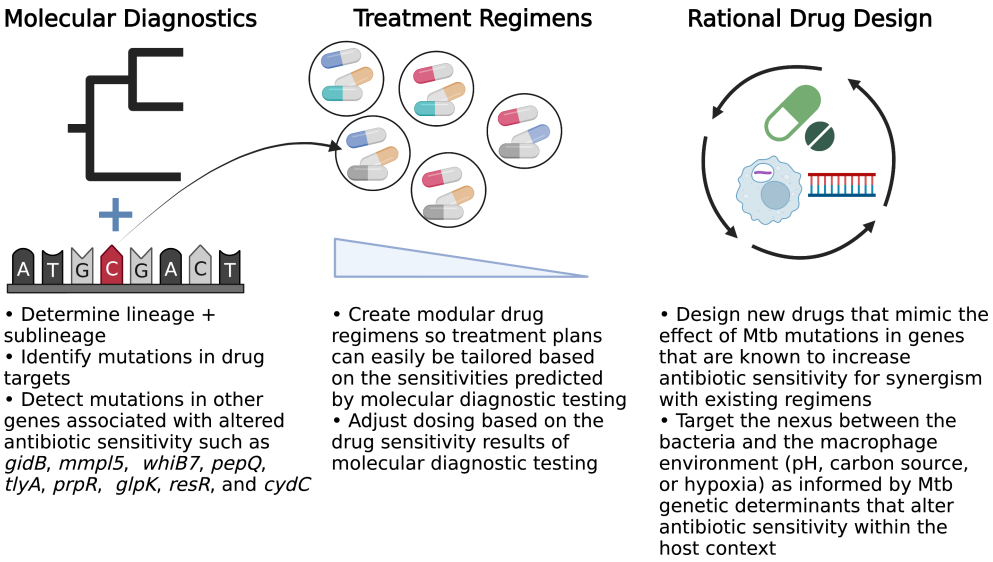


FIGURE 1
Opportunities to implement precision medicine approaches to improve TB treatment. BioRender.com

prevalence and penetrance of potentially diagnostic mutations. This necessitates more studies that collect treatment outcome data and also WGS Mtb isolates. It is true that individual mutations may only have the power to predict resistance in a portion of strains, for example *gyrA* mutations only occur in 50-90% of fluoroquinolone-resistant clinical Mtb isolates (Brossier et al., 2010; Yin and Yu, 2010; Singh et al., 2015). To cover the lower end of the range, we should consider finding suites of mutations that together highly associate with resistance or clinical outcomes, a method that has already shown to be effective (Walker et al., 2015).

We can start by exploring mutations noted in a 2021 WHO catalogue of mutations associated with drug resistance, but in order to improve upon the growing collection of Mtb mutations with diagnostic potential, we should diversify our repertoire of phenotypes of clinical interest beyond just MIC (Walker et al., 2022). For example, further exploration into prevalent mutations in Mtb clinical isolates that modulate MBC, rather than just MIC, could reveal interesting predictors of host-relevant antibiotic tolerance phenotypes (Kalia et al., 2017; Sarathy et al., 2018; Dutta et al., 2019; Kreutzfeldt et al., 2022). Further, we should integrate Mtb strain characteristics with information regarding patient factors such as genetics, metabolism, environment, and geographic location to find novel ways to optimize treatment regimens and dosing. Given the presence of sublineage-defining mutations in genes associated with resistance and the endemic nature of these strains, even-high level patient information such as country or region of origin could inform which antibiotics may confer the maximum benefit (Manson et al., 2017; Walker et al., 2018; Hicks et al., 2020; Merker et al., 2020; Li et al., 2022). In the future, exploiting what we know and what we discover regarding host-pathogen interactions and altered antibiotic sensitivity can inform rational drug and rational drug regimen design. But in the meantime, more effort should go towards repurposing existing drugs by making TB antibiotic regimens and dosing modular so that treatment plans can be easily tuned for precise care.

We can no longer afford to take a one-size fits all approach to TB. It is our responsibility to leverage bench science advancement to improve patient outcomes *via* precision medicine.

Author contributions

SS, SF, and QL contributed to the conceptual framework of the review. SS wrote the first draft of the manuscript and SF wrote additional sections. SS, SF, and QL completed edits and revisions. All authors contributed to the article and approved the submitted version.

Funding

This study was funded by NIH/NIAID (P01AI132130, T32AI049928, T32AI132120).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Almeida, D., Ioerger, T., Tyagi, S., Li, S. Y., Mdluli, K., Andries, K., et al. (2016). Mutations in pepQ confer low-level resistance to bedaquiline and clofazimine in mycobacterium tuberculosis. *Antimicrobial Agents chemotherapy* 60 (8), 4590–4599. doi: 10.1128/AAC.00753-16

Andries, K., Villellas, C., Coeck, N., Thys, K., Gevers, T., Vranckx, L., et al. (2014). Acquired resistance of mycobacterium tuberculosis to bedaquiline. *PloS One* 9 (7), e102135. doi: 10.1371/journal.pone.0102135

An East African and British Medical Research Council Co-operative Investigation (1978). Tuberculosis in Kenya: A second national sampling survey of drug resistance and other factors, and a comparison with the prevalence data from the first national sampling survey. *Tubercle* 59 (3), 155–177. doi: 10.1016/0041-3879(78)90023-5

CRyPTIC consortium and the 100,000 genomes project (2018). Prediction of susceptibility to first-line tuberculosis drugs by DNA sequencing. *New Engl. J. Med.* 379 (15), 1403–1415. doi: 10.1056/NEJMoa1800474

Bellerose, M. M., Baek, S. H., Huang, C. C., Moss, C. E., Koh, E. I., Proulx, M. K., et al. (2019). Common variants in the glycerol kinase gene reduce tuberculosis drug efficacy. *MBio* 10 (4), e00663-19. doi: 10.1128/mBio.00663-19

Blowers, D. D., and Cooke, D. J. (1954). Changes in isoniazid resistance of tubercle bacilli after cessation of treatment. *Thorax* 9, 254. doi: 10.1136/thx.9.4.254

Boehme, C. C., Nabeta, , Hillemann, D., Nicol, M. , Shenai, S., Krapp, F., et al. (2010). Rapid molecular detection of tuberculosis and rifampin resistance. *New Engl. J. Med.* 363 (11), 1005–1015. doi: 10.1056/NEJMoa0907847

Boldrin, F., Provvedi, R., Cioetto Mazzabò, L., Segafreddo, G., and Manganelli, R. (2020). Tolerance and persistence to drugs: a main challenge in the fight against mycobacterium tuberculosis. *Front. Microbiol.* 11, 1924. doi: 10.3389/fmicb.2020.01924

Borrell, S., and Gagneux, S. (2011). Strain diversity, epistasis and the evolution of drug resistance in mycobacterium tuberculosis. *Clin. Microbiol. Infection* 17 (6), 815–820. doi: 10.1111/j.1469-0691.2011.03556.x

Brossier, F., Veziris, N., Aubry, A., Jarlier, V., and Sougakoff, W. (2010). Detection by GenoType MTBDR sl test of complex mechanisms of resistance to second-line drugs and ethambutol in multidrug-resistant mycobacterium tuberculosis complex isolates. *J. Clin. Microbiol.* 48 (5), 1683–1689. doi: 10.1128/JCM.01947-09

Bryant, J. M., Harris, S. R., Parkhill, J., Dawson, R., Diacon, A. H., van Helden, , et al. (2013). Whole-genome sequencing to establish relapse or re-infection with mycobacterium tuberculosis: A retrospective observational study. *Lancet Respir. Med.* 1 (10), 786–792. doi: 10.1016/S2213-2600(13)70231-5

Cao, Y., Parmar, H., Gaur, R. L., Lieu, D., Raghunath, S., Via, N., et al. (2021). Xpert MTB/XDR: A 10-color reflex assay suitable for point-of-care settings to detect isoniazid, fluoroquinolone, and second-line-injectable-drug resistance directly from mycobacterium tuberculosis-positive sputum. *J. Clin. Microbiol.* 59 (3), e02314-20. doi: 10.1128/JCM.02314-20

Carey, A. F., Rock, J. M., Krieger, I. V., Chase, M. R., Fernandez-Suarez, M., Gagneux, S., et al. (2018). TnSeq of mycobacterium tuberculosis clinical isolates reveals strain-specific antibiotic liabilities. *PloS Pathog.* 14 (3), e1006939. doi: 10.1371/journal.ppat.1006939

Chengalroyen, M. D., Beukes, G. M., Gordhan, B. G., Streicher, E. M., Churchyard, G., Hafner, R., et al. (2016). Detection and quantification of differentially culturable tubercle bacteria in sputum from patients with tuberculosis. *Am. J. Respir. Crit. Care Med.* 194 (12), 1532–1540. doi: 10.1164/rccm.201604-0769OC

Chigutsa, E., Pasipanodya, J. G., Visser, M. E., Van Helden, D., Smith, J., Sirgel, F. A., et al. (2015). Impact of nonlinear interactions of pharmacokinetics and MICs on sputum bacillary kill rates as a marker of sterilizing effect in tuberculosis. *Antimicrobial Agents chemotherapy* 59 (1), 38–45. doi: 10.1128/AAC.03931-14

Cohen, K. A., Abeel, T., Manson McGuire, A., Desjardins, C. A., Munsamy, V., Shea, T. , et al. (2015). Evolution of extensively drug-resistant tuberculosis over four decades: Whole genome sequencing and dating analysis of mycobacterium tuberculosis isolates from KwaZulu-natal. *PloS Med.* 12 (9), e1001880. doi: 10.1371/journal.pmed.1001880

Cohen, K. A., Manson, A. L., Desjardins, C. A., Abeel, T., and Earl, A. M. (2019). Deciphering drug resistance in mycobacterium tuberculosis using whole-genome sequencing: Progress, promise, and challenges. *Genome Med.* 11 (1), 1–pp18. doi: 10.1186/s13073-019-0660-8

Cohen, K. A., Stott, K. E., Munsamy, V., Manson, A. L., Earl, A. M., and Pym, A. S. (2020). Evidence for expanding the role of streptomycin in the management of drug-resistant mycobacterium tuberculosis. *Antimicrobial Agents chemotherapy* 64 (9), e00860–20. doi: 10.1128/AAC.00860-20

Colangeli, R., Jedrey, H., Kim, S., Connell, R., Ma, S., Chippada Venkata, U. D., et al. (2018). Bacterial factors that predict relapse after tuberculosis therapy. *New Engl. J. Med.* 379 (9), 823–833. doi: 10.1056/NEJMoa1715849

Coll, F., Phelan, J., Hill-Cawthorne, G. A., Nair, M. B., Mallard, K., Ali, S., et al. (2018). Genome-wide analysis of multi-and extensively drug-resistant mycobacterium tuberculosis. *Nat. Genet.* 50 (2), 307–316. doi: 10.1038/s41588-017-0029-0

Daniel, J., Maamar, H., Deb, C., Sirakova, T. D., and Kolattukudy, E. (2011). Mycobacterium tuberculosis uses host triacylglycerol to accumulate lipid droplets and acquires a dormancy-like phenotype in lipid-loaded macrophages. *PloS Pathog.* 7 (6), e1002093. doi: 10.1371/journal.ppat.1002093

Desjardins, C. A., Cohen, K. A., Munsamy, V., Abeel, T., Maharaj, K., Walker, B. J., et al. (2016). Genomic and functional analyses of mycobacterium tuberculosis strains implicate ald in d-cycloserine resistance. *Nat. Genet.* , 48 (5), 544–551. doi: 10.1038/ng.3548

Dhar, N., and McKinney, J. D. (2010). Mycobacterium tuberculosis persistence mutants identified by screening in isoniazid-treated mice. *Proc. Natl. Acad. Sci.* 107 (27), 12275–12280. doi: 10.1073/pnas.1003219107

Dorman, S. E., Nahid, , Kurbatova, E. V., Phillips, , Bryant, K., Dooley, K. E., et al. (2021). Four-month rifapentine regimens with or without moxifloxacin for tuberculosis. *New Engl. J. Med.* 384 (18), 1705–1718. doi: 10.1056/NEJMoa2033400

Dutta, N. K., Klinkenberg, L. G., Vazquez, M. J., Segura-Carro, D., Colmenarejo, G., Ramon, F., et al. (2019). Inhibiting the stringent response blocks mycobacterium tuberculosis entry into quiescence and reduces persistence. *Sci. Adv.* 5 (3), eaav2104. doi: 10.1126/sciadv.aav2104

Edokimov, K., Yamada, Y., Dary, C., Miow, Q. H., Hsu, L. Y., Ong, R. T. H., et al. (2022). Whole-genome sequencing of mycobacterium tuberculosis from Cambodia. *Sci. Re* 12 (1), 1–8. doi: 10.1038/s41598-022-10964-9

Farhat, M. R., Freschi, L., Calderon, R., Ioerger, T., Snyder, M., Meehan, C. J., et al. (2019). GWAS for quantitative resistance phenotypes in mycobacterium tuberculosis reveals resistance genes and regulatory regions. *Nat. Commun.* 10 (1), 1–pp11. doi: 10.1038/s41467-019-10110-6

Fenner, L., Egger, M., Bodmer, T., Altpeter, E., Zwahlen, M., Jaton, K., et al. (2012). Effect of mutation and genetic background on drug resistance in mycobacterium tuberculosis. *Antimicrobial Agents chemotherapy* 56 (6), 3047–3053. doi: 10.1128/AAC.06460-11

Finken, M., Kirschner, , Meier, A., Wrede, A., and Böttger, E. C. (1993). Molecular basis of streptomycin resistance in mycobacterium tuberculosis: Alterations of the ribosomal protein S12 gene and point mutations within a functional 16S ribosomal RNA pseudoknot. *Mol. Microbiol.* 9 (6), 1239–1246. doi: 10.1111/j.1365-2958.1993.tb01253.x

Ford, C. B., Shah, R. R., Maeda, M. K., Gagneux, S., Murray, M. B., Cohen, T., et al. (2013). Mycobacterium tuberculosis mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nat. Genet.* 45 (7), 784–790. doi: 10.1038/ng.2656

Fox, W., Ellard, G. A., and Mitchison, D. A. (1999). Studies on the treatment of tuberculosis undertaken by the British medical research council tuberculosis units, 1946–1986, with relevant subsequent publications. *Int. J. Tuberculosis Lung Dis.* 3 (10), S231–S279.

Fox, W., Sutherland, I., and Daniels, M. (1954). A five-year assessment of patients in a controlled trial of streptomycin in pulmonary tuberculosis. *Q. J. Med.* 23 (91), 347–366.

Garton, N. J., Waddell, S. J., Sherratt, A. L., Lee, S. M., Smith, R. J., Senner, C., et al. (2008). Cytological and transcript analyses reveal fat and lazy persister-like

bacilli in tuberculous sputum. *PloS Med.* 5 (4), e75. doi: 10.1371/journal.pmed.0050075

Gygli, S. M., Borrell, S., Trauner, A., and Gagneux, S. (2017). Antimicrobial resistance in mycobacterium tuberculosis: Mechanistic and evolutionary perspectives. *FEMS Microbiol. Rev.* 41 (3), 354–373. doi: 10.1093/femsre/fux011

Hazbón, M. H., Brimacombe, M., Bobadilla del Valle, M., Cavatore, M., Guerrero, M. I., Varma-Basil, M., et al. (2006). Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant mycobacterium tuberculosis. *Antimicrobial Agents chemotherapy* 50 (8), 2640–2649. doi: 10.1128/AAC.00112-06

Hicks, N. D., Carey, A. F., Yang, J., Zhao, Y., and Fortune, S. M. (2019). Bacterial genome-wide association identifies novel factors that contribute to ethionamide and prothionamide susceptibility in mycobacterium tuberculosis. *MBio* 10 (2), e00616–19. doi: 10.1128/mBio.00616-19

Hicks, N. D., Giffen, S. R., Culviner, H., Chao, M. C., Dulberger, C. L., Liu, Q., et al. (2020). Mutations in dnaA and a cryptic interaction site increase drug resistance in mycobacterium tuberculosis. *PloS Pathog.* 16 (11), e1009063. doi: 10.1371/journal.ppat.1009063

Hicks, N. D., Yang, J., Zhang, X., Zhao, B., Grad, Y. H., Liu, L., et al. (2018). Clinically prevalent mutations in mycobacterium tuberculosis alter propionate metabolism and mediate multidrug tolerance. *Nat. Microbiol.* 3 (9), 1032–1042. doi: 10.1038/s41564-018-0218-3

Hong Kong Government Tuberculosis ServiceBritish Medical Research Council Co-operative Investigation, A. (1964). Drug-resistance in patients with pulmonary tuberculosis presenting at chest clinics in Hong Kong. *Tubercle* 45 (2), 77–95. doi: 10.1016/S0041-3879(64)80064-7

Jindani, A., Harrison, T. S., Nunn, A. J., Phillips, , Churchyard, G. J., Charalambous, S., et al. (2014). High-dose rifapentine with moxifloxacin for pulmonary tuberculosis. *N Engl. J. Med.* 371, 1599–1608. doi: 10.1056/NEJMoa1314210

Juréen, , Werngren, J., Toro, J. C., and Hoffner, S. (2008). Pyrazinamide resistance and pncA gene mutations in mycobacterium tuberculosis. *Antimicrobial Agents chemotherapy* 52 (5), 1852–1854. doi: 10.1128/AAC.00110-08

Kalia, N. , Hasenoehrl, E. J., Ab Rahman, N. B., Koh, V. H., Ang, M. L., Sajorda, D. R., et al. (2017). Exploiting the synthetic lethality between terminal respiratory oxidases to kill mycobacterium tuberculosis and clear host infection. *Proc. Natl. Acad. Sci.* 114 (28), 7426–7431. doi: 10.1073/pnas.1706139114

Kreutzfeldt, K. M., Jansen, R. S., Hartman, T. E., Gouzy, A., Wang, R., Krieger, I. V., et al. (2022). CinA mediates multidrug tolerance in mycobacterium tuberculosis. *Nat. Commun.* 13 (1), 1–pp11. doi: 10.1038/s41467-022-29832-1

Lai, Y. , and Ioerger, T. R. (2020). Exploiting homoplasy in genome-wide association studies to enhance identification of antibiotic-resistance mutations in bacterial genomes. *Evolutionary Bioinf.* 16, 1176934320944932. doi: 10.1177/1176934320944932

Lange, C., Aarnoutse, R., Chesov, D., Van Crevel, R., Gillespie, S. H., Grobbel, H. , et al. (2020). Perspective for precision medicine for tuberculosis. *Front. Immunol.* 11, 566608. doi: 10.3389/fimmu.2020.566608

Lavender, C., Globan, M., Sievers, A., Billman-Jacobe, H., and Fyfe, J. (2005). Molecular characterization of isoniazid-resistant mycobacterium tuberculosis isolates collected in Australia. *Antimicrobial Agents chemotherapy* 49 (10), 4068–4074. doi: 10.1128/AAC.49.10.4068-4074.2005

Lee, A. S., Teo, A. S., and Wong, S. Y. (2001). Novel mutations in ndh in isoniazid-resistant mycobacterium tuberculosis isolates. *Antimicrobial Agents chemotherapy* 45 (7), 2157–2159. doi: 10.1128/AAC.45.7.2157-2159.2001

Lempens, , Meehan, C. J., Vandelannoote, K., Fissette, K., de Rijk, , Van Deun, A., et al. (2018). Isoniazid resistance levels of mycobacterium tuberculosis can largely be predicted by high-confidence resistance-conferring mutations. *Sci. Re* 8 (1), 1–9. doi: 10.1038/s41598-018-21378-x

Lewis, K., and Shan, Y. (2017). Why tolerance invites resistance. *Science* 355 (6327), 796–796. doi: 10.1126/science.aam7926

Lim, J., Lee, J. J., Lee, S. K., Kim, S., Eum, S. Y., and Eoh, H. (2021). Phosphoenolpyruvate depletion mediates both growth arrest and drug tolerance of mycobacterium tuberculosis in hypoxia. *Proc. Natl. Acad. Sci.* 118 (35), e2105800118. doi: 10.1073/pnas.2105800118

Li, S., Poulton, N. C., Chang, J. S., Azadian, Z. A., Dejusus, M. A., Ruecker, N., et al. (2022). CRISPRi chemical genetics and comparative genomics identify genes mediating drug potency in Mycobacterium tuberculosis. *Nature Microbiology* 1–14. doi: 10.1101/2021.11.27.469863

Liu, Z., Gao, Y., Yang, H., Bao, H., Qin, L., Zhu, C., et al. (2016). Impact of hypoxia on drug resistance and growth characteristics of mycobacterium tuberculosis clinical isolates. *PloS One* 11 (11), e0166052. doi: 10.1371/journal.pone.0166052

Liu, Q., Yang, D., Qiu, B., Martinez, L., Ji, Y., Song, H., et al. (2021). Drug resistance gene mutations and treatment outcomes in MDR-TB: A prospective study in Eastern China. *PloS Negl. Tro Dis.* 15 (1), e0009068. doi: 10.1371/journal.pntd.0009068

Liu, Q., Zhu, J., Dulberger, C. L., Stanley, S., Wilson, S., Chung, E. S., et al. (2022). Tuberculosis treatment failure associated with evolution of antibiotic resilience. *bioRxiv*. doi: 10.1101/2022.03.29.486233

Malik, S., Willby, M., Sikes, D., Tsodikov, O. V., and Posey, J. E. (2012). New insights into fluoroquinolone resistance in mycobacterium tuberculosis: Functional genetic analysis of gyrA and gyrB mutations. *PloS One* 7 (6), e39754. doi: 10.1371/journal.pone.0039754

Manson, A. L., Abeel, T., Galagan, J. E., Sundaramurthi, J. C., Salazar, A., Gehrmann, T., et al. (2017). Mycobacterium tuberculosis whole genome sequences from southern India suggest novel resistance mechanisms and the need for region-specific diagnostics. *Clin. Infect. Dis.* 64 (11), 1494–1501. doi: 10.1093/cid/cix169

Marshall, G., Blacklock, J. S., Cameron, C., Capon, N. B., Cruickshank, R., Gaddum, J. H., et al. (1948). Streptomycin treatment of tuberculous meningitis. *Lancet* 254, 582–596.

Martini, M. C., Hicks, N. D., Xiao, J., Alonso, M. N., Barbier, T., Sixsmith, J., et al. (2022). Loss of RNase J leads to multi-drug tolerance and accumulation of highly structured mRNA fragments in mycobacterium tuberculosis. *PloS Pathog.* 18 (7), e1010705. doi: 10.1371/journal.ppat.1010705

Medical Research Council (1953). Laboratory techniques for the determination of sensitivity of tubercle bacilli to isoniazid, streptomycin and PAS. *Lancet* 2, 213.

Merker, M., Kohl, T. A., Barilar, I., Andres, S., Fowler, W., Chryssanthou, E., et al. (2020). Phylogenetically informative mutations in genes implicated in antibiotic resistance in mycobacterium tuberculosis complex. *Genome Med.* 12 (1), 1–8. doi: 10.1186/s13073-020-00726-5

Mitchison, D. A. (1949). Tests for streptomycin sensitivity of tubercle bacilli in tween 80 albumin liquid medium. *Lancet* 254, 694–696. doi: 10.1016/S0140-6736(49)91327-6

National Committee for Clinical Laboratory Standards, Barry, A. L. (1999). *Methods for determining bactericidal activity of antimicrobial agents: Approved guideline* Vol. Vol. 19, No. 18) (Wayne, PA: National Committee for Clinical Laboratory Standards).

Oppong, Y. E., Phelan, J., Perdigão, J., Machado, D., Miranda, A., Portugal, I., et al. (2019). Genome-wide analysis of mycobacterium tuberculosis polymorphisms reveals lineage-specific associations with drug resistance. *BMC Genomics* 20 (1), 1–pp15. doi: 10.1186/s12864-019-5615-3

Palomino, J. C., and Martin, A. (2014). Drug resistance mechanisms in mycobacterium tuberculosis. *Antibiotics* 3 (3), 317–340. doi: 10.3390/antibiotics3030317

Penn-Nicholson, A., Georghiou, S. B., Ciobanu, N., Kazi, M., Bhalla, M., David, A., et al. (2022). Detection of isoniazid, fluoroquinolone, ethionamide, amikacin, kanamycin, and capreomycin resistance by the xpert MTB/XDR assay: a cross-sectional multicentre diagnostic accuracy study. *Lancet Infect. Dis.* 22 (2), 242–249. doi: 10.1016/S1473-3099(21)00452-7

Ramachandran, G., Agibothu Kupparam, H. K., Vedhachalam, C., Thiruvengadam, K., Rajagandhi, V., Dusthackeer, A., et al. (2017). Factors influencing tuberculosis treatment outcome in adult patients treated with thrice-weekly regimens in India. *Antimicrobial Agents chemotherapy* 61 (5), e02464-16. doi: 10.1128/AAC.02464-16

Ramaswamy, S. V., Reich, R., Dou, S. J., Jasperse, L., Pan, X., Wanger, A., et al. (2003). Single nucleotide polymorphisms in genes associated with isoniazid resistance in mycobacterium tuberculosis. *Antimicrobial Agents chemotherapy* 47 (4), 1241–1250. doi: 10.1128/AAC.47.4.1241-1250.2003

Rozwarski, D. A., Grant, G. A., Barton, D. H., Jacobs, W. R.Jr., and Sacchettini, J. C. (1998). Modification of the NADH of the isoniazid target (InhA) from mycobacterium tuberculosis. *Science* 279 (5347), 98–pp102. doi: 10.1126/science.279.5347.98

Ruesen, C., Riza, A. L., Florescu, A., Chaidir, L., Editoiu, C., Aalders, N., et al. (2018). Linking minimum inhibitory concentrations to whole genome sequence-predicted drug resistance in mycobacterium tuberculosis strains from Romania. *Sci. Re* 8 (1), 1–8. doi: 10.1038/s41598-018-27962-5

Safi, H., Gopal, , Lingaraju, S., Ma, S., Levine, C., Dartois, V., et al. (2019). Phase variation in mycobacterium tuberculosis glpK produces transiently heritable drug tolerance. *Proc. Natl. Acad. Sci.* 116 (39), 19665–19674. doi: 10.1073/pnas.1907631116

Sarathy, J. , Via, L. E., Weiner, D., Blanc, L., Boshoff, H., Eugenin, E. A., et al. (2018). Extreme drug tolerance of mycobacterium tuberculosis in caseum. *Antimicrobial Agents chemotherapy* 62 (2), e02266–e02217. doi: 10.1128/AAC.02266-17

Schatz, A., Bugle, E., and Waksman, S. A. (1944). Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. *Proc. Soc. Ex Biol. Med.* 55 (1), 66–69. doi: 10.3181/00379727-55-14461

Schön, T., Werngren, J., Machado, D., Borroni, E., Wijkander, M., Lina, G., et al. (2020). Antimicrobial susceptibility testing of mycobacterium tuberculosis complex isolates–the EUCAST broth microdilution reference method for MIC determination *Clin Microbiol Infect.* 26(11), 1488–92. doi: 10.1016/j.cmi.2020.07.036

Schrader, S. M., Vaubourgeix, J., and Nathan, C. (2020). Biology of antimicrobial resistance and approaches to combat it. *Sci. Trans. Med.* 12 (549), eaaz6992. doi: 10.1126/scitranslmed.aaz6992

Scorpio, A., Lindholm-Levy, , Heifets, L., Gilman, R., Siddiqi, S., Cynamon, M., et al. (1997). Characterization of pncA mutations in pyrazinamide-resistant mycobacterium tuberculosis. *Antimicrobial Agents chemotherapy* 41 (3), 540–543. doi: 10.1128/AAC.41.3.540

Shanmugam, S. K., Kumar, N., Sembulingam, T., Ramalingam, S. B., Selvaraj, A., Rajendhiran, U., et al. (2022). Mycobacterium tuberculosis lineages associated with mutations and drug resistance in isolates from India. *Microbiol. Spectr.* 10 e01594-21. doi: 10.1128/spectrum.01594-21

Singh, , Jain, A., Dixit, , Prakash, S., Jaiswal, I., Venkatesh, V., et al. (2015). Prevalence of gyrA and b gene mutations in fluoroquinolone-resistant and-sensitive clinical isolates of mycobacterium tuberculosis and their relationship with MIC of ofloxacin. *J. antibiotics* 68 (1), 63–66. doi: 10.1038/ja.2014.95

Slayden, R. A., Dawson, C. C., and Cummings, J. E. (2018). Toxin–antitoxin systems and regulatory mechanisms in mycobacterium tuberculosis. *Pathog. Dis.* 76 (4), fty039. doi: 10.1093/femspd/fty039

Spies, F. S., Ribeiro, A. W., Ramos, D. F., Ribeiro, M. O., Martin, A., Palomino, J. C., et al. (2011). Streptomycin resistance and lineage-specific polymorphisms in mycobacterium tuberculosis gidB gene. *J. Clin. Microbiol.* 49 (7), 2625–2630. doi: 10.1128/JCM.00168-11

Sreevatsan, S., Stockbauer, K. E., Pan, X. I., Kreiswirth, B. N., Moghazeh, S. L., Jacobs, W. R.Jr., et al. (1997). Ethambutol resistance in mycobacterium tuberculosis: Critical role of embB mutations. *Antimicrobial Agents chemotherapy* 41 (8), 1677–1681. doi: 10.1128/AAC.41.8.1677

The World Health Organization (2018) *Technical report on critical concentrations for drug susceptibility testing of medicines used in the treatment of drug-resistant tuberculosis*. Available at: https://www.who.int/publications/i/item/WHO-CDS-TB-2018.5.

The World Health Organization (2021) *TB disease burden*. Available at: https://www.who.int/publications/digital/global-tuberculosis-report-2021/tb-disease-burden.

Torres Ortiz, A., Coronel, J., Vidal, J. R., Bonilla, C., Moore, D. A., Gilman, R. H., et al. (2021). Genomic signatures of pre-resistance in mycobacterium tuberculosis. *Nat. Commun.* 12 (1), 1–pp13. doi: 10.21203/rs.3.rs-364747/v1

Turapov, O., O'Connor, B. D., Sarybaeva, A. A., Williams, C., Patel, H., Kadyrov, A. S., et al. (2016). Phenotypically adapted mycobacterium tuberculosis populations from sputum are tolerant to first-line drugs. *Antimicrobial Agents chemotherapy* 60 (4), 2476–2483. doi: 10.1128/AAC.01380-15

Turkova, A., Wills, G. H., Wobudeya, E., Chabala, C., Palmer, M., Kinikar, A., et al. (2022). Shorter treatment for nonsevere tuberculosis in African and Indian children. *New Engl. J. Med.* 386 (10), 911–922. doi: 10.1056/NEJMoa2104535

Vargas, R.Jr., and Farhat, M. R. (2020). Antibiotic treatment and selection for glpK mutations in patients with active tuberculosis disease. *Proc. Natl. Acad. Sci.* 117 (8), 3910–3912. doi: 10.1073/pnas.1920788117

Walker, T. M., Kohl, T. A., Omar, S. V., Hedge, J., Elias, C. D. O., Bradley, , et al. (2015). Whole-genome sequencing for prediction of mycobacterium tuberculosis drug susceptibility and resistance: a retrospective cohort study. *Lancet Infect. Dis.* 15 (10), 1193–1202. doi: 10.1016/S1473-3099(15)00062-6

Walker, T. M., Merker, M., Knoblauch, A. M., Helbling, , Schoch, O. D., van der Werf, M. J., et al. (2018). A cluster of multidrug-resistant mycobacterium tuberculosis among patients arriving in Europe from the horn of Africa: A molecular epidemiological study. *Lancet Infect. Dis.* 18 (4), 431–440. doi: 10.1016/S1473-3099(18)30004-5

Walker, T. M., Miotto, , Köser, C. U., Fowler, W., Knaggs, J., Iqbal, Z., et al. (2022). The 2021 WHO catalogue of mycobacterium tuberculosis complex mutations associated with drug resistance: a genotypic analysis. *Lancet Microbe* 3 (4), e265–e273. doi: 10.1016/S2666-5247(21)00301-3

Wong, S. Y., Lee, J. S., Kwak, H. K., Via, L. E., Boshoff, H. I., and Barry, C. E.III (2011). Mutations in gidB confer low-level streptomycin resistance in mycobacterium tuberculosis. *Antimicrobial Agents chemotherapy* 55 (6), 2515–2522. doi: 10.1128/AAC.01814-10

Yin, X., and Yu, Z. (2010). Mutation characterization of gyrA and gyrB genes in levofloxacin-resistant mycobacterium tuberculosis clinical isolates from guangdong province in China. *J. Infection* 61 (2), 150–154. doi: 10.1016/j.jinf.2010.05.001

Zhang, H., Li, D., Zhao, L., Fleming, J., Lin, N., Wang, T., et al. (2013). Genome sequencing of 161 mycobacterium tuberculosis isolates from China identifies genes and intergenic regions associated with drug resistance. *Nat. Genet.* 45 (10), 1255–1260. doi: 10.1038/ng.2735

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 05 October 2022
DOI 10.3389/fcimb.2022.1027394

OPEN ACCESS

EDITED BY
Ben Gold,
Weill Cornell Medicine, United States

REVIEWED BY
Thomas Dick,
Hackensack Meridian Health, United States
Anna D. Tischler,
University of Minnesota Twin Cities, United States
Alexandre Gouzy,
NewYork-Presbyterian, United States

*CORRESPONDENCE
David R. Sherman
dsherman@uw.edu

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 24 August 2022
ACCEPTED 20 September 2022
PUBLISHED 05 October 2022

CITATION
Jones RM, Adams KN, Eldesouky HE and Sherman DR (2022) The evolving biology of *Mycobacterium tuberculosis* drug resistance. *Front. Cell. Infect. Microbiol.* 12:1027394. doi: 10.3389/fcimb.2022.1027394

The evolving biology of *Mycobacterium tuberculosis* drug resistance

Richard M. Jones, Kristin N. Adams, Hassan E. Eldesouky and David R. Sherman*

Department of Microbiology, University of Washington, Seattle, WA, United States

Tuberculosis, caused by *Mycobacterium tuberculosis* (Mtb) is an ancient disease that has remained a leading cause of infectious death. Mtb has evolved drug resistance to every antibiotic regimen ever introduced, greatly complicating treatment, lowering rates of cure and menacing TB control in parts of the world. As technology has advanced, our understanding of antimicrobial resistance has improved, and our models of the phenomenon have evolved. In this review, we focus on recent research progress that supports an updated model for the evolution of drug resistance in Mtb. We highlight the contribution of drug tolerance on the path to resistance, and the influence of heterogeneity on tolerance. Resistance is likely to remain an issue for as long as drugs are needed to treat TB. However, with technology driving new insights and careful management of newly developed resources, antimicrobial resistance need not continue to threaten global progress against TB, as it has done for decades.

KEYWORDS

tuberculosis, mycobacteria, resistance, tolerance, heterogeneity, antibiotics

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), has remained a centrally important cause of morbidity and mortality for centuries, but our understanding of TB disease, what causes it and how to combat it has evolved substantially over that time. In some of the earliest written records of the disease, the ancient Greeks recognized that TB was especially deadly, with the venerable "Father of Medicine" Hippocrates warning other physicians against treating advanced cases because the inevitable bad outcomes would damage the doctor's reputation (Herzog, 1998). Eventually, the 19th century discovery of the TB bacillus and the 20th century introduction of effective chemotherapies seemed to promise a new era in which TB was tamed if not eliminated (Daniel, 2006; Barberis et al., 2017). However, the emergence of drug-resistant isolates was noted in the very first TB chemotherapy trials, and

resistance has appeared whenever a new anti-TB agent is introduced (Crofton and Mitchison, 1948; Gillespie, 2002). With an estimated 1.3 million deaths in 2020 (WHO, 2021b), and given the dramatic change in world population, TB may claim as many total lives today as in the years before TB chemotherapy was available. This dismal situation has several causes, including co-morbidities like HIV and diabetes, and gaps in timely diagnosis and treatment, but drug resistance stands widely recognized as one of the major challenges to effective TB control worldwide.

The classical model of drug treatment and resistance

Just as ideas about TB have changed, thinking about drug resistance has undergone a significant evolution over time. Antibiotics were initially hailed as "magic bullets", capable of stopping even lethal infectious diseases in their tracks, but the emergence of resistance-fueled treatment failures led to deeper investigations into the biology of drug response. Driven by powerful advances in genetics and the emergence of molecular biology in the latter half of the 20th century, a concise model of antibiotic action and resistance developed over decades. Briefly, this model proposes that antibiotics work by inhibiting some essential target, generally an enzyme, in the pathogen. Mutations occur at random and exist in each population prior to antibiotic pressure. Resistance emerges when pre-existing mutations promote growth or survival in the presence of the drug (Figure 1, left side). A corollary of this model is that resistance is a numbers game. Any pathogen population of sufficient size will harbor at least one mutation conferring resistance to each agent that can be selected by drug exposure, so adding additional drugs to a regimen serves to reduce the rate at which resistance emerges.

Grounded in Darwinian natural selection, this model has long provided a useful framework for exploring antibiotic action and resistance. However, the available data have never all fit comfortably within this paradigm. For example, while current treatment for drug-susceptible TB is ~95% effective, most treatment failures are due to relapses with drug sensitive bacilli (Colangeli et al., 2018). These relapses are hard to explain with the classical model of drug treatment and resistance, since any drug-susceptible bacterium exposed to antibiotics should be eliminated. This phenomenon is not unique to TB – studies with many different bacteria have long revealed curious phenotypes of enhanced survival in the absence of any heritable resistance that are not readily explained by the classical model (Bigger, 1944; Levin and Rozen, 2006). Further, the rate of spontaneous resistance to a three-drug combination has been estimated at greater than 1 in 10^{18} bacteria (Gillespie, 2007), many orders of magnitude greater than the number of TB

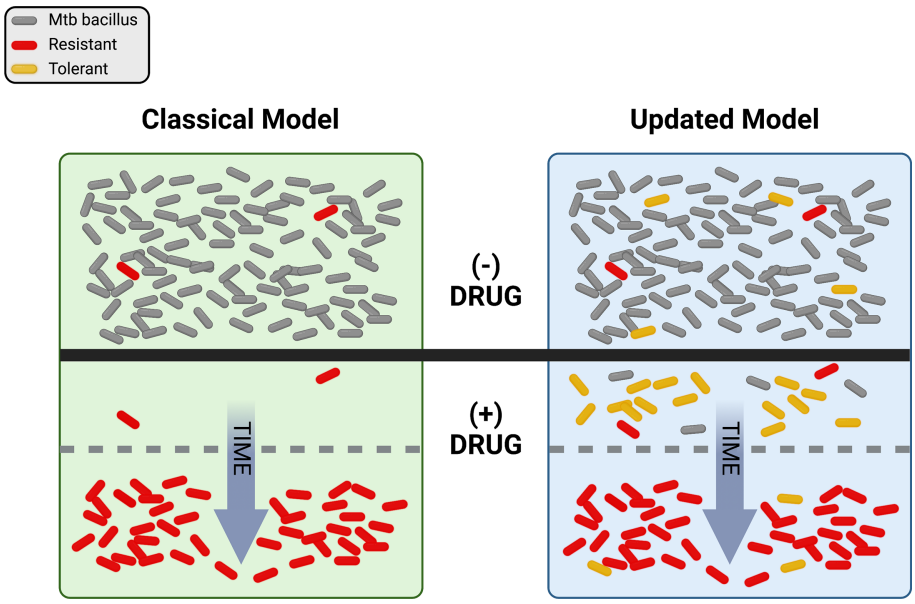


FIGURE 1
Visual representation of the classical and updated models for development of antibiotic resistance in Mtb. The top portion shows a population of Mtb in the absence of drug selection (- DRUG). In the classical model, pre-existing resistant bacilli (red) in the population grow despite the addition of drug (+ DRUG) and susceptible bacteria (gray) are eliminated over time until only resistant cells remain. In the updated model, pre-existing resistance may still be present, but tolerant bacilli (yellow) may also be present and/or be induced by drug exposure. With drug selection, susceptible bacteria are again eventually eliminated, with resistance dominating the population over time. Figure created with BioRender.com.

bacilli in any one patient, possibly more than in all current patients combined. Even considering how some drugs are excluded from some niches *in vivo* (Strydom et al., 2019), it is difficult to reconcile the not-infrequent emergence of widespread resistance to 3 or more agents using the classical model alone. Now, driven again by advances in molecular genetics and especially sequencing, thinking about the evolution of resistance in TB has itself evolved. Here we explore how new observations and insights are promoting an updated model of TB drug resistance, with the promise of new ways to combat the resistance problem.

Classical resistance determinants in Mtb

Antibiotic resistance is defined as a heritable trait that enables a bacterial population to both survive and replicate in the presence of an otherwise inhibitory antibiotic treatment (Box 1). Bacteria can evolve resistance through several well-known mechanisms including alteration of sequence or expression of the target (Ince and Hooper, 2003), alteration of drug modification enzymes (Robicsek et al., 2006), drug efflux (Nikaido, 2009), or gene amplification (Andersson and Hughes, 2009; Sandegren and Andersson, 2009). However, unlike in other bacteria, genetic resistance in Mtb is not known to be associated with horizontal gene transfer and instead is commonly the result of single nucleotide polymorphisms and insertions or deletions (Namouchi et al., 2012; Dookie et al., 2018). This lack of promiscuous gene transfer somewhat simplifies the process of uncovering genetic determinants of drug resistance in Mtb, however complexities remain at every level.

Resistance to first- and second-line anti-TB drugs is generally associated with known mutations at particular loci. These mutations have been reviewed previously (Almeida Da Silva and Palomino, 2011; Cohen et al., 2019) and are summarized in Table 1. Nearly all of these mutations confer resistance through the alteration of a drug target or activator. For example, the target of rifampin (RIF) is the β subunit of bacterial RNA polymerase (RNAP), which is encoded by the *rpoB* gene (Goldstein, 2014). Mutations in *rpoB* have been used to predict resistance to RIF with such a high degree of success (Goldstein, 2014) that an 81-bp region of the *rpoB* gene is now designated the RIF resistance-determining region (Ohno et al., 1996; Ramaswamy and Musser, 1998). However, not all cases of RIF resistance are so straightforward. Distinctions have been drawn between high- and low-level RIF resistance, phenotypes caused by mutations within and outside of the known RIF resistance-determining region of *rpoB* (Shea et al., 2021). Similarly, the presence of mutations in *rpoA* and *rpoC* (Andersson, 2006) as well as secondary site mutations in *rpoB* (Brandis and Hughes, 2013; Meftahi et al., 2016) are now known to play a role in the Mtb RIF resistance profile. Additionally, mutation, and therefore resistance, can be induced under drug pressure. For example, the mutation frequency to RIF was found to increase more than a thousand-fold during 14-days of monotherapy (Kayigire et al., 2017).

The classical model can shed light on most drug resistant Mtb strains circulating today. Indeed, identifying point mutations in specific loci is the basis of highly successful PCR-based tests for Mtb drug resistance (Stevens et al., 2017). Recently, the WHO has catalogued whole genome sequences and drug resistance profiles of 38,215 Mtb clinical strains (WHO, 2021a). This catalogue makes clear the value of the classical resistance model, while also revealing many mutations of unknown mechanism are linked to resistance. Indeed, for every anti-TB agent, there are resistant strains that continue to elude molecular genetic characterization. In addition, the pre-existing mutation model sheds little light on how drug resistance evolves in Mtb. However, in recent years an updated model has emerged that seeks to incorporate older, seemingly anomalous observations with newer, technology-driven insights to explain more completely the global Mtb drug resistance landscape. To take the 18th century writer and polymath Johann Wolfgang von Goethe badly out of context, “tolerance comes of age”.

The updated model: Tolerance on the pathway to resistance

In 1944, Joseph Bigger described a subpopulation of *Staphylococci* that survived exposure to penicillin without generating heritable resistance. When those cells were cultured in fresh media and then re-exposed to penicillin, they retained

Box 1. Definitions

Antibiotic resistance is a stably heritable trait that enables a bacterial population to both survive and replicate in the presence of an otherwise inhibitory antibiotic concentration. Mechanisms of antibiotic resistance are tightly associated with mutations in drug targets, activating enzymes, efflux systems, or membrane porins. Of these, mutations in targets and activators are by far most common in Mtb.

Antibiotic tolerance. We refer to bacteria that survive high or prolonged concentrations of antibiotics in the absence of stably heritable mechanisms of resistance as drug tolerant persisters. Progeny of tolerant cells exhibit a drug susceptibility profile similar to that of the parental strain. Of note, the nomenclature of drug tolerance can be challenging, as some researchers employ different terms (eg. – phenotypic resistance; non-heritable resistance, antibiotic indifference), and others define tolerance and persistence in more limited ways e.g. (Brauner et al., 2016; Balaban et al., 2019; Urbaniec et al., 2022). In general, these nomenclature distinctions serve to highlight particular subsets of tolerance. Mechanisms of drug tolerance are varied, including reduced metabolic activities, low ATP levels, toxin-antitoxin systems, and stringent response. Note that the definition we employ for drug tolerant persisters is agnostic as to form of tolerance or mechanism.

TABLE 1 Examples of resistance mutations and compensatory mechanisms in Mtb.

Drug	Major resistance mutation	Compensatory mechanism	References
Rifampin (RIF)	*rpoB*	*rpoA, rpoC*	(Telenti et al., 1993; Ohno et al., 1996; Ramaswamy and Musser, 1998; Comas et al., 2011; Shea et al., 2021)
Pyrazinamide (PZA)	*pncA*	*pnaB2* (epistatic)	(Konno et al., 1967; Scorpio and Zhang, 1996; Muzondiwa et al., 2021)
Para-aminosalicylic acid (PAS)	*thyA*	*thyX–hsdS.1* intergenic region associated, but not shown to be compensatory	(Rengarajan et al., 2004; Zhang et al., 2013; Coll et al., 2018)
Ethambutol (EMB)	*embCAB* operon	*aftA* (Rv3792)	(Alcaide et al., 1997; Telenti et al., 1997; Safi et al., 2013)
Isoniazid (INH)	*katG*, *inhA*	*ahpC* promoter	(Zhang et al., 1992; Heym et al., 1995; Sherman et al., 1996)
Fluoroquinolones (FQ)	*gyrA*	Extragenic *Rv0890c*, Insertions in *glgC* in *Mycobacterium aurum*	(Takiff et al., 1994; Pi et al., 2020)
Bedaquiline (BDQ)	*mmpR (Rv0678), atpE, pepQ*	*atpB*? (suggested)	(Andries et al., 2005; de Jonge et al., 2007; Huitric et al., 2010; Andries et al., 2014; Nieto Ramirez et al., 2020)
Clofazimine (CFZ)	*pepQ, mmpR*	Unknown	(Almeida et al., 2016)
Pretomanid (PA-824)/ Delaminid (DLM)	*ddn, fgd1, fbiA, fbiB, fbiC*, and *fbiD*	Unknown	(Haver et al., 2015; Gomez-Gonzalez et al., 2021)
Linezolid (LZD)	*rrl, rplC*	Unknown	(Hillemann et al., 2008; Beckert et al., 2012)
Capreomycin (CAP)	A1408G mutation in 16S rRNA gene (*rrs*)	Increased expression of *tlyA* leading to methylation of C1409	(Maus et al., 2005; Freihofer et al., 2016)
Streptomycin (STR)	*rpsL, rrs, gidB*	*rpsD*?, *rpsE*? (proposed)	(Nair et al., 1993; Meier et al., 1994)

the parent strain's level of susceptibility (Bigger, 1944). Since then, non-heritable survival in the face of antibiotics has been noted in a variety of bacteria exposed to different agents, including Mtb and the phenomenon has been given many names (McCune and Tompsett, 1956; Levin and Rozen, 2006). Here we refer to bacteria that do not harbor stably heritable resistance and yet survive significant antibiotic exposure as drug tolerant persisters (see Box 1). Note that our definition is agnostic as to the mechanism(s) by which tolerance occurs.

A possible link between tolerance and the evolution of resistance immediately suggests itself – other things being equal, the longer bacteria survive, the greater the opportunity to mutate to a stably antibiotic resistant state. This reasoning underpins mathematical models that link the two phenomena (Levin-Reisman et al., 2019), and explains why we discuss tolerance in a review about drug resistance. However, it is important to bear in mind that experimental validation for this link is so far limited to a very small number of examples (Levin-Reisman et al., 2017; Sebastian et al., 2017).

Tolerance and heterogeneity

It was recognized early on that most drugs are less effective on Mtb during infection than they are *in vitro* (McCune and Tompsett, 1956). One important reason is that most antibiotics work best on actively dividing cells, but a robust immune response is one of several mechanisms to slow the Mtb replication rate (Gill et al., 2009; Ford et al., 2011; Colangeli et al., 2014). More recently it has become evident that Mtb has evolved multiple strategies to generate subpopulations of phenotypically distinct bacteria, each with separate growth rates and levels of drug tolerance (Aldridge et al., 2012; Sarathy et al., 2018; Richards et al., 2019). In any given mycobacterial population, variations in replication, DNA repair, transcription, translation, metabolism, and efflux all promote bacterial heterogeneity and are also linked to drug tolerance (Szumowski et al., 2013; Chung et al., 2022). A similar phenomenon is evident within the human lung, where some lesions can support Mtb growth and expand during drug treatment even as other lesions shrink and the patient improves overall (Akira et al., 2000; Xie et al., 2021). Finally, heterogeneity exists among Mtb strains worldwide, driving differences in the accumulation and spread of drug resistance. Recently appreciation has been growing for how widely different mechanisms that promote and maintain bacterial heterogeneity are linked to drug tolerance, treatment failure, and ultimately the evolution of resistance. While this review makes no attempt to be comprehensive, some relevant examples of these mechanisms are provided below (Figure 2).

DNA replication, growth and division

Unlike other bacterial species, mycobacteria elongate from the cell poles, providing a straightforward opportunity for asymmetric growth and division leading to daughter cells with different sizes and growth rates (Aldridge et al., 2012; Santi et al.,

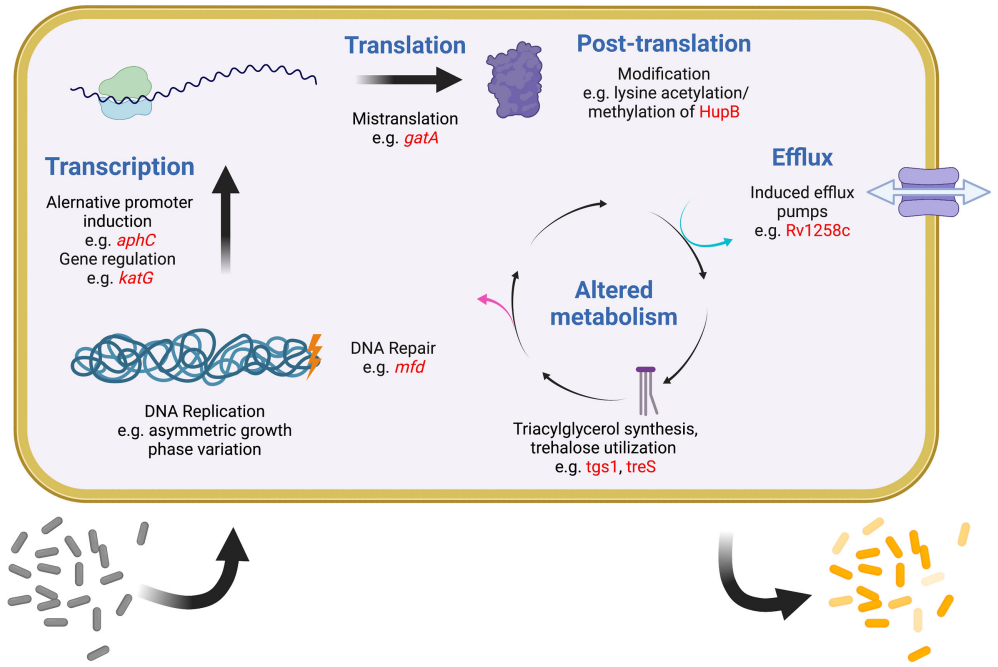


FIGURE 2
Examples of intracellular mechanisms to generate heterogeneity and tolerance in Mtb. Shown are various cellular processes (blue) at which heterogeneity and tolerance can be generated. Examples of known mechanisms are given at each level with examples in red. One expected outcome of these mechanisms is shown at the bottom, with a sensitive population (gray) becoming differentially tolerant (shades of yellow) after induction of any of these systems. Figure created with BioRender.com.

2013; Chung et al., 2022). A key member of mycobacterial divisome complex, LamA has been identified as a mediator of asymmetric growth by inhibiting cell-wall synthesis at the new poles (Rego et al., 2017). In the absence of *lamA*, daughter cells are less heterogeneous in size and also display increased sensitivity to RIF and vancomycin compared to wild-type cells (Rego et al., 2017). During *in vitro* growth, Mtb cells shorter in length were found to be more susceptible to RIF, along with being more sensitive to oxidative and nitrite stress (Vijay et al., 2017). Clinically, Mtb with an increased cell length has been associated with host stresses such as iron deficiency and oxidative stress along with an increase in severe pulmonary disease (Vijay et al., 2017). Multidrug-resistant (MDR)-Mtb strains were also found to exhibit increased heterogeneity in cell length compared to drug-sensitive strains during intramacrophage growth and during RIF exposure, which when combined, was further associated with an increase in cell length (Vijay et al., 2017).

In a separate genome-wide association study of clinical Mtb strains, variants in the essential DNA replication initiation factor, *dnaA*, were found to be associated with drug resistance (Hicks et al., 2020). These *dnaA* variants enhance Mtb survival during isoniazid (INH) treatment by interacting with the *Rv0010c-Rv0011c* intergenic region and reduced expression of *katG*, the activator of INH. However, the connection between *Rv0010c-Rv0011c* and altered *katG* expression is not currently understood (Hicks et al., 2020).

Phase variation

Phase variation is an adaptive mechanism that mediates reversible switching of a gene by genotypic changes, which in turn can lead to reversible or transient drug resistance. Phase variation results from insertions or deletions in a homopolymeric tract (HT) located within the promoter or open reading frame of a gene. Reversible frameshift mutations in HTs are thought to be a result of slipped-strand mispairing errors during replication. Inactivating transient frameshift mutations in the HT of 7 cytosines in the *glpK* gene, which encodes a glycerol-3-kinase, were found to confer heritable drug resistance to INH, RIF, ethambutol (EMB), pyrazinamide (PZA) and moxifloxacin (MOX), but these changes were reversible with additional insertions or deletions in the same HT (Bellerose et al., 2019; Safi et al., 2019). These frameshift mutations were also identified in Mtb-infected mice and in clinical Mtb strains (Bellerose et al., 2019; Safi et al., 2019).

In another example, reversible frameshift mutations in the Mtb *orn* gene in combination with low-level EMB-resistant double *embB-aftA* mutant produced a small colony variant

with a significantly higher MIC and further culture led to a rapid reversion of the *orn* frameshift (Safi et al., 2020).

Genetic regulators mediating tolerance and resistance

Heterogeneity in levels or activity of proteins and flux of metabolites is often facilitated by heterogeneity in gene expression. Recent work has provided examples of transcription factors and regulatory circuits that directly impact drug susceptibility. For instance, bedaquiline (BDQ) is a newer antitubercular drug that inhibits the F_1F_0-ATP synthase of Mtb. Network analysis of the Mtb transcriptional response to BDQ suggested that regulators Rv0324 and Rv0880 played important roles in the response to this drug (Peterson et al., 2016). Subsequent work showed that deletion of either Rv0324 or Rv0880 led to improved killing by BDQ but not other drugs. Exposure to capreomycin and MOX also induced Rv0324 suggesting potential antagonism with BDQ, while exposure to pretomanid decreased expression of the Rv0880 BDQ response regulon (Peterson et al., 2016). The addition of pretomanid to novel BDQ-containing regimens was found to improve clearance and reduce relapse in several murine models of TB (Xu et al., 2019), consistent with the synergistic effect of BDQ and pretomanid predicted by the transcriptional response and network analysis.

In another example, a network-based genetic screening approach, the transcriptional regulator-induced phenotype (TRIP) screen, was used to identify regulators that alter susceptibility to INH. Expression of *mce3R*, a TetR family transcription factor, was found to mediate heightened sensitivity to INH (Ma et al., 2021), which was linked to repression of *ctpD*, a gene encoding a metal binding protein (Raimunda et al., 2014; Patel et al., 2016; Ma et al., 2021) not previously known to play a role in INH susceptibility. Disruption of *ctpD* conferred hypersusceptibilty to INH, with increased intracellular accumulation of INH and INH-NAD adduct.

Drug induced tolerance

Drug exposure has also been shown to induce transcriptional changes conferring tolerance. When exposed to low RIF concentrations, transcription from one promoter is inhibited, allowing increased *rpoB* expression from a second promoter and after a delay, growth can resume despite ongoing drug exposure (Zhu et al., 2018). Subsequent studies have found that the absence of a functional *lepA*, a translation-associated elongation factor, increased RIF tolerance by the upregulation of *rpoB* expression (Wang et al., 2020). Mutations in *lepA* found in clinical Mtb strains were also found to have increased tolerance to RIF (Wang et al., 2020).

DNA repair

Environmental stresses such as hypoxia, nutrient deprivation, and host immune effectors can generate genome-wide mutations in subsets of cells, leading to heritable genetic resistance (Sebastian et al., 2017; Ragheb et al., 2019; Hicks et al., 2020; Jakkala et al., 2020; Swaminath et al., 2020). The activity and efficiency of different DNA repair pathways influences the rate at which these cells are a reservoir from which resistant isolates emerge.

A recently described link between DNA repair and drug resistance involved the DNA translocase protein Mfd. Mfd has long been thought to initiate nucleotide excision repair at sites of stalled RNAPs, though Mtb cells deficient in Mfd are not more sensitive to DNA-damaging agents. However, Mfd is found to associate with RNAP in the absence of DNA damage and has also been identified as an anti-backtracking factor for RNAP. Interactions of Mfd with the β-subunit of RNAP promote mutagenesis leading to drug resistance in several bacterial pathogens, including Mtb. Deletion of *mfd* led to a decrease in the number of spontaneous resistant mutants to RIF, EMB and ciprofloxacin (Ragheb et al., 2019). Recently, Rv1019, a transcriptional regulator of the TetR family, was found to negatively regulate *mfd* expression. Overexpression of Rv1019 leads to the downregulation of *mfd* and decreased Mtb survival under oxidative stress (Pushparajan et al., 2020). Since Rv1019 is differently expressed during hypoxia and reactivation (Schubert et al., 2015), it would be interesting to know if Rv1019 is the key regulator of Mfd-mediated changes in Mtb leading to drug resistance.

In an *in vitro* persistence model, Mtb was found to develop resistance to RIF or MOX at a much higher frequency than predicted when exposed to continuous lethal concentrations of RIF (Sebastian et al., 2017). Mtb in the RIF persistence-phase was found to carry elevated levels of hydroxyl radicals leading to genome-wide random mutagenesis, generating not only mutants in *rpoB*, but also in *gyrA* demonstrating that exposure to one antibiotic can generate genetic resistance to a different antibiotic (Sebastian et al., 2017). Similarly, exposure to lethal concentrations of MOX also generated high levels of hydroxyl radicals leading to resistance not only to MOX but also to EMB and INH (Swaminath et al., 2020).

Mistranslation during protein synthesis

Mistranslation happens when an error occurs during the protein synthesis yielding either incorporation of an incorrect amino acid or a truncated protein product. Generally deleterious, mistranslation can be adaptive in the presence of RIF. As noted above, most RIF resistance is due to mutation in a small region of *rpoB*, the RIF resistance determining region (Gagneux et al., 2006b). Increasing the mistranslation rate in *M.*

smegmatis led to substitutions of glutamate for glutamine and aspartate for asparagine within the same region, which improved survival during RIF exposure (Javid et al., 2014). This effect is regulated by levels of the GatCAB enzyme complex, where reduced expression results in increased mistranslation and RIF tolerance (Su et al., 2016). Clinical strains with mutations in *gatA*, cause a partial loss of function of the complex along with increased mistranslation and increased RIF tolerance (Su et al., 2016; Li et al., 2021).

Metabolic regulation

Mtb can utilize a wide variety of carbon sources to support *in vitro* growth. However, Mtb resides *in vivo* within intracellular and extracellular niches where the nutrient composition is thought to be sparse and growth-limiting (Berney and Berney-Meyer, 2017; Sarathy et al., 2018). This slowed growth has traditionally been associated with drug tolerance, as most antibiotics target metabolically active Mtb (Schaefer, 1954). In Mtb, the regulation of several interconnected pathways that control carbon and lipid metabolism contribute to this metabolic slowdown. Rerouting pathways from energy-generation towards energy storage is associated with growth arrest and reduced drug susceptibility. Importantly, even stochastic differences in expression or activity of regulators and rate-limiting steps in these pathways results in bacterial heterogeneity and differential susceptibility to drugs.

For instance, under stress conditions (including drug pressure), Mtb can shift from the growth-promoting TCA cycle to carbon storage in fatty acids *via* triacylglycerol (TAG) synthesis *via* the upregulation of the triglyceride synthase *tgs1* (Sirakova et al., 2006). In the absence of *tgs1*, drug tolerance induced during hypoxia is reversed and Mtb remains sensitive to INH, streptomycin (STR), fluoroquinolones (FQ) and EMB (Baek et al., 2011). The slowing of the TCA cycle alters the turnover of alpha-ketoglutarate, oxaloacetate and reducing agents such as NADH are diminished, resulting in reduced amino acid synthesis and protein translation. Additionally, enzymes such as isocitrate lysases, which are involved in the the glyoxylate bypass, are induced upon exposure to INH, RIF and STR conferring cross-tolerance (Nandakumar et al., 2014). Deficiency in the gluconeogenic enzyme phosphoenolpyruvate carboxykinase encoded by *pckA*, has been implicated in enhanced drug tolerance to INH and BDQ, with this tolerance associated with the overaccumulation of methylcitrate cycle (MCC) intermediates (Quinonez et al., 2022). Similarly, depletion of phosphoenolpyruvate during hypoxia confers tolerance to INH (Lim et al., 2021). In *prpR* mutants, defective regulation of MCC leads to an accumulation of propionyl-CoA conferring tolerance to INH, RIF and ofloxacin (OFX) (Hicks et al., 2018).

In another example, trehalose in Mtb serves as both a carbohydrate store as well as a component of the cell surface glycolipids trehalose monomycolate (TMM) and trehalose dimycolate (TDM). During hypoxia, Mtb has been shown to down-regulate TMM and TDM and channel trehalose into the biosynthesis of central carbon metabolism (CCM) intermediates. In a biofilm model, drug-tolerant persisters were shown to shift trehalose metabolism towards CCM intermediates (Lee et al., 2019). *treS* deletion mutants were unable to shift trehalose metabolism to CMM and showed a rapid depletion of ATP and were also significantly more susceptible to BDQ (Lee et al., 2019).

Efflux

The Mtb genome encodes a significant number of efflux pumps with a known role in intrinsic and acquired drug resistance, and many of these pumps are also induced during infection (Schnappinger et al., 2003; Rengarajan et al., 2005; Gupta et al., 2010; Szumowski et al., 2013; Pule et al., 2016). Since efflux pumps can have broad substrate specificities, their induction under varying environmental conditions or drug exposure may confer tolerance or resistance to multiple drugs, as is seen with RIF exposure resulting in tolerance to OXF (Louw et al., 2011). Exposure to INH has been shown to induce efflux pumps mediating its tolerance, which then promote the emergence of genetically resistant INH strains (Machado et al., 2012). Additionally, upon infection of macrophages, Mtb has been shown to induce tolerance to numerous antitubercular drugs including INH, RIF, MOX and BDQ that is not tied to reduced growth rate and is sensitive to efflux pump inhibitors such as verapamil (Adams et al., 2011; Adams et al., 2014). Macrophage-induced tolerance to RIF was shown to be mediated by Rv1258c/Tap, an efflux pump also important for intracellular growth (Adams et al., 2011). Further study revealed that strains from all tested global lineages developed macrophage-induced tolerance to RIF except lineage 2 Beijing isolates, which harbor a natural loss-of-function mutation in Rv1258c (Villellas et al., 2013; Adams et al., 2019). In addition, mutations in Rv1258c that were identified in clinical isolates have been linked with resistance to INH, PZA, and STR (Liu et al., 2019).

Within-host Mtb differences affect drug response

One limitation with the classical model of resistance is that it does not consider within-host variation during TB infection; however, recent studies are beginning to bring evidence of heterogeneity in the Mtb response during drug treatment to light (Borrell and Gagneux, 2009; McGrath et al., 2014). Clinical Mtb strains were collected from patients with delayed culture

conversion and WGS was performed. Exposing these strains to RIF *in vitro* revealed drug tolerant variants undetected by bulk WGS-analysis (Genestet et al., 2021). One variant identified by RIF treatment was also enriched during macrophage infection and was found to have a mutation in the gene *mas*, altering its cell surface lipids (Genestet et al., 2021). Characterizing these tolerant sub-populations may help identify patients at risk for treatment failure and the evolution of resistance.

In another study, Mtb isolates from treatment-naïve patients were subjected to WGS to assess within-host diversity. The accumulation of identified mutations varied substantially between isolates from the same individual and were elevated in HIV-negative patients, suggesting that the host immune environment may influence mutation rates (Liu et al., 2020). These results argue that the risk of developing new drug resistance mutations *in vivo* may vary with the host immune environment (Liu et al., 2020). This idea is consistent with evidence from TB patients that host gene signatures exhibiting heightened inflammatory and immune gene expression correlate with longer times to cure and a reduced cure rate (DiNardo et al., 2022).

Lineage-specific Mtb differences and drug response

It has become clear in the last several years that global variations in Mtb strains have strong impacts on drug response and the evolution of drug resistance. Worldwide Mtb has been separated into seven lineages and many sub-lineages with distinct characteristics that co-evolved with the human populations in which they are present (Brites and Gagneux, 2015). Global lineages differ in their ability to respond to drugs and develop resistance. Members of the modern lineages (2, 3 and 4) are associated with greater disease burden and drug resistance than the ancient lineages, possibly due to an increased spontaneous mutation rate (Borrell and Gagneux, 2009; McGrath et al., 2014). Further, differences *in vitro* and *in vivo*, the genetic background of the strain and the nature of the specific resistance mutation both influence outcomes. For example, there is an association between strain lineage and the type of resistance mutation identified, suggesting that certain Mtb lineages may have characteristics that encourage different routes to resistance (Gagneux et al., 2006a). One study using TnSeq showed that clinically distinct strains have different requirements for *in vitro* growth, including *katG* and *glcB* (Carey et al., 2018). The differences in TnSeq phenotypes of these strains were found to predict their drug resistance rates (Carey et al., 2018).

Compensatory mutations

Antibiotics, by their nature, target important functions of the bacterial cell. Thus, any mutation that renders a strain resistant has good potential to also reduce the strain's fitness. This observation once led to the hope that simply reducing the use of antibiotics would lead to fitter, susceptible strains outcompeting resistant ones. However, reduced fitness can also be addressed by compensatory evolution and genetic co-selection (Andersson and Levin, 1999; Andersson, 2006; Andersson and Hughes, 2010).

The fitness cost of a resistance mutation can be measured *in vitro* with isogenic strains serially passaged or grown continuously in chemostats. Such results do not always translate into a host setting, so it is important to also consider how virulence and pathogen transmission are affected. For example, in the 1950s Middlebrook and colleagues found that many INH-resistant Mtb strains were less virulent in Guinea pigs (Middlebrook, 1954; Widelock et al., 1955; Wolinsky et al., 1956). Later, it was revealed that INH-resistant Mtb lacking KatG catalase-peroxidase activity could compensate by overexpressing an alkyl hydroperoxidase (Sherman et al., 1996). Similarly, with regard to RIF resistance, it has been shown that prolonged treatment can result in multidrug resistant strains that have no measurable fitness defect (Gagneux et al., 2006b). These examples illustrate the complex relationship of drug resistance and fitness, where initial costs can be corrected by compensatory mutations that retain the resistance phenotype. It is an important consideration, as such low and no cost mutations have been seen in clinical isolates (Sander et al., 2002). Specific compensatory mutations are shown in Table 1 and have been reviewed elsewhere (Alame Emane et al., 2021).

One non-canonical form of compensatory mutation that was recently described in Mtb involves restoring fitness of a capreomycin (CAP)-resistant mutant. CAP binds to 16S rRNA and inhibits translation. CAP resistance is conferred by 16S rRNA mutation that also reduces translation efficiency. However, translation can be largely restored by increased expression of an enzyme that methylates a nearby site on the 16S rRNA, significantly reducing the fitness cost of CAP resistance (Freihofer et al., 2016). This is a striking example of a compensatory mutation that relies on changes in expression but acts through post-transcriptional modification. Evidence of these 'multi-level' mechanisms of fitness alterations are rare, but it seems likely that more will be discovered and shown to be relevant in other contexts.

Epistasis

Epistasis refers to a phenomenon where the phenotypic effect of a particular gene allele depends on its genomic background (Hughes and Andersson, 2017). In the context of antibiotic resistance, epistatic interactions between resistance-conferring mutations have a major influence on the fitness of the multidrug-resistant (MDR) isolates and hence their evolution.

Epistasis can have either positive or negative outcomes depending on the net effect on bacterial fitness in the absence of antibiotic pressure. Positive epistasis occurs when the fitness cost associated with multiple resistance-conferring mutations is lower than the anticipated additive cost of these mutations if calculated independently. Positive epistasis is a common phenomenon in mycobacteria and in other bacteria as illustrated in numerous studies. For example, a study by Borrel et al. showed that MDR isolates harboring double mutations in *rpoB* H526Y and *gyrA* D94G, conferring resistance to RIF and ofloxacin respectively, were associated with enhanced fitness as opposed to their respective single mutants (Borrell et al., 2013). Similarly, Sun et al. reported a positive epistatic interaction in MDR isolates with double mutations in *rpsL* K43M and *gyrA* D94Y, which confer resistance to STR and fluoroquinolones respectively (Sun et al., 2018). Another example of positive epistasis was also reported by Li et al. where MDR isolates with dual mutations in *rpoB* C531T and *katG* 315C were associated with enhanced fitness (Li et al., 2017). Importantly, those MDR isolates where positive epistasis conferred fitness benefits were associated with better transmissibility and thus were frequently encountered in clinical settings, which supports the idea that positive epistasis plays an important role in the evolution of MDR isolates (Trindade et al., 2009; Borrell et al., 2013).

In addition, positive lineage-specific epistatic interactions were found to be associated with particular Mtb clades. One study identified two epistatic interactions that were exclusively observed in lineage 4 (Coll et al., 2018). Compensatory mutations in *pnaB2* and *thyX–hsdS.1* promoter were found to be associated with resistances to PZA and para-aminosalicylic acid (PAS), due to mutations in *pncA* and *thyA*, respectively.

On the other hand, negative epistasis occurs when the interaction between two or more resistance-conferring mutations aggravates the fitness cost associated with these mutations. For example, FQ resistant isolates with double mutations in *gyrA* and *gyrB* were associated with diminished fitness (Luo et al., 2017). Similarly, in the Borrel et al. study, negative epistasis was observed in MDR isolates with double mutations in *rpoB* H526P and *gyrA* G88C (Borrell et al., 2013). In contrast to positive epistasis, MDR isolates where epistatic interactions resulted in diminished fitness were associated with low transmission rates and were rarely encountered in clinics.

Epistasis and the evolution of resistance

Several studies have revealed a strong correlation between Mtb lineages and particular drug resistance-conferring mutations, highlighting the major influence of the genetic background on the evolution of drug resistance. For example, one study reported lineage-specific differences in the level of INH resistance due to mutations in *katG* and *inhA* (Fenner et al., 2012). The *katG* mutations were more prevalent in lineage 2 isolates and conferred a high level of INH resistance, whereas *inhA* mutations were more prevalent in lineage 1 and were associated with low levels of INH resistance. Another study found that fluoroquinolone resistance due to mutated *gyrA* occurred more frequently in lineages 2 and 4 (Castro et al., 2020). Similarly, *katG* and *rpoB* mutations occur more frequently in modern Beijing sublineages compared to the ancient strains (Li et al., 2017). These examples demonstrate the major influence the genetic background could have on the evolution of drug response and also may explain why some Mtb lineages, particularly Beijing isolates, are often associated with multidrug resistance (Fenner et al., 2012; Nieto Ramirez et al., 2020; Fursov et al., 2021).

Epistasis and the level of drug resistance

The classical resistance model relies on using specific genetic determinants to define drug resistance. However, a key limitation of this model is that it tends to ignore the effect of epistasis on the level of drug resistance. Several studies have recently shown that bacterial cells can epistatically exhibit enhanced drug susceptibility despite the presence of a resistance-conferring mutation. For example, a study showed that a loss of function mutation in the *eis* coding region was able to restore amikacin susceptibility in resistant isolates harboring *eis* C-14T mutation (Vargas et al., 2021). Moreover, the same study questioned the validity of *mmpR* mutations as a determinant of bedaquiline and clofazimine resistances if loss of function mutations in *mmpS5* and *mmpL5* were present concomitantly (Vargas et al., 2021).

Summary and conclusions

So where does drug resistance in Mtb come from, and where is it going? Historically, the classical model (Figure 1, left side), in which pre-existing mutations are selected by drug pressure, has proven a very useful framework for our evolving understanding of resistance. However, the updated model (Figure 1, right side), with non-obligatory steps through tolerance on the path to resistance, does a better job of describing the rates, types, and patterns of drug resistance within communities and around the world. It is clear that pre-existing mutations conferring resistance do exist in any population of sufficient size, and that resistance does not require a tolerant pre-step. In practice however, with so many different routes to a tolerant state, it is entirely possible that the majority of resistant isolates worldwide today emerged from drug-tolerant precursors.

As described above, tolerance can be stochastic or genetically programmed, and it is frequently linked with the formation and

maintenance of heterogeneous sub-populations. Heterogeneity can be recognized at all levels of TB disease, including bacterial subpopulations within individual lesions and across lesions in a single patient, within a single patient over time, within communities, and in different lineages across the globe. In all these cases, we should expect that heterogeneity contributes both to treatment failure and evolution of resistance. Further, since the drug tolerance spawned by heterogeneity is adaptive, we might anticipate that future work will demonstrate that the production of heterogeneous sub-populations is itself under genetic selection and control. In fact, multiple recent reports already point in that direction (Bellerose et al., 2019; Safi et al., 2019; Safi et al., 2020; Ma et al., 2021; Carey et al., 2022; Martini et al., 2022).

The updated model has implications for how we track drug susceptibility and resistance. Increasingly around the world, slow and labor-intensive microbiological drug susceptibility testing is being replaced by faster DNA-based methods, either PCR or next-generation sequencing (NGS) (Dookie et al., 2022; Rowlinson and Musser, 2022). For example, a recent study reported the whole genome sequences of more than 12,000 Mtb clinical isolates, along with sensitivity data for 13 different drugs (The CRyPTIC Consortium, 2022). DNA-based methods offer important advantages in speed, throughput, and safety, as well as altogether novel insights into drug resistance mechanisms (Hicks et al., 2019; The CRyPTIC Consortium, 2022). Catalogues of sequencing results should be widely available and analyzed regularly for potential associations and emerging mutations of interest. However, it is important that these methods are implemented with stringent controls for DNA extraction, sequencing and data handling. NGS sequencing and analysis are not always straightforward (Villellas et al., 2017; Kaniga et al., 2022), and global standards for the application of NGS data to drug susceptibility testing and data reporting are still emerging. Further, there are cases of discordance between whole genome sequencing and drug sensitivity data (Dookie et al., 2022), though these are rare and the extent to which they are due to experimental errors is not yet clear. Finally, NGS generally provides only a snapshot of a bulk sample, without conveying the subtleties of the heterogeneous subpopulations described above. Technologies are in development (Box 2) for the identification and characterization of subpopulations, but these are not yet commonplace, and are certainly not yet employed for drug susceptibility testing. Altogether, DNA-based approaches are revolutionizing how we monitor drug susceptibility and resistance and show much promise for further advances but making good on that promise will require both the development of new tools and the rigorous application of those tools in the lab and the clinic.

The updated model also suggests new approaches to combat the emergence of drug resistance. If resistance frequently emerges from tolerant cells, then strategies to kill drug tolerant persisters or restrict their formation should slow the emergence of resistance. While not the topic of this review, eliminating persisters may also shorten the course of current therapy (Chung et al., 2022), so efforts to develop anti-persister therapy should receive high priority. Assays that identify small molecules targeting specific persister subpopulations have been reported (Sukheja et al., 2017; Gold et al., 2021). Hits from these screens could be combined with recent work to identify synergistic drug combinations (Cokol et al., 2017; Katzir et al., 2019; Ma et al., 2019) that can target multiple subpopulations at once. It may also be possible to directly target the machinery that promotes tolerance and resistance. For example, small molecules that inhibit the action of *mfd* (Ragheb et al., 2019) or DNA repair enzymes (Reiche et al., 2017) should reduce the rate at which resistance to other drugs emerge. Also, since small expression changes can have substantial effects on drug tolerance (Ma et al., 2021) and treatment outcome (Colangeli et al., 2018), it should be possible to identify small molecules that specifically alter Mtb gene expression away from tolerance-promoting states. When combined with NGS to characterize individual strains and efforts to uncover host-directed therapies, it is possible to imagine these

Box 2. Technology and our understanding of antibiotic resistance

As is common in biology, technological advances have been critical in updating our concepts of antibiotic action and the evolution of resistance. Important advances have occurred in:

Visualization – Advanced visualization tools such as multiparameter confocal microscopy (Gern et al., 2021; Plumlee et al., 2021) and mass cytometry (Xu et al., 2021) are helping to uncover the complexity of the host response to TB infection. Positron emission tomography (PET) imaging has brought to light the heterogeneity of TB lesions in live animals and humans (Lenaerts et al., 2015). Microfluidics (Molloy et al., 2021) and time-lapse microscopy (Herricks et al., 2020) are revealing the complexity of Mtb populations *in vitro*, and reporter gene technology (Huang et al., 2019) is providing similar insights *in vivo*.

Next-generation sequencing – High throughput sequencing has revolutionized to study of drug resistance. With thousands of Mtb genomes sequenced, the diversity of the Mtb pan genome is now evident. Many novel mutations have been linked with resistance to particular drugs, either alone or in association with known resistance loci (Zhang et al., 2013; Zeng et al., 2018; The CRyPTIC Consortium, 2022). Each new mutation must then be studied to see if it truly confers resistance or compensates for fitness defects imposed by mutations at other sites. Single-cell RNA-seq (Pisu et al., 2021) and dual-seq that simultaneously captures transcriptomes of Mtb and host (Pisu et al., 2020) have become important tools to study rare cell types and sub-populations *in vivo*. In addition, next-gen sequencing is central to the updated genetic screens described below.

Molecular genetics – Updated approaches in molecular genetics are also shedding new light on antibiotic action and resistance. Tn-seq is not really new technology, but it is being used to gain new insights into resistance mechanisms (Carey et al., 2018). Similarly, CRISPRi screens are identifying new loci associated with resistance to different agents (Li et al., 2022). Also, network-based TRIP screens have been employed to identify novel regulons and effector genes linked to drug sensitivity, tolerance and resistance (Ma et al., 2021).

approaches promoting an era of personalized TB therapy to achieve both improved outcomes and diminished resistance.

In conclusion, the history of drug development argues that resistance will emerge following the introduction of virtually any new agent. However, as the field has evolved and a new model of resistance has emerged, new strategies to protect and preserve agents can be envisioned. With careful monitoring and thoughtful development, we need not tolerate the loss of new agents to TB drug resistance any longer.

Author contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Funding

This review was supported by grants to DS: U19 AI 135976, U19 AI 1625598, and R01 AI 146194. The funders had no role in the decision to publish or prepare this manuscript.

Acknowledgments

The authors wish to thank David Alland and Shuyi Ma for helpful discussions and suggestions, and Eleanor Lamont for critical reading of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Adams, K. N., Szumowski, J. D., and Ramakrishnan, L. (2014). Verapamil, and its metabolite norverapamil, inhibit macrophage-induced, bacterial efflux pump-mediated tolerance to multiple anti-tubercular drugs. *J. Infect. Dis.* 210 (3), 456–466. doi: 10.1093/infdis/jiu095

Adams, K. N., Takaki, K., Connolly, L. E., Wiedenhoft, H., Winglee, K., Humbert, O., et al. (2011). Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell* 145 (1), 39–53. doi: 10.1016/j.cell.2011.02.022

Adams, K. N., Verma, A. K., Gopalaswamy, R., Adikesavalu, H., Singhal, D. K., Tripathy, S., et al. (2019). Diverse clinical isolates of *Mycobacterium tuberculosis* develop macrophage-induced rifampin tolerance. *J. Infect. Dis.* 219 (10), 1554–1558. doi: 10.1093/infdis/jiy710

Akira, M., Sakatani, M., and Ishikawa, H. (2000). Transient radiographic progression during initial treatment of pulmonary tuberculosis: CT findings. *J. Comput. Assist. Tomogr* 24 (3), 426–431. doi: 10.1097/00004728-200005000-00014

Alame Emane, A. K., Guo, X., Takiff, H. E., and Liu, S. (2021). Drug resistance, fitness and compensatory mutations in mycobacterium tuberculosis. *Tuberculosis (Edinb)* 129, 102091. doi: 10.1016/j.tube.2021.102091

Alcaide, F., Pfyffer, G. E., and Telenti, A. (1997). Role of *embB* in natural and acquired resistance to ethambutol in mycobacteria. *Antimicrob. Agents Chemother.* 41 (10), 2270–2273. doi: 10.1128/AAC.41.10.2270

Aldridge, B. B., Fernandez-Suarez, M., Heller, D., Ambravaneswaran, V., Irimia, D., Toner, M., et al. (2012). Asymmetry and aging of mycobacterial cells lead to variable growth and antibiotic susceptibility. *Science* 335 (6064), 100–104. doi: 10.1126/science.1216166

Almeida Da Silva, P. E., and Palomino, J. C. (2011). Molecular basis and mechanisms of drug resistance in *Mycobacterium tuberculosis*: classical and new drugs. *J. Antimicrob. Chemother.* 66 (7), 1417–1430. doi: 10.1093/jac/dkr173

Almeida, D., Ioerger, T., Tyagi, S., Li, S. Y., Mdluli, K., Andries, K., et al. (2016). Mutations in *pepQ* confer low-level resistance to bedaquiline and clofazimine in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 60 (8), 4590–4599. doi: 10.1128/AAC.00753-16

Andersson, D. I. (2006). The biological cost of mutational antibiotic resistance: any practical conclusions? *Curr. Opin. Microbiol.* 9 (5), 461–465. doi: 10.1016/j.mib.2006.07.002

Andersson, D. I., and Hughes, D. (2009). Gene amplification and adaptive evolution in bacteria. *Annu. Rev. Genet.* 43, 167–195. doi: 10.1146/annurev-genet-102108-134805

Andersson, D. I., and Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat. Rev. Microbiol.* 8 (4), 260–271. doi: 10.1038/nrmicro2319

Andersson, D. I., and Levin, B. R. (1999). The biological cost of antibiotic resistance. *Curr. Opin. Microbiol.* 2 (5), 489–493. doi: 10.1016/S1369-5274(99)00005-3

Andries, K., Verhasselt, P., Guillemont, J., Gohlmann, H. W., Neefs, J. M., Winkler, H., et al. (2005). A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. *Science* 307 (5707), 223–227. doi: 10.1126/science.1106753

Andries, K., Villellas, C., Coeck, N., Thys, K., Gevers, T., Vranckx, L., et al. (2014). Acquired resistance of *Mycobacterium tuberculosis* to bedaquiline. *PloS One* 9 (7), e102135. doi: 10.1371/journal.pone.0102135

Baek, S. H., Li, A. H., and Sassetti, C. M. (2011). Metabolic regulation of mycobacterial growth and antibiotic sensitivity. *PloS Biol.* 9 (5), e1001065. doi: 10.1371/journal.pbio.1001065

Balaban, N. Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D. I., et al. (2019). Definitions and guidelines for research on antibiotic persistence. *Nat. Rev. Microbiol.* 17 (7), 441–448. doi: 10.1038/s41579-019-0196-3

Barberis, I., Bragazzi, N. L., Galluzzo, L., and Martini, M. (2017). The history of tuberculosis: from the first historical records to the isolation of koch's bacillus. *J. Prev. Med. Hyg* 58 (1), E9–E12. doi: 10.15167/2421-4248/jpmh2017.58.1.728

Beckert, P., Hillemann, D., Kohl, T. A., Kalinowski, J., Richter, E., Niemann, S., et al. (2012). *rplC* T460C identified as a dominant mutation in linezolid-resistant *Mycobacterium tuberculosis* strains. *Antimicrob. Agents Chemother.* 56 (5), 2743–2745. doi: 10.1128/AAC.06227-11

Bellerose, M. M., Baek, S. H., Huang, C. C., Moss, C. E., Koh, E. I., Proulx, M. K., et al. (2019). Common variants in the glycerol kinase gene reduce tuberculosis drug efficacy. *mBio* 10 (4), 1–15. doi: 10.1128/mBio.00663-19

Berney, M., and Berney-Meyer, L. (2017). *Mycobacterium tuberculosis* in the face of host-imposed nutrient limitation. *Microbiol. Spectr.* 5 (3), 1–17. doi: 10.1128/microbiolspec.TBTB2-0030-2016

Bigger, J. W. (1944). Treatment of staphylococcal infections with penicillin by intermittent sterilisation. *Lancet* 244 (6320), 497–500. doi: 10.1016/s0140-6736(00)74210-3

Borrell, S., and Gagneux, S. (2009). Infectiousness, reproductive fitness and evolution of drug-resistant *Mycobacterium tuberculosis*. *Int. J. tuberculosis Lung Dis.* 13 (12), 1456–1466.

Borrell, S., Teo, Y., Giardina, F., Streicher, E. M., Klopper, M., Feldmann, J., et al. (2013). Epistasis between antibiotic resistance mutations drives the evolution of extensively drug-resistant tuberculosis. *Evolution Medicine Public Health* 2013 (1), 65–74. doi: 10.1093/emph/eot003

Brandis, G., and Hughes, D. (2013). Genetic characterization of compensatory evolution in strains carrying *rpoB* Ser531Leu, the rifampicin resistance mutation most frequently found in clinical isolates. *J. Antimicrob. Chemother.* 68 (11), 2493–2497. doi: 10.1093/jac/dkt224

Brauner, A., Fridman, O., Gefen, O., and Balaban, N. Q. (2016). Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat. Rev. Microbiol.* 14 (5), 320–330. doi: 10.1038/nrmicro.2016.34

Brites, D., and Gagneux, S. (2015). Co-Evolution of *Mycobacterium tuberculosis* and *Homo sapiens*. *Immunol. Rev.* 264 (1), 6–24. doi: 10.1111/imr.12264

Carey, A. F., Rock, J. M., Krieger, I. V., Chase, M. R., Fernandez-Suarez, M., Gagneux, S., et al. (2018). TnSeq of *Mycobacterium tuberculosis* clinical isolates reveals strain-specific antibiotic liabilities. *PloS Pathog.* 14 (3), e1006939. doi: 10.1371/journal.ppat.1006939

Carey, A. F., Wang, X., Cicchetti, N., Spaulding, C. N., Liu, Q., Hopkins, F., et al. (2022). Multiplexed strain phenotyping defines consequences of genetic diversity in *Mycobacterium tuberculosis* for infection and vaccination outcomes. *mSystems* 7 (3), e0011022. doi: 10.1128/msystems.00110-22

Castro, R. A. D., Ross, A., Kamwela, L., Reinhard, M., Loiseau, C., Feldmann, J., et al. (2020). The genetic background modulates the evolution of fluoroquinolone-resistance in *Mycobacterium tuberculosis*. *Mol. Biol. Evol.* 37 (1), 195–207. doi: 10.1093/molbev/msz214

Chung, E. S., Johnson, W. C., and Aldridge, B. B. (2022). Types and functions of heterogeneity in mycobacteria. *Nat. Rev. Microbiol* 20, 529–541. doi: 10.1038/s41579-022-00721-0

Cohen, K. A., Manson, A. L., Desjardins, C. A., Abeel, T., and Earl, A. M. (2019). Deciphering drug resistance in *Mycobacterium tuberculosis* using whole-genome sequencing: progress, promise, and challenges. *Genome Med.* 11 (1), 45. doi: 10.1186/s13073-019-0660-8

Cokol, M., Kuru, N., Bicak, E., Larkins-Ford, J., and Aldridge, B. B. (2017). Efficient measurement and factorization of high-order drug interactions in *Mycobacterium tuberculosis*. *Sci. Adv.* 3 (10), e1701881. doi: 10.1126/sciadv.1701881

Colangeli, R., Arcus, V. L., Cursons, R. T., Ruthe, A., Karalus, N., Coley, K., et al. (2014). Whole genome sequencing of *Mycobacterium tuberculosis* reveals slow growth and low mutation rates during latent infections in humans. *PloS One* 9 (3), e91024. doi: 10.1371/journal.pone.0091024

Colangeli, R., Jedrey, H., Kim, S., Connell, R., Ma, S., Venkata, U. D. C., et al. (2018). Bacterial factors that predict relapse after tuberculosis therapy. *New Engl. J. Med.* 379 (9), 823–833. doi: 10.1056/nejmoa1715849

Coll, F., Phelan, J., Hill-Cawthorne, G. A., Nair, M. B., Mallard, K., Ali, S., et al. (2018). Genome-wide analysis of multi- and extensively drug-resistant *Mycobacterium tuberculosis*. *Nat. Genet.* 50 (2), 307–316. doi: 10.1038/s41588-017-0029-0

Comas, I., Borrell, S., Roetzer, A., Rose, G., Malla, B., Kato-Maeda, M., et al. (2011). Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. *Nat. Genet.* 44 (1), 106–110. doi: 10.1038/ng.1038

Crofton, J., and Mitchison, D. A. (1948). Streptomycin resistance in pulmonary tuberculosis. *Br. Med. J.* 2 (4588), 1009–1015. doi: 10.1136/bmj.2.4588.1009

Daniel, T. M. (2006). The history of tuberculosis. *Respir. Med.* 100 (11), 1862–1870. doi: 10.1016/j.rmed.2006.08.006

de Jonge, M. R., Koymans, L. H., Guillemont, J. E., Koul, A., and Andries, K. (2007). A computational model of the inhibition of *Mycobacterium tuberculosis* ATPase by a new drug candidate R207910. *Proteins* 67 (4), 971–980. doi: 10.1002/prot.21376

DiNardo, A. R., Gandhi, T., Heyckendorf, J., Grimm, S. L., Rajapakshe, K., Nishiguchi, T., et al. (2022). Gene expression signatures identify biologically and clinically distinct tuberculosis endotypes. *Eur. Respir. J* 60 (3), 1–15. doi: 10.1183/13993003.02263-2021

Dookie, N., Khan, A., Padayatchi, N., and Naidoo, K. (2022). Application of next generation sequencing for diagnosis and clinical management of drug-resistant tuberculosis: Updates on recent developments in the field. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.775030

Dookie, N., Naidoo, K., and Padayatchi, N. (2018). Whole-genome sequencing to guide the selection of treatment for drug-resistant tuberculosis. *Antimicrob. Agents Chemother.* 62 (8), 1–2. doi: 10.1128/AAC.00574-18

Fenner, L., Egger, M., Bodmer, T., Altpeter, E., Zwahlen, M., Jaton, K., et al. (2012). Effect of mutation and genetic background on drug resistance in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 56 (6), 3047–3053. doi: 10.1128/AAC.06460-11

Ford, C. B., Lin, P. L., Chase, M. R., Shah, R. R., Iartchouk, O., Galagan, J., et al. (2011). Use of whole genome sequencing to estimate the mutation rate of *Mycobacterium tuberculosis* during latent infection. *Nat. Genet.* 43 (5), 482–486. doi: 10.1038/ng.811

Freihofer, P., Akbergenov, R., Teo, Y., Juskeviciene, R., Andersson, D. I., and Böttger, E. C. (2016). Nonmutational compensation of the fitness cost of antibiotic resistance in mycobacteria by overexpression of *tlyA* rRNA methylase. *RNA* 22 (12), 1836–1843. doi: 10.1261/rna.057257.116

Fursov, M. V., Shitikov, E. A., Lagutkin, D. A., Fursova, A. D., Ganina, E. A., Kombarova, T. I., et al. (2021). MDR and pre-XDR clinical mycobacterium tuberculosis Beijing strains: Assessment of virulence and host cytokine response in mice infectious model. *Microorganisms* 9 (8), 1–17. doi: 10.3390/microorganisms9081792

Gagneux, S., Burgos, M. V., DeRiemer, K., Enciso, A., Muñoz, S., Hopewell, P. C., et al. (2006a). Impact of bacterial genetics on the transmission of isoniazid-resistant *Mycobacterium tuberculosis*. *PloS Pathog.* 2 (6), e61. doi: 10.1371/journal.ppat.0020061

Gagneux, S., Long, C. D., Small, P. M., Van, T., Schoolnik, G. K., and Bohannan, B. J. M. (2006b). The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. *Science* 312 (5782), 1944–1946. doi: 10.1126/science.1124410

Genestet, C., Hodille, E., Barbry, A., Berland, J. L., Hoffmann, J., Westeel, E., et al. (2021). Rifampicin exposure reveals within-host *Mycobacterium tuberculosis* diversity in patients with delayed culture conversion. *PloS Pathog.* 17 (6), e1009643. doi: 10.1371/journal.ppat.1009643

Gern, B. H., Adams, K. N., Plumlee, C. R., Stoltzfus, C. R., Shehata, L., Moguche, A. O., et al. (2021). TGFbeta restricts expansion, survival, and function of T cells within the tuberculous granuloma. *Cell Host Microbe* 29 (4), 594–606.e596. doi: 10.1016/j.chom.2021.02.005

Gillespie, S. H. (2002). Evolution of drug resistance in *Mycobacterium tuberculosis*: clinical and molecular perspective. *Antimicrob. Agents Chemother.* 46 (2), 267–274. doi: 10.1128/AAC.46.2.267-274.2002

Gillespie, S. H. (2007). Tuberculosis: evolution in millennia and minutes. *Biochem. Soc. Trans.* 35 (Pt 5), 1317–1320. doi: 10.1042/BST0351317

Gill, W. P., Harik, N. S., Whiddon, M. R., Liao, R. P., Mittler, J. E., and Sherman, D. R. (2009). A replication clock for *Mycobacterium tuberculosis*. *Nat. Med.* 15 (2), 211–214. doi: 10.1038/nm.1915

Goldstein, B. P. (2014). Resistance to rifampicin: a review. *J. Antibiot (Tokyo)* 67 (9), 625–630. doi: 10.1038/ja.2014.107

Gold, B., Warrier, T., and Nathan, C. (2021). A multistress model for high throughput screening against nonreplicating *Mycobacterium tuberculosis*. *Methods Mol. Biol.* 2314, 611–635. doi: 10.1007/978-1-0716-1460-0_27

Gomez-Gonzalez, P. J., Perdigao, J., Gomes, P., Puyen, Z. M., Santos-Lazaro, D., Napier, G., et al. (2021). Genetic diversity of candidate loci linked to *Mycobacterium tuberculosis* resistance to bedaquiline, delamanid and pretomanid. *Sci. Rep.* 11 (1), 19431. doi: 10.1038/s41598-021-98862-4

Gupta, A. K., Katoch, V. M., Chauhan, D. S., Sharma, R., Singh, M., Venkatesan, K., et al. (2010). Microarray analysis of efflux pump genes in multidrug-resistant *Mycobacterium tuberculosis* during stress induced by common anti-tuberculous drugs. *Microb. Drug Resist.* 16 (1), 21–28. doi: 10.1089/mdr.2009.0054

Haver, H. L., Chua, A., Ghode, P., Lakshminarayana, S. B., Singhal, A., Mathema, B., et al. (2015). Mutations in genes for the F420 biosynthetic pathway and a nitroreductase enzyme are the primary resistance determinants in spontaneous *in vitro*-selected PA-824-resistant mutants of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 59 (9), 5316–5323. doi: 10.1128/AAC.00308-15

Herricks, T., Donczew, M., Mast, F. D., Rustad, T., Morrison, R., Sterling, T. R., et al. (2020). ODELAM, rapid sequence-independent detection of drug resistance in isolates of *Mycobacterium tuberculosis*. *Elife* 9, 1–23. doi: 10.7554/eLife.56613

Herzog, H. (1998). History of tuberculosis. *Respiration* 65 (1), 5–15. doi: 10.1159/000029220

Heym, B., Alzari, P. M., Honore, N., and Cole, S. T. (1995). Missense mutations in the catalase-peroxidase gene, *katG*, are associated with isoniazid resistance in *Mycobacterium tuberculosis*. *Mol. Microbiol.* 15 (2), 235–245. doi: 10.1111/j.1365-2958.1995.tb02238.x

Hicks, N. D., Carey, A. F., Yang, J., Zhao, Y., and Fortune, S. M. (2019). Bacterial genome-wide association identifies novel factors that contribute to ethionamide and prothionamide susceptibility in mycobacterium tuberculosis. *mBio* 10 (2), 1–14. doi: 10.1128/mBio.00616-19

Hicks, N. D., Giffen, S. R., Culviner, P. H., Chao, M. C., Dulberger, C. L., Liu, Q., et al. (2020). Mutations in *dnaA* and a cryptic interaction site increase drug resistance in *Mycobacterium tuberculosis*. *PloS Pathog.* 16 (11), e1009063. doi: 10.1371/journal.ppat.1009063

Hicks, N. D., Yang, J., Zhang, X., Zhao, B., Grad, Y. H., Liu, L., et al. (2018). Clinically prevalent mutations in *Mycobacterium tuberculosis* alter propionate metabolism and mediate multidrug tolerance. *Nat. Microbiol.* 3 (9), 1032–1042. doi: 10.1038/s41564-018-0218-3

Hillemann, D., Rusch-Gerdes, S., and Richter, E. (2008). *In vitro*-selected linezolid-resistant *Mycobacterium tuberculosis* mutants. *Antimicrob. Agents Chemother.* 52 (2), 800–801. doi: 10.1128/AAC.01189-07

Huang, L., Nazarova, E. V., and Russell, D. G. (2019). *Mycobacterium tuberculosis*: Bacterial fitness within the host macrophage. *Microbiol. Spectr.* 7 (2), 1–12. doi: 10.1128/microbiolspec.BAI-0001-2019

Hughes, D., and Andersson, D. I. (2017). Evolutionary trajectories to antibiotic resistance. *Annu. Rev. Microbiol.* 71, 579–596. doi: 10.1146/annurev-micro-090816-093813

Huitric, E., Verhasselt, P., Koul, A., Andries, K., Hoffner, S., and Andersson, D. I. (2010). Rates and mechanisms of resistance development in *Mycobacterium tuberculosis* to a novel diarylquinoline ATP synthase inhibitor. *Antimicrob. Agents Chemother.* 54 (3), 1022–1028. doi: 10.1128/AAC.01611-09

Ince, D., and Hooper, D. C. (2003). Quinolone resistance due to reduced target enzyme expression. *J. Bacteriol* 185 (23), 6883–6892. doi: 10.1128/JB.185.23.6883-6892.2003

Jakkala, K., Paul, A., Pradhan, A., Nair, R. R., Sharan, D., Swaminath, S., et al. (2020). Unique mode of cell division by the mycobacterial genetic resister clones emerging *De novo* from the antibiotic-surviving population. *mSphere.* 5 (6), 1–25. doi: 10.1128/mSphere.00994-20

Javid, B., Sorrentino, F., Toosky, M., Zheng, W., Pinkham, J. T., Jain, N., et al. (2014). Mycobacterial mistranslation is necessary and sufficient for rifampicin phenotypic resistance. *Proc. Natl. Acad. Sci. U.S.A.* 111 (3), 1132–1137. doi: 10.1073/pnas.1317580111

Kaniga, K., Lounis, N., Zhuo, S., Bakare, N., and Andries, K. (2022). Impact of Rv0678 mutations on patients with drug-resistant TB treated with bedaquiline. *Int. J. Tuberc Lung Dis.* 26 (6), 571–573. doi: 10.5588/ijtld.21.0670

Katzir, I., Cokol, M., Aldridge, B. B., and Alon, U. (2019). Prediction of ultra-high-order antibiotic combinations based on pairwise interactions. *PloS Comput. Biol.* 15 (1), e1006774. doi: 10.1371/journal.pcbi.1006774

Kayigire, X. A., Friedrich, S. O., van der Merwe, L., and Diacon, A. H. (2017). Acquisition of rifampin resistance in pulmonary tuberculosis. *Antimicrob. Agents Chemother.* 61 (4), 1–5. doi: 10.1128/AAC.02220-16

Konno, K., Feldmann, F. M., and McDermott, W. (1967). Pyrazinamide susceptibility and amidase activity of tubercle bacilli. *Am. Rev. Respir. Dis.* 95 (3), 461–469. doi: 10.1164/arrd.1967.95.3.461

Lee, J. J., Lee, S. K., Song, N., Nathan, T. O., Swarts, B. M., Eum, S. Y., et al. (2019). Transient drug-tolerance and permanent drug-resistance rely on the trehalose-catalytic shift in *Mycobacterium tuberculosis*. *Nat. Commun.* 10 (1), 2928. doi: 10.1038/s41467-019-10975-7

Lenaerts, A., Barry, C. E.3rd, and Dartois, V. (2015). Heterogeneity in tuberculosis pathology, microenvironments and therapeutic responses. *Immunol. Rev.* 264 (1), 288–307. doi: 10.1111/imr.12252

Levin-Reisman, I., Brauner, A., Ronin, I., and Balaban, N. Q. (2019). Epistasis between antibiotic tolerance, persistence, and resistance mutations. *Proc. Natl. Acad. Sci. U.S.A.* 116 (29), 14734–14739. doi: 10.1073/pnas.1906169116

Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., and Balaban, N. Q. (2017). Antibiotic tolerance facilitates the evolution of resistance. *Science* 355 (6327), 826–830. doi: 10.1126/science.aaj2191

Levin, B. R., and Rozen, D. E. (2006). Non-inherited antibiotic resistance. *Nat. Rev. Microbiol.* 4 (7), 556–562. doi: 10.1038/nrmicro1445

Li, Y. Y., Cai, R. J., Yang, J. Y., Hendrickson, T. L., Xiang, Y., and Javid, B. (2021). Clinically relevant mutations of mycobacterial GatCAB inform regulation of translational fidelity. *mBio* 12 (4), e0110021. doi: 10.1128/mBio.01100-21

Li, Q.-j., Jiao, W.-w., Yin, Q.-q., Li, Y.-j., Li, J.-q., Xu, F., et al. (2017). Positive epistasis of major low-cost drug resistance mutations rpoB531-TTG and katG315-ACC depends on the phylogenetic background of *Mycobacterium tuberculosis* strains. *Int. J. Antimicrobial Agents* 49 (6), 757–762. doi: 10.1016/j.ijantimicag.2017.02.009

Lim, J., Lee, J. J., Lee, S. K., Kim, S., Eum, S. Y., and Eoh, H. (2021). Phosphoenolpyruvate depletion mediates both growth arrest and drug tolerance of *Mycobacterium tuberculosis* in hypoxia. *Proc. Natl. Acad. Sci. U.S.A.* 118 (35), 1–12. doi: 10.1073/pnas.2105800118

Li, S., Poulton, N. C., Chang, J. S., Azadian, Z. A., DeJesus, M. A., Ruecker, N., et al. (2022). CRISPRi chemical genetics and comparative genomics identify genes mediating drug potency in *Mycobacterium tuberculosis*. *Nat. Microbiol.* 7 (6), 766–779. doi: 10.1038/s41564-022-01130-y

Liu, J., Shi, W., Zhang, S., Hao, X., Maslov, D. A., Shur, K. V., et al. (2019). Mutations in efflux pump Rv1258c (Tap) cause resistance to pyrazinamide, isoniazid, and streptomycin in *M. tuberculosis*. *Front. Microbiol.* 10. doi: 10.3389/fmicb.2019.00216

Liu, Q., Wei, J., Li, Y., Wang, M., Su, J., Lu, Y., et al. (2020). *Mycobacterium tuberculosis* clinical isolates carry mutational signatures of host immune environments. *Sci. Adv.* 6 (22), eaba4901. doi: 10.1126/sciadv.aba4901

Louw, G. E., Warren, R. M., Gey van Pittius, N. C., Leon, R., Jimenez, A., Hernandez-Pando, R., et al. (2011). Rifampicin reduces susceptibility to ofloxacin in rifampicin-resistant *Mycobacterium tuberculosis* through efflux. *Am. J. Respir. Crit. Care Med.* 184 (2), 269–276. doi: 10.1164/rccm.201011-1924OC

Luo, T., Yuan, J., Peng, X., Yang, G., Mi, Y., Sun, C., et al. (2017). Double mutation in DNA gyrase confers moxifloxacin resistance and decreased fitness of mycobacterium smegmatis. *J. Antimicrobial Chemotherapy* 72 (7), 1893–1900. doi: 10.1093/jac/dkx110

Machado, D., Couto, I., Perdigao, J., Rodrigues, L., Portugal, I., Baptista, P., et al. (2012). Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. *PloS One* 7 (4), e34538. doi: 10.1371/journal.pone.0034538

Ma, S., Jaipalli, S., Larkins-Ford, J., Lohmiller, J., Aldridge, B. B., Sherman, D. R., et al. (2019). Transcriptomic signatures predict regulators of drug synergy and clinical regimen efficacy against tuberculosis. *mBio* 10 (6), 1–16. doi: 10.1128/mBio.02627-19

Ma, S., Morrison, R., Hobbs, S. J., Soni, V., Farrow-Johnson, J., Frando, A., et al. (2021). Transcriptional regulator-induced phenotype screen reveals drug potentiators in *Mycobacterium tuberculosis*. *Nat. Microbiol.* 6 (1), 44–50. doi: 10.1038/s41564-020-00810-x

Martini, M. C., Hicks, N. D., Xiao, J., Alonso, M. N., Barbier, T., Sixsmith, J., et al. (2022). Loss of RNase J leads to multi-drug tolerance and accumulation of highly structured mRNA fragments in *Mycobacterium tuberculosis*. *PloS Pathog.* 18 (7), e1010705. doi: 10.1371/journal.ppat.1010705

Maus, C. E., Plikaytis, B. B., and Shinnick, T. M. (2005). Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 49 (8), 3192–3197. doi: 10.1128/AAC.49.8.3192-3197.2005

McCune, R. M.Jr., and Tompsett, R. (1956). Fate of *Mycobacterium tuberculosis* in mouse tissues as determined by the microbial enumeration technique. i. the persistence of drug-susceptible tubercle bacilli in the tissues despite prolonged antimicrobial therapy. *J. Exp. Med.* 104 (5), 737–762. doi: 10.1084/jem.104.5.737

McGrath, M., Pittius, N., Helden, P., Warren, R. M., and Warner, D. F. (2014). Mutation rate and the emergence of drug resistance in *Mycobacterium tuberculosis*. *J. Antimicrobial Chemotherapy.* 69 (2), 292–302. doi: 10.1093/jac/dkt364

Meftahi, N., Namouchi, A., Mhenni, B., Brandis, G., Hughes, D., and Mardassi, H. (2016). Evidence for the critical role of a secondary site *rpoB* mutation in the compensatory evolution and successful transmission of an MDR tuberculosis outbreak strain. *J. Antimicrob. Chemother.* 71 (2), 324–332. doi: 10.1093/jac/dkv345

Meier, A., Kirschner, P., Bange, F. C., Vogel, U., and Bottger, E. C. (1994). Genetic alterations in streptomycin-resistant *Mycobacterium tuberculosis*: mapping of mutations conferring resistance. *Antimicrob. Agents Chemother.* 38 (2), 228–233. doi: 10.1128/AAC.38.2.228

Middlebrook, G. (1954). Isoniazid-resistance and catalase activity of tubercle bacilli; a preliminary report. *Am. Rev. tuberculosis.* 69 (3), 471–472. doi: 10.1164/art.1954.69.3.471

Molloy, A., Harrison, J., McGrath, J. S., Owen, Z., Smith, C., Liu, X., et al. (2021). Microfluidics as a novel technique for tuberculosis: From diagnostics to drug discovery. *Microorganisms* 9 (11), 1–24. doi: 10.3390/microorganisms9112330

Muzondiwa, D., Hlanze, H., and Reva, O. N. (2021). The epistatic landscape of antibiotic resistance of different clades of *Mycobacterium tuberculosis*. *Antibiotics (Basel)* 10 (7), 1–14. doi: 10.3390/antibiotics10070857

Nair, J., Rouse, D. A., Bai, G. H., and Morris, S. L. (1993). The *rpsL* gene and streptomycin resistance in single and multiple drug-resistant strains of *Mycobacterium tuberculosis*. *Mol. Microbiol.* 10 (3), 521–527. doi: 10.1111/j.1365-2958.1993.tb00924.x

Namouchi, A., Didelot, X., Schock, U., Gicquel, B., and Rocha, E. P. (2012). After the bottleneck: Genome-wide diversification of the *Mycobacterium tuberculosis* complex by mutation, recombination, and natural selection. *Genome Res.* 22 (4), 721–734. doi: 10.1101/gr.129544.111

Nandakumar, M., Nathan, C., and Rhee, K. Y. (2014). Isocitrate lyase mediates broad antibiotic tolerance in *Mycobacterium tuberculosis*. *Nat. Commun.* 5, 4306. doi: 10.1038/ncomms5306

Nieto Ramirez, L. M., Quintero Vargas, K., and Diaz, G. (2020). Whole genome sequencing for the analysis of drug resistant strains of *Mycobacterium tuberculosis*:

A systematic review for bedaquiline and delamanid. *Antibiotics (Basel)* 9 (3), 1–14. doi: 10.3390/antibiotics9030133

Nikaido, H. (2009). Multidrug resistance in bacteria. *Biochemistry* 78 (1), 119–146. doi: 10.1146/annurev.biochem.78.082907

Ohno, H., Koga, H., Kohno, S., Tashiro, T., and Hara, K. (1996). Relationship between rifampin MICs for and *rpoB* mutations of *Mycobacterium tuberculosis* strains isolated in Japan. *Antimicrob. Agents Chemother.* 40 (4), 1053–1056. doi: 10.1128/AAC.40.4.1053

Patel, S. J., Lewis, B. E., Long, J. E., Nambi, S., Sassetti, C. M., Stemmler, T. L., et al. (2016). Fine-tuning of substrate affinity leads to alternative roles of *Mycobacterium tuberculosis* Fe2+-ATPases. *J. Biol. Chem.* 291 (22), 11529–11539. doi: 10.1074/jbc.M116.718239

Peterson, E. J. R., Ma, S., Sherman, D. R., and Baliga, N. S. (2016). Network analysis identifies Rv0324 and Rv0880 as regulators of bedaquiline tolerance in *Mycobacterium tuberculosis*. *Nat. Microbiol.* 1 (8), nmicrobiol201678. doi: 10.1038/nmicrobiol.2016.78

Pi, R., Liu, Q., Takiff, H. E., and Gao, Q. (2020). Fitness cost and compensatory evolution in levofloxacin-resistant *Mycobacterium aurum*. *Antimicrob. Agents Chemother.* 64 (8), 1–5. doi: 10.1128/AAC.00224-20

Pisu, D., Huang, L., Grenier, J. K., and Russell, D. G. (2020). Dual RNA-seq of mtb-infected macrophages *In vivo* reveals ontologically distinct host-pathogen interactions. *Cell Rep.* 30 (2), 335–350.e334. doi: 10.1016/j.celrep.2019.12.033

Pisu, D., Huang, L., Narang, V., Theriault, M., Le-Bury, G., Lee, B., et al. (2021). Single cell analysis of *M. tuberculosis* phenotype and macrophage lineages in the infected lung. *J. Exp. Med.* 218 (9), 1–25. doi: 10.1084/jem.20210615

Plumlee, C. R., Duffy, F. J., Gern, B. H., Delahaye, J. L., Cohen, S. B., Stoltzfus, C. R., et al. (2021). Ultra-low dose aerosol infection of mice with *Mycobacterium tuberculosis* more closely models human tuberculosis. *Cell Host Microbe* 29 (1), 68–82.e65. doi: 10.1016/j.chom.2020.10.003

Pule, C. M., Sampson, S. L., Warren, R. M., Black, P. A., van Helden, P. D., Victor, T. C., et al. (2016). Efflux pump inhibitors: targeting mycobacterial efflux systems to enhance TB therapy. *J. Antimicrob. Chemother.* 71 (1), 17–26. doi: 10.1093/jac/dkv316

Pushparajan, A. R., Ramachandran, R., Gopi Reji, J., and Ajay Kumar, R. (2020). Mycobacterium tuberculosis TetR family transcriptional regulator Rv1019 is a negative regulator of the *mfd-mazG* operon encoding DNA repair proteins. *FEBS Lett.* 594 (17), 2867–2880. doi: 10.1002/1873-3468.13861

Quinonez, C. G., Lee, J. J., Lim, J., Odell, M., Lawson, C. P., Anyogu, A., et al. (2022). The role of fatty acid metabolism in drug tolerance of *Mycobacterium tuberculosis*. *mBio* 13 (1), e0355921. doi: 10.1128/mbio.03559-21

Ragheb, M. N., Thomason, M. K., Hsu, C., Nugent, P., Gage, J., Samadpour, A. N., et al. (2019). Inhibiting the evolution of antibiotic resistance. *Mol. Cell* 73 (1), 157–165.e155. doi: 10.1016/j.molcel.2018.10.015

Raimunda, D., Long, J. E., Padilla-Benavides, T., Sassetti, C. M., and Argüello, J. M. (2014). Differential roles for the Co(2+) /Ni(2+) transporting ATPases, CtpD and CtpJ, in *Mycobacterium tuberculosis* virulence. *Mol. Microbiol.* 91 (1), 185–197. doi: 10.1111/mmi.12454

Ramaswamy, S., and Musser, J. M. (1998). Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tuber Lung Dis.* 79 (1), 3–29. doi: 10.1054/tuld.1998.0002

Rego, E. H., Audette, R. E., and Rubin, E. J. (2017). Deletion of a mycobacterial divisome factor collapses single-cell phenotypic heterogeneity. *Nature* 546 (7656), 153–157. doi: 10.1038/nature22361

Reiche, M. A., Warner, D. F., and Mizrahi, V. (2017). Targeting DNA replication and repair for the development of novel therapeutics against tuberculosis. *Front. Mol. Biosci.* 4. doi: 10.3389/fmolb.2017.00075

Rengarajan, J., Bloom, B. R., and Rubin, E. J. (2005). Genome-wide requirements for *Mycobacterium tuberculosis* adaptation and survival in macrophages. *Proc. Natl. Acad. Sci. U.S.A.* 102 (23), 8327–8332. doi: 10.1073/pnas.0503272102

Rengarajan, J., Sassetti, C. M., Naroditskaya, V., Sloutsky, A., Bloom, B. R., and Rubin, E. J. (2004). The folate pathway is a target for resistance to the drug para-aminosalicylic acid (PAS) in mycobacteria. *Mol. Microbiol.* 53 (1), 275–282. doi: 10.1111/j.1365-2958.2004.04120.x

Richards, J. P., Cai, W., Zill, N. A., Zhang, W., and Ojha, A. K. (2019). Adaptation of *Mycobacterium tuberculosis* to biofilm growth is genetically linked to drug tolerance. *Antimicrob. Agents Chemother.* 63 (11), 1–17. doi: 10.1128/AAC.01213-19

Robicsek, A., Strahilevitz, J., Jacoby, G. A., Macielag, M., Abbanat, D., Park, C. H., et al. (2006). Fluoroquinolone-modifying enzyme: a new adaptation of a common aminoglycoside acetyltransferase. *Nat. Med.* 12 (1), 83–88. doi: 10.1038/nm1347

Rowlinson, M.-C., and Musser, K. A. (2022). Current methods and role of next-generation sequencing in the diagnosis of antimicrobial resistance in tuberculosis. *Clin. Microbiol. Newslett.* 44 (1), 1–12. doi: 10.1016/j.clinmicnews.2021.12.001

Safi, H., Gopal, P., Lingaraju, S., Ma, S., Levine, C., Dartois, V., et al. (2019). Phase variation in *Mycobacterium tuberculosis glpK* produces transiently heritable drug tolerance. *Proc. Natl. Acad. Sci. U.S.A.* 116 (39), 19665–19674. doi: 10.1073/pnas.1907631116

Safi, H., Lingaraju, S., Amin, A., Kim, S., Jones, M., Holmes, M., et al. (2013). Evolution of high-level ethambutol-resistant tuberculosis through interacting mutations in decaprenylphosphoryl-beta-D-arabinose biosynthetic and utilization pathway genes. *Nat. Genet.* 45 (10), 1190–1197. doi: 10.1038/ng.2743

Safi, H., Lingaraju, S., Ma, S., Husain, S., Hoque, M., Soteropoulos, P., et al. (2020). Rapidly correcting frameshift mutations in the *Mycobacterium tuberculosis orn* gene produce reversible ethambutol resistance and small-Colony-Variant morphology. *Antimicrob. Agents Chemother.* 64 (9), 1–11. doi: 10.1128/AAC.00213-20

Sandegren, L., and Andersson, D. I. (2009). Bacterial gene amplification: implications for the evolution of antibiotic resistance. *Nat. Rev. Microbiol.* 7 (8), 578–588. doi: 10.1038/nrmicro2174

Sander, P., Springer, B., Prammananan, T., Sturmfels, A., Kappler, M., Pletschette, M., et al. (2002). Fitness cost of chromosomal drug resistance-conferring mutations. *Antimicrobial Agents Chemotherapy* 46 (5), 1204–1211. doi: 10.1128/aac.46.5.1204-1211.2002

Santi, I., Dhar, N., Bousbaine, D., Wakamoto, Y., and McKinney, J. D. (2013). Single-cell dynamics of the chromosome replication and cell division cycles in mycobacteria. *Nat. Commun.* 4, 2470. doi: 10.1038/ncomms3470

Sarathy, J. P., Via, L. E., Weiner, D., Blanc, L., Boshoff, H., Eugenin, E. A., et al. (2018). Extreme drug tolerance of *Mycobacterium tuberculosis* in caseum. *Antimicrob. Agents Chemother.* 62 (2), 1–11. doi: 10.1128/AAC.02266-17

Schaefer, W. B. (1954). The effect of isoniazid on growing and resting tubercle bacilli. *Am. Rev. Tuberc* 69 (1), 125–127. doi: 10.1164/art.1954.69.1.125

Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., et al. (2003). Transcriptional adaptation of mycobacterium tuberculosis within macrophages: Insights into the phagosomal environment. *J. Exp. Med.* 198 (5), 693–704. doi: 10.1084/jem.20030846

Schubert, O. T., Ludwig, C., Kogadeeva, M., Zimmermann, M., Rosenberger, G., Gengenbacher, M., et al. (2015). Absolute proteome composition and dynamics during dormancy and resuscitation of *Mycobacterium tuberculosis*. *Cell Host Microbe* 18 (1), 96–108. doi: 10.1016/j.chom.2015.06.001

Scorpio, A., and Zhang, Y. (1996). Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. *Nat. Med.* 2 (6), 662–667. doi: 10.1038/nm0696-662

Sebastian, J., Swaminath, S., Nair, R. R., Jakkala, K., Pradhan, A., and Ajitkumar, P. (2017). *De novo* emergence of genetically resistant mutants of *Mycobacterium tuberculosis* from the persistence phase cells formed against antituberculosis drugs *In vitro*. *Antimicrobial Agents Chemotherapy* 61 (2), 1–25. doi: 10.1128/aac.01343-16

Shea, J., Halse, T. A., Kohlerschmidt, D., Lapierre, P., Modestil, H. A., Kearns, C. H., et al. (2021). Low-level rifampin resistance and *rpoB* mutations in *Mycobacterium tuberculosis*: an analysis of whole-genome sequencing and drug susceptibility test data in new York. *J. Clin. Microbiol.* 59 (4), 1–11. doi: 10.1128/JCM.01885-20

Sherman, D. R., Mdluli, K., Hickey, M. J., Arain, T. M., Morris, S. L., Iii, C. E. B., et al. (1996). Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. *Science* 272 (5268), 1641–1643. doi: 10.1126/science.272.5268.1641

Sirakova, T. D., Dubey, V. S., Deb, C., Daniel, J., Korotkova, T. A., Abomoelak, B., et al. (2006). Identification of a diacylglycerol acyltransferase gene involved in accumulation of triacylglycerol in *Mycobacterium tuberculosis* under stress. *Microbiol. (Reading)* 152 (Pt 9), 2717–2725. doi: 10.1099/mic.0.28993-0

Stevens, W. S., Scott, L., Noble, L., Gous, N., and Dheda, K. (2017). Impact of the GeneXpert MTB/RIF technology on tuberculosis control. *Microbiol. Spectr.* 5 (1), 1–21. doi: 10.1128/microbiolspec.TBTB2-0040-2016

Strydom, N., Gupta, S. V., Fox, W. S., Via, L. E., Bang, H., Lee, M., et al. (2019). Tuberculosis drugs' distribution and emergence of resistance in patient's lung lesions: A mechanistic model and tool for regimen and dose optimization. *PloS Med.* 16 (4), e1002773. doi: 10.1371/journal.pmed.1002773

Sukheja, P., Kumar, P., Mittal, N., Li, S. G., Singleton, E., Russo, R., et al. (2017). A novel small-molecule inhibitor of the *Mycobacterium tuberculosis* demethylmenaquinone methyltransferase MenG is bactericidal to both growing and nutritionally deprived persister cells. *mBio* 8 (1), 1–21. doi: 10.1128/mBio.02022-16

Sun, H., Zeng, J., Li, S., Liang, P., Zheng, C., Liu, Y., et al. (2018). Interaction between rpsL and gyrA mutations affects the fitness and dual resistance of *Mycobacterium tuberculosis* clinical isolates against streptomycin and fluoroquinolones. *Infection Drug Resistance* 11, 431–440. doi: 10.2147/idr.s152335

Su, H. W., Zhu, J. H., Li, H., Cai, R. J., Ealand, C., Wang, X., et al. (2016). The essential mycobacterial amidotransferase GatCAB is a modulator of specific translational fidelity. *Nat. Microbiol.* 1 (11), 16147. doi: 10.1038/nmicrobiol.2016.147

Swaminath, S., Paul, A., Pradhan, A., Sebastian, J., Nair, R. R., and Ajitkumar, P. (2020). *Mycobacterium smegmatis* moxifloxacin persister cells produce high levels of hydroxyl radical, generating genetic resisters selectable not only with moxifloxacin, but also with ethambutol and isoniazid. *Microbiol. (Reading)* 166 (2), 180–198. doi: 10.1099/mic.0.000874

Szumowski, J. D., Adams, K. N., Edelstein, P. H., and Ramakrishnan, L. (2013). Antimicrobial efflux pumps and *Mycobacterium tuberculosis* drug tolerance: evolutionary considerations. *Curr. Top. Microbiol. Immunol.* 374, 81–108. doi: 10.1007/82_2012_300

Takiff, H. E., Salazar, L., Guerrero, C., Philipp, W., Huang, W. M., Kreiswirth, B., et al. (1994). Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations. *Antimicrob. Agents Chemother.* 38 (4), 773–780. doi: 10.1128/AAC.38.4.773

Telenti, A., Imboden, P., Marchesi, F., Lowrie, D., Cole, S., Colston, M. J., et al. (1993). Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet* 341 (8846), 647–650. doi: 10.1016/0140-6736(93)90417-f

Telenti, A., Philipp, W. J., Sreevatsan, S., Bernasconi, C., Stockbauer, K. E., Wieles, B., et al. (1997). The *emb* operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. *Nat. Med.* 3 (5), 567–570. doi: 10.1038/nm0597-567

The CRyPTIC Consortium (2022). A data compendium associating the genomes of 12,289 *Mycobacterium tuberculosis* isolates with quantitative resistance phenotypes to 13 antibiotics. *PloS Biol.* 20 (8), e3001721. doi: 10.1371/journal.pbio.3001721

Trindade, S., Sousa, A., Xavier, K. B., Dionisio, F., Ferreira, M. G., and Gordo, I. (2009). Positive epistasis drives the acquisition of multidrug resistance. *PloS Genet.* 5 (7), e1000578. doi: 10.1371/journal.pgen.1000578

Urbaniec, J., Xu, Y., Hu, Y., Hingley-Wilson, S., and McFadden, J. (2022). Phenotypic heterogeneity in persisters: a novel 'hunker' theory of persistence. *FEMS Microbiol. Rev.* 46 (1), 1–16. doi: 10.1093/femsre/fuab042

Vargas, R., Freschi, L., Spitaleri, A., Tahseen, S., Barilar, I., Niemann, S., et al. (2021). Role of epistasis in amikacin, kanamycin, bedaquiline, and clofazimine resistance in *Mycobacterium tuberculosis* complex. *Antimicrobial Agents Chemotherapy* 65 (11), e01164-01121. doi: 10.1128/aac.01164-21

Vijay, S., Vinh, D. N., Hai, H. T., Ha, V. T. N., Dung, V. T. M., Dinh, T. D., et al. (2017). Influence of stress and antibiotic resistance on cell-length distribution in *Mycobacterium tuberculosis* clinical isolates. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.02296

Villellas, C., Aristimuno, L., Vitoria, M. A., Prat, C., Blanco, S., Garcia de Viedma, D., et al. (2013). Analysis of mutations in streptomycin-resistant strains reveals a simple and reliable genetic marker for identification of the *Mycobacterium tuberculosis* Beijing genotype. *J. Clin. Microbiol.* 51 (7), 2124–2130. doi: 10.1128/JCM.01944-12

Villellas, C., Coeck, N., Meehan, C. J., Lounis, N., de Jong, B., Rigouts, L., et al. (2017). Unexpected high prevalence of resistance-associated Rv0678 variants in MDR-TB patients without documented prior use of clofazimine or bedaquiline. *J. Antimicrob. Chemother.* 72 (3), 684–690. doi: 10.1093/jac/dkw502

Wang, B. W., Zhu, J. H., and Javid, B. (2020). Clinically relevant mutations in mycobacterial LepA cause rifampicin-specific phenotypic resistance. *Sci. Rep.* 10 (1), 8402. doi: 10.1038/s41598-020-65308-2

WHO (2021a). *Catalogue of mutations in mycobacterium tuberculosis complex and their association with drug resistance* (Geneva: World Health Organization). Licence: CC BY-NC-SA 3.0 IGO ed.

WHO (2021b). *Global tuberculosis report 2021* (Geneva: World Health Organization).

Widelock, D., Peizer, L. R., and Klein, S. (1955). Public health significance of tubercle bacilli resistant to isoniazid. *Am. J. Public Health Nations Health* 45 (1), 79–83. doi: 10.2105/AJPH.45.1.79

Wolinsky, E., Smith, M. M., and Steenken, W. (1956). Isoniazid susceptibility, catalase activity, and guinea pig virulence of recently isolated cultures of tubercle bacilli. *Am. Rev. tuberculosis* 73 (5), 768–772. doi: 10.1164/artpd.1956.73.5.768

Xie, Y. L., de Jager, V. R., Chen, R. Y., Dodd, L. E., Paripati, P., Via, L. E., et al. (2021). Fourteen-day PET/CT imaging to monitor drug combination activity in treated individuals with tuberculosis. *Sci. Transl. Med.* 13 (579), 1–11. doi: 10.1126/scitranslmed.abd7618

Xu, J., Li, S. Y., Almeida, D. V., Tasneen, R., Barnes-Boyle, K., Converse, P. J., et al. (2019). Contribution of pretomanid to novel regimens containing bedaquiline with either linezolid or moxifloxacin and pyrazinamide in murine models of tuberculosis. *Antimicrob. Agents Chemother.* 63 (5), 1–14. doi: 10.1128/AAC.00021-19

Xu, W., Snell, L. M., Guo, M., Boukhaled, G., Macleod, B. L., Li, M., et al. (2021). Early innate and adaptive immune perturbations determine long-term severity of chronic virus and *Mycobacterium tuberculosis* coinfection. *Immunity* 54 (3), 526–541.e527. doi: 10.1016/j.immuni.2021.01.003

Zeng, X., Kwok, J. S., Yang, K. Y., Leung, K. S., Shi, M., Yang, Z., et al. (2018). Whole genome sequencing data of 1110 *Mycobacterium tuberculosis* isolates identifies insertions and deletions associated with drug resistance. *BMC Genomics* 19 (1), 365. doi: 10.1186/s12864-018-4734-6

Zhang, Y., Heym, B., Allen, B., Young, D., and Cole, S. (1992). The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature* 358 (6387), 591–593. doi: 10.1038/358591a0

Zhang, H., Li, D., Zhao, L., Fleming, J., Lin, N., Wang, T., et al. (2013). Genome sequencing of 161 *Mycobacterium tuberculosis* isolates from China identifies genes and intergenic regions associated with drug resistance. *Nat. Genet.* 45 (10), 1255–1260. doi: 10.1038/ng.2735

Zhu, J. H., Wang, B. W., Pan, M., Zeng, Y. N., Rego, H., and Javid, B. (2018). Rifampicin can induce antibiotic tolerance in mycobacteria *via* paradoxical changes in *rpoB* transcription. *Nat. Commun.* 9 (1), 4218. doi: 10.1038/s41467-018-06667-3

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Perspective
PUBLISHED 06 October 2022
DOI 10.3389/fcimb.2022.1029044

OPEN ACCESS

EDITED BY
Helena Ingrid Boshoff,
National Institutes of Health (NIH),
United States

REVIEWED BY
Samantha Wellington Miranda,
University of Washington,
United States
Scott Gary Franzblau,
University of Illinois at Chicago,
United States

*CORRESPONDENCE
Christine Roubert
christine.roubert@evotec.com

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 26 August 2022
ACCEPTED 21 September 2022
PUBLISHED 06 October 2022

CITATION
Roubert C, Fontaine E and Upton AM (2022) "Upcycling" known molecules and targets for drug-resistant TB. *Front. Cell. Infect. Microbiol.* 12:1029044.
doi: 10.3389/fcimb.2022.1029044

"Upcycling" known molecules and targets for drug-resistant TB

Christine Roubert*, Evelyne Fontaine and Anna M Upton

Evotec ID (Lyon), Lyon, France

Despite reinvigorated efforts in Tuberculosis (TB) drug discovery over the past 20 years, relatively few new drugs and candidates have emerged with clear utility against drug resistant TB. Over the same period, significant technological advances and learnings around target value have taken place. This has offered opportunities to re-assess the potential for optimization of previously discovered chemical matter against *Mycobacterium tuberculosis* (M.tb) and for reconsideration of clinically validated targets encumbered by drug resistance. A re-assessment of discarded compounds and programs from the "golden age of antibiotics" has yielded new scaffolds and targets against TB and uncovered classes, for example beta-lactams, with previously unappreciated utility for TB. Leveraging validated classes and targets has also met with success: booster technologies and efforts to thwart efflux have improved the potential of ethionamide and spectinomycin classes. Multiple programs to rescue high value targets while avoiding cross-resistance are making progress. These attempts to make the most of known classes, drugs and targets complement efforts to discover new chemical matter against novel targets, enhancing the chances of success of discovering effective novel regimens against drug-resistant TB.

KEYWORDS

drug resistance, target, antibiotics, phenotypic screening, golden age of antibiotics

Abbreviations: TB, Tuberculosis; WHO, World Health Organization; DMPK, Drug metabolism and pharmacokinetics; M. tb, Mycobacterium tuberculosis; BacPROTACs, Bacterial-Proteolysis Targeting Chimeras; MDR, Multidrug-resistant; XDR, Extensively drug-resistant; InhA, Enoyl Acyl Carrier Protein Reductase; PZA, pyrazinamide; RIF, Rifampicin; INH, Isoniazid; ETH, ethambutol; FQ, fluoroquinolones; CID, Compound identification number.

Introduction

Despite 20 years of new vigor in Tuberculosis (TB) drug discovery, the number of novel compounds that have potential utility against drug resistant TB, and that have successfully advanced to clinical studies, is low. The Target Regimen Profiles for TB, for example those set out by the World Health Organization (WHO, 2016), set a high bar for contributing drugs, aiming for regimens that shorten treatment compared to the standard of care and are improved with respect to route of administration (oral replacing non-oral), tolerability, drug-drug interactions and with a limited need for monitoring. Considering these goals and the desire to de-risk drug candidate properties prior to clinical development, it is understandable that significant attrition is seen across discovery and early development, especially when considering novel chemical series for which precise safety, pharmaceutical and distribution, metabolism and pharmacokinetics (DMPK) profiles are, a priori, unknown. Programs seeking to discover and develop novel treatments suitable for drug-resistant TB face an additional constraint that can limit options when considering novel chemical matter - that is the requirement that the series of interest exhibit very limited or no pre-existing resistance (Miotto et al., 2017; Coll et al., 2018; Walker et al., 2022).

Most TB drug resistance is drug-target related, so significant efforts have been expended towards discovery of modulators of new targets. However, new targets, by definition, are not yet clinically validated. So far, for the small number of development compounds against new targets, the success rate in demonstrating proof of concept efficacy in Early Bactericidal Activity clinical trial trials has been high (Diacon et al., 201). This is likely due to the care that has been taken by teams to evaluate and preclinically-validate new targets, as well as the good overall performance of the tools available to enable prediction of clinical efficacy, including animal models of TB. However, a key desirable property for novel TB treatments is the ability to shorten treatment duration to cure, compared to the standard of care. For this specific property, due to the limited clinical data available, it is more challenging to predict the degree to which a drug against a new target will contribute.

Over recent years, several strategies have emerged, to address the risks inherent in progressing novel chemical matter against novel targets. These involve a second look at what may be already-existing possibilities: to leverage known antibacterial classes for TB (with the advantage of their known safety and DMPK profiles, but lack of pre-existing resistance), and to "rescue" clinically validated TB drug targets (with known clinical efficacy profiles) that have been compromised by resistance. In both cases, this second-life brings together pre-existing opportunities with new technologies and thinking, against drug-resistant TB (Lohrasbi et al., 2018; Bandodkar et al., 2020; Singh and Chibale, 2021).

This perspective summarizes the progress of these approaches to date, together with evident advantages and limitations of these strategies.

Revisiting known classes and abandoned programs from the golden age of antibiotics

Prior to the renewed efforts of the past 20 years, the last phase of major activity in TB drug discovery was during what is now called "the golden age of antibiotics" (Lewis, 2013). Multiple successful TB drugs emerged from this period, including Streptomycin (1943), para-aminosalicylic acid (1946), isoniazid (INH, 1952), pyrazinamide (PZA, 1952), ethambutol (ETH 1961) and rifampicin (RIF, 1966) (Chakraborty & Rhee, 2015). Many other TB drug research projects began and were then discontinued during this time, often for scientific and/or business reasons. In some cases, based on anti-M.tb activity data available at the time, classes such as beta-lactams were developed for other antibacterial indications but were not progressed for TB. In other cases, compounds were advanced to early clinical studies but were discontinued due to low probability of commercial success judged based on the contemporary landscape and capabilities. A second, new-millennium look at cases of these types has brought new insights to the TB drug target space as well as progression of known classes and molecules re-directed for potential utility against TB.

Maximizing utility of known classes

Beta-lactams are an exceptionally safe class of antibiotics. They kill bacteria by inhibiting the transpeptidase that catalyzes the final step in cell wall biosynthesis - a source of several clinically validated TB drug targets. However, this class was long considered to be ineffective against M.tb, due to rapid hydrolysis by the M.tb beta lactamase and was never used to treat TB. Therefore, unlike the situation for other cell wall targeting TB drugs, no clinical record of beta-lactam resistance exists. In an example of a successful strategy to look again at a well-known class, evaluated the activity of carbapenems combined with clavulanic acid, a beta-lactamase inhibitor, was evaluated against M.tb, demonstrating potent activity of the meropenem-clavulonate combination (Watt et al., 1992; Huggonet et al., 2009). This opened the door to clinical exploration of this class for TB, with early bactericidal activity of meropenem, administered intravenously combined with amoxicillin–clavulanic acid, demonstrated in 2016 (Diacon et al., 2016). This clinical proof of concept motivated a search for an oral beta-lactam with utility for TB, resulting in the repurposing of the tricyclic beta-lactam Sanfetrinem, cilexetil, the oral prodrug of sanfetrinem, developed by GlaxoSmithKline

in the 1990s (Singh et al., 1996; Iavarone et al., 1997). This drug is currently in early development (Phase 2, NCT05388448) following preclinical demonstration of anti-M.tb activity (Ramon-Garcia, 2019). In parallel, in an extraordinary example of the use of pharmaceutical companies' patrimony in a collaborative approach, an initiative led by the Tuberculosis Drug Accelerator consortium screened about 8900 beta-lactams from GSK, Sanofi, Lilly, and MSD (Gold et al., 2022), looking for safe, *in vivo* active and possibly beta-lactamase inhibitor independent compounds.

In another example of successful revisiting of known classes, spectinamides, semisynthetic analogs of Spectinomycin, which was discovered in 1961 (Mason et al., 1961; Bergy et al., 1961), have demonstrated efficacy against TB in mouse models and MBX-488A has progressed to preclinical development as a potential TB drug. Spectinomycin exhibits poor activity against M.tb (Lee et al., 2014), despite targeting protein synthesis – a validated TB drug target - *via* the 30S subunit of the bacterial ribosome. It is used to treat gonorrheal infections but demonstrates limited tolerability and is dosed with intramuscular injections. The semisynthetic spectinamides, on the other hand, demonstrate improved selectivity as well as more potent anti-M.tb activity by avoiding efflux through the M.tb efflux pump Rv1258c. In a remarkable medicinal chemistry effort, Lee and colleagues optimized the series to avoid the Rv1258c efflux pump resulting in leads that demonstrate significant activity in acute and chronic mouse models of TB and contribute to combinations of TB drugs (Lee et al., 2014; Bruhn et al., 2015; Gonzalez-Juarrero et al., 2021).

Macrolides represent another class of antibacterial protein synthesis inhibitors that were thought to lack potency and utility against TB. This thinking has now been challenged by, for instance, the SEQ-503 macrolide from the Sanofi Natural Product patrimony, discovered in 1962 in Vitry-sur Seine and named after sequana, the seine goddess in the Gallo-Roman religion models (Lair et al., 2015). Optimization of SEQ-503 has given rise to SEQ9 which has a lower MIC (0.6 μM) than previously tested macrolides, for example Clarithromycin (8 μM), and is similarly potent to the more active macrolides reported by Falzari and colleagues (Falzari et al., 2005). Similar to the substituted 11,12 carbazate macrolide reported by Falzari et al, SEQ9 is active in mouse TB models (Lair et al., 2015); another demonstration of a new activity for a revisited class of antibiotics.

Bringing today's technologies to yesterday's discoveries

Beyond rethinking potential utility of known drug classes, like ghosts from the past, compounds discovered in the golden age of antibiotics but not advanced to market can be identified, retrieved and re-addressed with a new view. Such activities have been invigorated in the informatics era by document scanning and digitization of archives, providing the possibility to perform extensive searches of databases to select forgotten compounds for improvement or repurposing. Recent examples include the unexplored cyclohexapeptide natural product Desotamide (Miao et al., 1997), or Wollamides A and B (Khazil et al., 2014), which exhibit antimycobacterial activity including inhibition of intracellular M.tb in murine bone marrow-derived macrophages. These were optimized from 2017 resulting in improved pharmacokinetic properties but no description of vivo efficacy has been reported yet (Asfaw et al., 2017; Asfaw et al., 2018; Khalil et al., 2019) (Table 1).

In the meantime, TB drug discovery also benefited from the progresses made in peptide chemistry. After Merrifield's discovery (Merryfield, 1963) on solid-phase peptide synthesis in 1963 and the introduction of basolabile 9-fluorenylmethyloxycarbonyl (Fmoc) able to protect orthogonal side-chain groups (Carpino and Han, 1970), the automation of the process combined to the use of new types of resins have successfully improved the speed and possibility to explore SAR on natural compounds with total or hemisynthesis (Kimmerlin and Sebach, 2005).

Considering the different mergers of pharmaceutical companies, searching and analyzing the archive (patrimony) and compound libraries of pharma companies, not available to the public, might represent a golden opportunity inherited from the past. This strategy was used by Sanofi with the revival of natural product scaffolds) discovered by Rhône-Poulenc-Rorer in the 1960-1980 era. Griselimycin, discovered in 1964, showed success in treating TB, but with poor ADME properties (Hénazet 1966; Noufflard-Gyu-Noé and Berteaux, 1965). The discovery of its unique mechanism of action (Kling et al., 2015) through DnaN, supported by earlier reports of the effectiveness of Griselimycin against drug-resistant *M. tuberculosis* (Toyohara, 1987) led to a new drug discovery program addressing liabilities of this molecule. Advances in peptide chemistry, as described above, were instrumental in taking the original natural product further to produce the lead hexyl compound.

There are many further examples of TB active compounds uncovered from patent or literature searches that revealed antibiotic activity on M.tb and that have been, or have potential for optimization using modern techniques. These include Viomycin and Capreomycin (Youmans & Youmans, 1951; Bycroft, 1972), Histogranin (Lemaire et al., 1993) Amiclenomycin (Okami et al., 1974; Mann and Ploux, 2006; Dey et al., 2010), Isoxazoline (Tangallapally et al., 2007; Phanumartwiwath et al., 2021), and Pleuromutilin (Kavanagh et al., 1951; Lemieux et al., 2018). In some cases, a close look at such compounds using new technologies has also yielded targets of interest, for example cyclomarin A (Renner et al., 1999; Schmitt et al., 2011; Kiefer et al., 2019), which targets the Clp protease complex and was recently used in a proof of concept for chimeric small-molecule degraders, the bacterial-Proteolysis Targeting Chimeras (BacPROTACs) in M.tb (Morreale et al., 2022).

10.3389/fcimb.2022.1029044

TABLE 1 Compounds described in this review.

Coumpound	Suspected Target	Target Class	Pubmed CID	Reported Minimal Inhibitory Concentration MIC (µM) * author generated data	Efficasacy demonstrated in an animal model results can be limited by adm routes/ poor Pk/ animal models	Discovery	TB patients
Isoniazed	InhA	Cell wall	3767	0.3*	Yes	1912	1952
Rifampicin	RNA polymerase	Transcription	135398735	0.09	Yes	1957	1971
Meropenem	L,D-transpeptidase	Cell wall	441130	6.5	Yes	1976	2016
Sanfetrinem	L,D-transpeptidase	Cell wall	71452	5.3	Yes	1976?	2022 Phase 2, NCT05388448
Spectinomycin	30S subunit of the bacterial ribosome	Protein synthesis inhibitor	15541	150	low/no activity	1961	
Spectinamides 1599	30S subunit of the bacterial ribosome	Protein synthesis inhibitor	60173108	3.3	Yes	2014	
Clarithromycin	23S rRNA	Protein synthesis inhibitor	84029	8*	No	1980	
SEQ9	23S rRNA	Protein synthesis inhibitor		0.7	Yes	2015	
Desotamide			181446	NA		1997	
Wollamides B	Unknown		102341742	3		2014	
Viomycin	23S/16S	Translation	135398671	5.8-12		1950	
Capreomycin	23S/16S	Translation	3000502	5.8-12	Yes	1960	1966
Histogranine	Unknown/ATP		16131189	6.9		1993	
Amiclenomycin	Biotin pathway	Metabolism	99594	16		1975	
Pyridomycin	InhA	Cell wall	3037036	0.55-3.0		1953	
CPZEN-45	MraY/murX	Cell wall	674119859	2.3	Yes	2003	Pre-Clinical (Non-GLP)
FNDR 20364	inhibiting ribosome associated Gtpase activity	translation		Unknown	Yes	Unknown	GLP toxicology
Cyclomarin A	ClpC1	proteostasis	10772429			1999	
Pleuromutilin	50S subunit	Translation	9886081	2.2	Yes	1951	
Griselimycin	DnaN	Replication inhibitor	429055	0.09	Yes	1964	1964
Ethionamide	ethA	Cell wall	2761171	4.5*		1956	1965
Pretomanid			456199	0.3*		2000	2019
Kanglemycin A	RNA polymerase	Transcription	6443924	0.36	Yes	2018	
Sorangicin A	RNA polymerase	Transcription	657059	Unknown		1985	
Corallopyronin	RNA polymerase	Transcription	90477824	30		1985	
Fidaxomycin	RNA polymerase	Transcription	10034073	0.24		1987	
PUM	RNA polymerase	Transcription	72792467	inactive		2017	
D-AAP1	RNA polymerase	Transcription		4		2017	
Nargenicin	DnaE1	Cell wall	6326334	12.5		1980	
Compound 22 (Spiropyrimidinetrione)	DNA Gyrase	Replication inhibitor		1.7-5.2	Yes	2015	
VXc-486/SPR720	DNA Gyrase	Replication inhibitor			Yes	2014	Phase 2, NCT04553406

(Continued)

TABLE 1 Continued

Coumpound	Suspected Target	Target Class	Pubmed CID	Reported Minimal Inhibitory Concentration MIC (μM) * author generated data	Efficasacy demonstrated in an animal model results can be limited by adm routes/ poor Pk/ animal models	Discovery	TB patients
Compound 17 (Thiazolopyridone ureas)	DNA Gyrase	Replication inhibitor		2	Yes	2014	
Gepopidacin (GSK2140944)	DNA Gyrase	Replication inhibitor	25101874	0.38		2015	

CID, pubchem compound identification number; DprE1, decaprenylphosphoryl-β-D-ribose 2'-epimerase (Rv3790); EthA, monooxygenase EthA (Rv3854c); DnaN, DNA polymerase III DnaN (Rv0002); ClpC1, TP-dependent Clp protease ATP-binding subunit ClpC (Rv3596c); InhA, NADH-dependent enoyl-[acyl-carrier-protein] reductase (Rv1484); MmpL3, ransmembrane transporter (Rv0206c); LeuRS, leucyl-tRNA synthetase (Rv0041); DnaE1, DNA polymerase III alpha subunit (Rv1547); DHFR, dihydrofolate reductase DfrA (Rv2763c).

Many of these golden era natural products suffer from lower oral bioavailability. Newer drug delivery systems and formulation technologies can be brought to bear to improve oral bioavailability or even increase specific targeting, once again harnessing modern technology to make use of known or re-discovered TB actives. New formulation adapted to classical TB drugs in the hope to overcome both toxicity and resistance has been reviewed elsewhere (Singh et al., 2016; Mazlan et al., 2021). Combinations of clinically validated antibiotics, encapsulated in nanoparticles, have been investigated in macrophages and this concept could be opening a new path to combinations of drugs with different PK/PD parameters (Jiang et al., 2022). Furthermore, the recent examples of FNDR 20364 (Working Group for New TB Drugs) or CPZEN-45, a caprazamycin derived compound (Salomon et al., 2013) indicates potential for success using these approaches.

Approaches to address highly validated drugs and targets compromised by resistance

Besides the possible forgotten classes and missed opportunities from the Golden Age, several antibiotics were indeed developed and successfully used for TB from that time and beyond. Unfortunately, most of these are now compromised by resistance. With proven efficacy, these drugs and their targets are well validated and a variety of approaches have been pursued to "rescue" the targets and the drugs themselves for further use including against resistant TB.

Rethinking known marketed drug classes for TB

Towards rescuing known TB drugs to which resistance has emerged, a potential "short-cut" or repurposing strategy is to reconsider dosing of efficacious clinical anti-TB drugs, to evaluate whether higher exposures than currently used can safely and effectively treat TB due to infections with Mtb strains resistant to that drug. Indeed, the first line drug RIF could be given at higher doses and although current clinical trials are aiming to shorten treatment of drug sensitive TB, the outcome of a safe higher dose of RIF could also impact MDR TB (RIFASHORT, NCT02581527; HIRIF, NCT01408914). The same thinking has been applied to INH with the INHindsight study, a phase 2A dose-ranging trial of INH for patients with pulmonary MDR-tuberculosis and inhA mutations (Dooley et al., 2020; Wasserman and Furin, 2020). Alternatively, members of the same class as existing TB drugs can be evaluated where added value on moderately resistant clinical strains has been implied. An example is the rifampicin analog, rifabutin, which demonstrates activity on some RIF- resistant clinical strains (Yan et al., 2015; Berrada et al., 2016; Alfarisi et al., 2017).

For drugs to which high level resistance has emerged, such dose optimization is often not an option, and alternative efforts are needed. In a noteworthy example of innovation, utilizing a known marketed drug with a "resistance bypassing" strategy, the potentiation of Ethionamide has been achieved, to overcome its deleterious side effects and resistance. Ethionamide, discovered in 1956, is an intra-bacterial-prodrug that requires bioactivation within M.tb to acquire its antibacterial effect. Screening for "Ethionamide boosters" was conceptualized by the Institut Pasteur of Lille (Willand et al., 2009), and this group and collaborators have conducted fragment-based screening and structure-based optimization efforts (Prevet et al., 2019; Villemagne et al., 2020) towards achieving molecules inactivating repressors of the enzymes responsible for the bioactivation of drugs within M.tb, referred to as Small Molecules Aborting Resistance (SMARt) (Blondiaux et al., 2017) with the subsequent discovery of the phase 1 compound BVL-GSK098 (Working Group on New TB Drugs). Now, this group is actively working on this concept for other intrabacterial-prodrugs with low-level pre-existing resistance that may rise to significant clinical resistance in the future, such as pretonamid (Djaout, 2022).

The discovery of specific pathways and underlying druggable targets involved in M.tb's adaptation to and the subsequent reduction of efficacy of clinically validated anti-TB drugs might

be of great value. Recently, ingenious use of a set of inducible transcription factors strains, the Transcriptional Regulator Induced Phenotype (TRIP), representing most annotated M.tb regulators unraveled new uncharacterized regulons and downstream genes involved in the adaptation to INH (Ma et al., 2021). Understanding these mechanisms may lead to strategies to intervene and reverse such drug tolerance that may be the gateway to drug resistance.

As the non-target mechanisms of TB drug resistance continue to be uncovered, there exists an opportunity to identify and develop modulators of these mechanisms, as potential companion drugs, potentially lending new life to further existing TB drugs.

How can we rescue compromised validated targets

Another approach to overcoming drug resistance is the identification of novel scaffolds that inhibit the few clinically validated targets of the first and second-line anti-TB drugs, which have already shown efficacy or even treatment-shortening behavior. Indeed, the exploitation of new or modified scaffolds against highly validated targets for which existing TB drugs are compromised by resistance can decrease biological and clinical failure risk associated with pursuing compounds against new targets.

An obvious opportunity towards this aim is classical target-based screenings and these have been conducted against validated TB targets, seeking new chemical matter against targets for which current drugs are compromised by resistance. However, these have not met with much success. Small molecule uptake into and metabolism within Mtb, as well as access to molecular targets in the complex lipid-rich cell wall of mycobacteria, has hindered this effort. Freely accessible algorithms have been developed and can be used to predict mycobacterial cell wall penetration (eg MycPermCheck) based on drug activity (Merget et al., 2013). However, it remains to be seen whether such tools can aid in optimizing whole cell penetration for target-based hits without whole cell activity. Alternative approaches to screening, conducted against validated targets and pathways, but in whole cells, may prove more successful (Abrahams et al., 2012; Bonnett et al., 2016; Naran et al., 2016; Abramovitch, 2018; Evans and Mizrahi, 2018; Johnson et al., 2019; Burke et al., 2020; Smith et al., 2020). However, the following outlines alternative and innovative approaches towards this drug and target "rescue" goal.

Besides target-based screening approaches, repurposing and lead optimization strategies have been applied for molecules inhibiting validated targets in a different way to, and without cross-resistance with, important TB drugs. As an example, treatment shortening behavior has been clinically demonstrated with rifamycins, making them key TB drugs. They target the beta-subunit of the RNA polymerase complex encoded by the rpoB gene and this represents a valuable target. However, it is unclear if RIFs are sterilizing due to their specific binding mode, their physicochemical and excellent pharmacokinetic-pharmacodynamic properties including lesion penetration (Sarathy et al., 2016), or a combination of these.

Efforts have been made to repurpose and optimize RNA polymerase inhibitors discovered to be active in other bacterial species. These have been assessed in M.tb. with moderate success *in vitro* (Table 1). Examples are Kanglemycin (Mosaei et al., 2018; Peek et al., 2020; Harbotte et al., 2021) and Sorangicin A (Lilic et al., 2020) as well as compounds binding to different pockets than RIF like Corallopyronin A (Haebich et al., 2009; Boyaci et al., 2019); Fidaxomycin (Kurabachew et al., 2008; Boyaci et al., 2018); PUM (Maffioli et al., 2017) and the small molecule D-AAP1 (Lin et al., 2017). Moreover, much progress has been made in the comprehension of M.tb's RNAP complex and the design of new biochemical and biophysical assays should soon answer this tricky question (Stefan et al., 2020).

New whole-cell phenotypic screenings focusing on the global protein synthesis pathway, addressing both RNA polymerase and the ribosome, may be the key of new discoveries in this field (Burke et al., 2020). Indeed, protein synthesis inhibitors have also shown their value as antibiotics (Kavčič et al., 2020) in TB treatment. For instance, oxazolidinones' contributions to regimen have proven their treatment shortening activity in mouse TB models (Zhao et al., 2014; Xu et al., 2019) as well as in the Nix-TB and ZeNix clinical trial (Conradie et al., 2020; Conradie et al., 2022). Drug discovery efforts to date have mostly focused on finding safer oxazolidinones because there is very little pre-existing resistance for this class.

Inhibition of nucleic acid synthesis by inhibiting M.tb's type II topoisomerase, responsible for ATP-driven introduction of negative supercoils into DNA, has proven to be a successful strategy for antibacterial drugs. However, high level resistance to fluoroquinolones (FQ), used as second-line TB treatments (moxifloxacin, levofloxacin, and gatifloxacin) is observed throughout targeted bacterial species. Significant efforts, including target-based screens have resulted in new antibacterial drugs that are effective against fluoroquinolone-resistant pathogens. These include Spiropyrimidinetriones which act against type II topoisomerase (Basarab et al., 2022; Govender et al., 2022) as well as compounds inhibiting DNA replication with different modes of action like the aminobenzimidazole, VXc-486, alias SPR720 (Locher et al., 2015); Thiazolopyridone ureas (Kale et al., 2014) and more recently, Gepotidacin analogs, members of the "novel bacterial topoisomerase inhibitors" (NBTIs) (Blanco et al., 2015; Gibson et al., 2018). As deeply reviewed by Reiche et al. (2017), the DNA replication machinery is encoded by essential mycobacterial genes. Thus, other replication inhibitors are being explored including compounds targeting topoisomerase I (Sandhaus et al., 2016; Ekins et al., 2017), DNA polymerase complex (by Nargenicin, targeting DnaE1) (Chengalroyen et al., 2022) or Griselymicin (DnaN) as described above. Whether or not these molecules targeting machinery other than the fluoroquinolone target – i.e. type II topoisomerase – will deliver efficacy similar to that of the fluoroquinolone class – remains to be seen. As discussed in Basarab et al., 2022, fluoroquinolones and other DNA-targeting agents that have a characteristic mode of action, by which they elicit an SOS response, demonstrate enhanced bactericidal killing versus those

that do not (and tend towards bacteriostatic behavior). It is to be determined whether specific modes of target engagement and/or specific DMPK properties, can lead to the specific efficacy profile of successful TB drugs such as moxifloxacin.

In addition to these efforts to rescue the targets of RIFs and FQs, multiple programs have sought to identify replacements for INH that inhibit its target. In the end, what may have appeared as the most simple of rescue efforts, was not: INH is a prodrug and most INH-resistant clinical isolates arise from mutations in KatG, an enzyme responsible for INH activation within M.tb. So, compounds that inhibit Enoyl Acyl Carrier Protein Reductase (InhA), an enzyme involved in fatty acid synthesis and mycolic acid biosynthesis, without first requiring KatG activation, should be active against most INH resistant strains. But identifying novel compounds that target InhA – so-called Direct InhA Inhibitors (DIIs)- has faced many hurdles like uncorrelated enzymatic inhibition and Mtb activity, or poor ADMET and PK properties (Rožman et al., 2017) and have not delivered efficacy similar to INH in mice. These DIIs might not recapitulate entirely INH's mode of action against M.tb. Recent knowledge and inhibitor classes have been extensively reviewed by Prasad et al. (Prasad et al., 2021).

Last but not least, the mysterious antibiotic PZA, a prodrug used as a first line anti-TB drug since the 1980s, will be the most challenging to address, since its mechanism of action is not completely understood. Adding PZA to RIF INH and ETH reduced the treatment time to a TB cure from 9 to 6 months and exploring PZA's mode of action (Ragunathan et al., 2021) may eventually facilitate efforts to discover new pathways involved in sterilization and treatment shortening.

Conclusion

With the increasing threat of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB, multiple innovative efforts have been conducted and are ongoing to identify novel chemical matter against M.tb using a variety of screening approaches that are agnostic to drug target. These aim to discover novel M.tb-active classes and, in addition, have a chance to reveal or pharmacologically validate novel targets (Li et al., 2017; Ballinger et al., 2019; Shetye et al., 2020; Nuermberger et al., 2022) that can be pursued. In addition, recent advances in the understanding of TB target vulnerability and in pharmacological and genetic validation of specific novel targets have highlighted additional targets for further work (Bosch et al., 2021; Koh et al., 2022; Smith et al., 2022). Target-based or pathway-specific screens in whole cell systems show particular promise. Multiple compounds representing the fruits of these approaches have entered the clinical TB pipeline. These are extensively reviewed elsewhere (Dartois and Rubin, 2022; Butler et al., 2022)

In this article, we highlight a different swath of activities in the TB drug discovery field; those that seek to leverage known drugs and drug classes, previously discovered but not optimized leads, or validated drug targets that are compromised by resistance. These activities have met with some success and demonstrate the power of collaborative approaches; of bringing new technologies and innovations to bear on old drugs and compounds, and of careful re-assessment of existing data associated with old drugs and abandoned concepts. It is noteworthy that about a third of the compounds reported to the Stop TB Partnership's Working Group on New TB Drugs as currently undergoing preclinical or clinical development for TB, have been developed through this type of program.

These approaches may in some cases avoid the considerable costs, time and risks associated with the discovery and clinical development of a totally new chemical entity and/or compounds against a completely novel target. However, potential pitfalls abound – the advantage of known properties and profile of an existing class is balanced by its known liabilities. Compounds from the Golden Age might represent forgotten possibilities but come with the challenges inherent to natural products, including possibly limited optimization opportunities due to the smaller number of skilled natural products chemists involved in TB drug discovery today. Finally, care should be taken in assessing the chance of success when attempting to recapitulate a specific efficacy profile of a known TB drug by seeking a novel modulator of the same target. There is still much to learn regarding the specific mode of target engagement, downstream events and DMPK properties that lead to the precise clinical efficacy profiles observed for TB drugs.

Finally, a new path to bypass resistance might not arise from small drug molecules but from RNA network regulation such as ncRNA modulation (Gerrick et al., 2018) or proteinaceous inhibitors that may disrupt important protein-protein interaction (Sala et al., 2014), or from antimicrobial peptides (Oliveira et al., 2021).

By conducting diverse drug discovery and development efforts that encompass both identification of novel series and targets and the optimization or rescuing of known classes and targets, the field may produce sufficient substrate for novel treatment-shortening regimens that are effective against drug-resistant TB. Directing our energies to all approaches in a balanced, innovative and collaborative manner will undeniably represent new hopes for fighting TB resistance.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Author contributions

CR and AU have designed and written the article. EF provided scientific advice and knowledge and carefully read and edited of the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

We acknowledge support from Eric Bacqué for scientific advice and for careful reading of the manuscript.

Conflict of interest

Authors CR, EF, and AU are employed by Evotec ID Lyon, France (CR and EF) and Evotec, US (AU).

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Abrahams, G. L., Kumar, A., Savvi, S., Hung, A. W., Wen, S., Abell, C., et al. (2012). Pathway-selective sensitization of mycobacterium tuberculosis for target-based whole-cell screening. *Chem. Biol.* 19 (7), 844–854. doi: 10.1016/j.chembiol.2012.05.020

Abramovitch, R. B. (2018). Mycobacterium tuberculosis reporter strains as tools for drug discovery and development. *IUBMB Life.* 70 (9), 818–825. doi: 10.1002/iub.1862

Alfarisi, O., Alghamdi, W. A., Al-Shaer, M. H., Dooley, K. E., and Peloquin, C. A. (2017). Rifampin vs. rifapentine: what is the preferred rifamycin for tuberculosis? *Expert Rev. Clin. Pharmacol.* 10 (10), 1027–1036. doi: 10.1080/17512433.2017.1366311

Asfaw, H., Laqua, K., Walkowska, A. M., Cunningham, F., Martinez-Martinez, M. S., Cuevas-Zurita, J. C., et al. (2017). Design, synthesis and structure-activity relationship study of wollamide b; a new potential anti TB agent. *PloS One* 12 (4), e0176088. doi: 10.1371/journal.pone.0176088

Asfaw, H., Wetzlar, T., Martinez-Martinez, M. S., and Imming, P. (2018). An efficient synthetic route for preparation of antimycobacterial wollamides and evaluation of their *in vitro* and *in vivo* efficacy. *Bioorg Med. Chem. Lett.* 28 (17), 2899–2905. doi: 10.1016/j.bmcl.2018.07.021

Ballinger, E., Mosior, J., Hartman, T., Burns-Huang, K., Gold, B., Morris, R., et al. (2019). Opposing reactions in coenzyme a metabolism sensitize mycobacterium tuberculosis to enzyme inhibition. *Science* 363 (6426), eaau8959. doi: 10.1126/science.aau8959

Bandodkar, B., Shandil, R. K., Bhat, J., and Balganesh, T. S. (2020). Two decades of TB drug discovery efforts–what have we learned? *Appl. Sci.* 10, 5704. doi: 10.3390/app10165704

Basarab, G. S., Ghorpade, S., Gibhard, L., Mueller, R., Njoroge, M., Peton, N., et al. (2022). Spiropyrimidinetriones: a class of DNA gyrase inhibitors with activity against mycobacterium tuberculosis and without cross-resistance to fluoroquinolones. *Antimicrob. Agents Chemother.* 66 (4), e0219221. doi: 10.1128/aac.02192-21

Bergy, M. E., Eble, T. E., and Herr, R. R. (1961). Actinospectacin, a new antibiotic. IV. isolation, purification, and chemical properties. *Antibiot Chemother (Northfield).* 11, 661–664.

Berrada, Z. L., Lin, S. Y., Rodwell, T. C., Nguyen, D., Schecter, G. F., Pham, L., et al. (2016). Rifabutin and rifampin resistance levels and associated rpoB mutations in clinical isolates of mycobacterium tuberculosis complex. *Diagn. Microbiol. Infect. Dis.* 85 (2), 177–181. doi: 10.1016/j.diagmicrobio.2016.01.019

Blanco, D., Perez-Herran, E., Cacho, M., Ballell, L., Castro, J., González del Río, R., et al. (2015). Mycobacterium tuberculosis gyrase inhibitors as a new class of antitubercular drugs. *Antimicrob. Agents Chemother.* 59, 1868–1875. doi: 10.1128/aac.03913-14

Blondiaux, N., Moune, M., Desroses, M., Frita, R., Flipo, M., Mathys, V., et al. (2017). Reversion of antibiotic resistance in mycobacterium tuberculosis by spiroisoxazoline SMARt-420. *Science* 355 (6330), 1206–1211. doi: 10.1126/science.aag1006

Bonnett, S. A., Ollinger, J., Chandrasekera, S., Florio, S., O'Malley, T., Files, M., et al. (2016). A target-based whole cell screen approach to identify potential inhibitors of mycobacterium tuberculosis signal peptidase. *ACS Infect. Dis.* 2 (12), 893–902. doi: 10.1021/acsinfecdis.6b00075

Bosch, B., DeJesus, M. A., Poulton, N. C., Zhang, W., Engelhart, C. A., Zaveri, A., et al. (2021). Genome-wide gene expression tuning reveals diverse vulnerabilities of m. tuberculosis. *Cell* 184 (17), 4579–4592. doi: 10.1016/j.cell.2021.06.033

Boyaci, H., Chen, J., Jansen, R., Darst, S. A., and Campbell, E. A. (2019). Structures of an RNA polymerase promoter melting intermediate elucidate DNA unwinding. *Nature* 565 (7739), 382–385. doi: 10.1038/s41586-018-0840-5

Boyaci, H., Chen, J., Lilic, M., Palka, M., Mooney, R. A., Landick, R., et al. (2018). Fidaxomicin jams mycobacterium tuberculosis RNA polymerase motions needed for initiation *via* RbpA contacts. *Elife* 7, e34823. doi: 10.7554/eLife.34823

Bruhn, D. F., Scherman, M. S., Liu, J., Scherbakov, D., Meibohm, B., Böttger, E. C., et al. (2015). *In vitro* and *In vivo* evaluation of synergism between anti-tubercular spectinamides and non-classical tuberculosis antibiotics. *Sci. Rep.* 5, 5:13985. doi: 10.1038/srep13985

Burke, C., Jankute, M., Moynihan, P., Gonzalez Del Rio, R, Li, X, Esquivias, J, et al. (2020). Development of a novel secondary phenotypic screen to identify hits within the mycobacterial protein synthesis pipeline. *FASEB Bioadv.* 2 (10), 600–612. doi: 10.1096/fba.2020-00022

Butler, M. S., Gigante, V., Sati, H., Paulin, S., Al-Sulaiman, L., Rex, J. H., et al. (2022). Analysis of the clinical pipeline of treatments for drug-resistant bacterial infections: Despite progress, more action is needed. *Antimicrob. Agents Chemother.* 66 (3), e0199121. doi: 10.1128/AAC.01991-21

Bycroft, B. W. (1972). The crystal structure of viomycin, a tuberculostatic antibiotic. *Chem. Commun.* 11), 660. doi: 10.1039/c39720000660

Carpino, L. A., and Han, G. Y. (1970). The 9-fluorenylmethoxycarbonyl function, a new base-sensitive amino-protecting group. *J. Am. Chem. Soc* 92, 5748–5749. doi: 10.1021/ja00722a043

Chakraborty, S., and Rhee, K. Y. (2015). Tuberculosis drug development: History and evolution of the mechanism-based paradigm. *Cold Spring Harb. Perspect. Med.* 5 (8), a021147. doi: 10.1101/cshperspect.a021147

Chengalroyen, M. D., Mason, M. K., Borsellini, A., Tassoni, R., Abrahams, G. L., Lynch, S., et al. (2022). DNA-Dependent binding of nargenicin to DnaE1 inhibits replication in mycobacterium tuberculosis. *ACS Infect. Dis.* 8 (3), 612–625. doi: 10.1021/acsinfecdis.1c00643

Coll, F., Phelan, J., Hill-Cawthorne, G. A., Nair, M. B., Mallard, K., Ali, S., et al. (2018). Genome-wide analysis of multi- and extensively drug-resistant mycobacterium tuberculosis. *Nat. Genet.* 50, 307–316. doi: 10.1038/s41588-017-0029-0

Conradie, F., Bagdasaryan, T. R., Borisov, S., Howell, P., Mikiashvili, L., Ngubane, N., et al. (2022). Bedaquiline-Pretomanid-Linezolid regimens for drug-resistant tuberculosis. *N Engl. J. Med.* 387 (9), 810–823. doi: 10.1056/NEJMoa2119430

Conradie, F., Diacon, A. H., Ngubane, N., Howell, P., Everitt, D., Crook, A. M., et al. (2020). Treatment of highly drug-resistant pulmonary tuberculosis. *N Engl. J. Med.* 382 (10), 893–902. doi: 10.1056/NEJMoa1901814

Dartois, V. A., and Rubin, E. J. (2022). Anti-tuberculosis treatment strategies and drug development: challenges and priorities. *Nat. Rev. Microbiol.* 27, 1–17. doi: 10.1038/s41579-022-00731-y

Dey, S., Lane, J. M., Lee, R. E., Rubin, E. J., and Sacchettini, J. C. (2010). Structural characterization of the mycobacterium tuberculosis biotin biosynthesis

enzymes 7,8-diaminopelargonic acid synthase and dethiobiotin synthetase. *Biochemistry* 49 (31), 6746–6760. doi: 10.1021/bi902097j

Diacon, A. H., van der Merwe, L., Barnard, M., von Groote-Bidlingmaier, F., Lange, C., García-Basteiro, A. L., et al. (2016). β-lactams against tuberculosis–new trick for an old dog? *N Engl. J. Med.* 375 (4), 393–394. doi: 10.1056/NEJMc1513236

Djaout, K. (2022). *Boosting pretomanid potency: towards reversion of resistance, mycodays*. C13, 17. Available at: https://www.sfm-microbiologie.org/wp-content/uploads/2022/06/MYCODAYSbook2022.pdf

Dooley, K. E., Miyahara, S., von Groote-Bidlingmaier, F., Sun, X., Hafner, R., Rosenkranz, S. L., et al. (2020). Early bactericidal activity of different isoniazid doses for drug-resistant tuberculosis (INHindsight): A randomized, open-label clinical trial. *Am. J. Respir. Crit. Care Med.* 201 (11), 1416–1424. doi: 10.1164/rccm.201910-1960OC

Ekins, S., Godbole, A. A., Kéri, G., Orfi, L., Pato, J., Bhat, R. S., et al. (2017). Machine learning and docking models for mycobacterium tuberculosis topoisomerase I. *Tuberculosis (Edinb).* 103, 52–60. doi: 10.1016/j.tube.2017.01.005

Evans, J. C., and Mizrahi, V. (2018). Priming the tuberculosis drug pipeline: new antimycobacterial targets and agents. *Curr. Opin. Microbiol.* 45, 39–46. doi: 10.1016/j.mib.2018.02.006

Falzari, K., Zhu, Z., Pan, D., Liu, H., Hongmanee, P., and Franzblau, S. G. (2005). *In vitro* and *in vivo* activities of macrolide derivatives against mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 49 (4), 1447–1454. doi: 10.1128/AAC.49.4.1447-1454.2005

Gerrick, E. R., Barbier, T., Chase, M. R., Xu, R., François, J., Lin, V. H., et al. (2018). Small RNA profiling in mycobacterium tuberculosis identifies MrsI as necessary for an anticipatory iron sparing response. *Proc. Natl. Acad. Sci. U. S. A.* 115 (25), 6464–6469. doi: 10.1073/pnas.1718003115

Gibson, E. G., Blower, T. R., Cacho, M., Bax, B., Berger, J. M., and Osheroff, N. (2018). Mechanism of action of mycobacterium tuberculosis gyrase inhibitors: A novel class of gyrase poisons. *ACS Infect. Dis.* 4 (8), 1211–1222. doi: 10.1021/acsinfecdis.8b00035

Gold, B., Zhang, J., Quezada, L. L., Roberts, J., Ling, Y., Wood, M., et al. (2022). Identification of β-lactams active against mycobacterium tuberculosis by a consortium of pharmaceutical companies and academic institutions. *ACS Infect. Dis.* 8 (3), 557–573. doi: 10.1021/acsinfecdis.1c00570

Gonzalez-Juarrero, M., Lukka, P. B., Wagh, S., Walz, A., Arab, J., Pearce, C., et al. (2021). Preclinical evaluation of inhalational spectinamide-1599 therapy against tuberculosis. *ACS Infect. Dis.* 7 (10), 2850–2863. doi: 10.1021/acsinfecdis.1c00213

Govender, P., Müller, R., Singh, K., Reddy, V., Eyermann, C. J., Fienberg, S., et al. (2022). Spiropyrimidinetrione DNA gyrase inhibitors with potent and selective antituberculosis activity. *J. Med. Chem.* 65 (9), 6903–6925. doi: 10.1021/acs.jmedchem.2c00266

Haebich, D., and von Nussbaum, F. (2009). Lost in transcription–inhibition of RNA polymerase. *Angew Chem. Int. Ed Engl.* 48 (19), 3397–3400. doi: 10.1002/anie.200900338

Harbottle, J., Mosaei, H., Allenby, N., and Zenkin, N. (2021). Kanglemycin a can overcome rifamycin resistance caused by ADP-ribosylation by arr protein. *Antimicrob. Agents Chemother.* 65 (12), e0086421. doi: 10.1128/AAC.00864-21

Hénazet, F., Cosar, C., Dubost, M., Guy-Loè, H., Julou, L., Mancy, D., et al. (1966). *Advances in research, production and clinical use: Proceedings of the congress on antibiotics held in Prague*. Eds. M. Herold and Z. Gabriel (London: Butterworths), 262–264.

Hugonnet, J. E., Tremblay, L. W., Boshoff, H. I., Barry, C. E.3rd, and Blanchard, J. S. (2009). Meropenem-clavulanate is effective against extensively drug-resistant mycobacterium tuberculosis. *Science* 323 (5918), 1215–1218. doi: 10.1126/science.1167498

Iavarone, L., Bottacini, M., Pugnaghi, F., Morandini, C., and Grossi, P. (1997). Sanfetrinem and sanfetrinem-cilexetil: disposition in rat and dog. *Xenobiotica* 27 (7), 693–709. doi: 10.1080/004982597240280

Jiang, H., Li, X., Xing, Z., Niu, Q., and Xu, J. (2022). Intracellular activity of poly (DL-lactide-co-glycolide) nanoparticles encapsulated with prothionamide, pyrazinamide, levofloxacin, linezolid or ethambutol on multidrug-resistant mycobacterium tuberculosis. *Curr. Drug Delivery*. doi: 10.2174/1567201819666220511120215

Johnson, E. O., LaVerriere, E., Office, E., Stanley, M., Meyer, E., Kawate, T., et al. (2019). Large-Scale chemical-genetics yields new m. tuberculosis inhibitor classes. *Nature* 571 (7763), 72–78. doi: 10.1038/s41586-019-1315-z

Kale, R. R., Kale, M. G., Waterson, D., Raichurkar, A., Hameed, S. P., Manjunatha, M. R., et al. (2014). Thiazolopyridone ureas as DNA gyrase b inhibitors: optimization of antitubercular activity and efficacy. *Bioorg Med. Chem. Lett.* 24 (3), 870–879. doi: 10.1016/j.bmcl.2013.12.080

Kavanagh, F., Hervey, A., and Robbins, W. J. (1951). Antibiotic substances from basidiomycetes: VIII. pleurotus multilus (Fr.) sacc. and pleurotus passeckerianus pilat. *Proc. Natl. Acad. Sci. U. S. A.* 37 (9), 570–574. doi: 10.1073/pnas.37.9.570

Kavčič, B., Tkačik, G., and Bollenbach, T. (2020). Mechanisms of drug interactions between translation-inhibiting antibiotics. *Nat. Commun.* 11 (1), 4013. doi: 10.1038/s41467-020-17734-z

Khalil, Z. G., Hill, T. A., De Leon Rodriguez, L. M., Lohman, R. J., Hoang, H. N., Reiling, N., et al. (2019). Structure-activity relationships of wollamide cyclic hexapeptides with activity against drug-resistant and intracellular mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 63 (3), e01773–e01718. doi: 10.1128/AAC.01773-18

Khalil, Z. G., Salim, A. A., Lacey, E., Blumenthal, A., and Capon, R. J. (2014). Wollamides: antimycobacterial cyclic hexapeptides from an Australian soil streptomyces. *Org Lett.* 16 (19), 5120–5123. doi: 10.1021/ol502472c

Kiefer, A., Bader, C. D., Held, J., Esser, A., Rybniker, J., Empting, M., et al. (2019). Synthesis of new cyclomarin derivatives and their biological evaluation towards mycobacterium tuberculosis and plasmodium falciparum. *Chemistry* 25 (37), 8894–8902. doi: 10.1002/chem.201901640

Kimmerlin, T., and Sebach, D. (2005). 100 years of peptide synthesis : ligation methods for peptide and protein synthesis with applications to beta-peptide assemblies. *J. Pept. Res.* 65, 229–260. doi: 10.1111/j.1399-3011.2005.00214.x

Kling, A., Lukat, P., Almeida, D. V., Bauer, A., Fontaine, E., Sordello, S., et al. (2015). Antibiotics. targeting DnaN for tuberculosis therapy using novel griselimycins. *Science* 348 (6239), 1106–1112. doi: 10.1126/science.aaa4690

Koh, E. I., Oluoch, P. O., Ruecker, N., Proulx, M. K., Soni, V., Murphy, K. C., et al. (2022). Chemical-genetic interaction mapping links carbon metabolism and cell wall structure to tuberculosis drug efficacy. *Proc. Natl. Acad. Sci. U. S. A.* 119 (15), e2201632119. doi: 10.1073/pnas.2201632119

Kurabachew, M., Lu, S. H., Krastel, P., Schmitt, E. K., Suresh, B. L., Goh, A., et al. (2008). Lipiarmycin targets RNA polymerase and has good activity against multidrug-resistant strains of mycobacterium tuberculosis. *J. Antimicrobial Chemother.* 62, 713–719. doi: 10.1093/jac/dkn269

Lair, C., Benedetti, Y., Blanc, I., Boulenc, X., Briot, C., Fraisse, L., et al. (2015). Anti-tuberculosis *in vitro* and *in vivo* activity of a new sequanamycin-like macrolide. *Interscience Conf. Antimicrobial Agents Chemother.*, F–23.

Lee, R. E., Hurdle, J. G., Liu, J., Bruhn, D. F., Matt, T., Scherman, M. S., et al. (2014). Spectinamides: a new class of semisynthetic antituberculosis agents that overcome native drug efflux. *Nat. Med.* 20 (2), 152–158. doi: 10.1038/nm.3458

Lemaire, S., Shukla, V. K., Rogers, C., Ibrahim, I. H., Lapierre, C., Parent, P., et al. (1993). Isolation and characterization of histogranin, a natural peptide with NMDA receptor antagonist activity. *Eur J Pharmacol.* 245(3), 247–256. doi: 10.1016/0922-4106(93)90104-h

Lemieux, M. R., Siricilla, S., Mitachi, K., Eslamimehr, S., Wang, Y., Yang, D., et al. (2018). An antimycobacterial pleuromutilin analogue effective against dormant bacilli. *Bioorg Med. Chem.* 26 (17), 4787–4796. doi: 10.1016/j.bmc.2018.07.034

Lewis, K. (2013). Platforms for antibiotic discovery. *Nat. Rev. Drug Discovery* 12 (5), 371–387. doi: 10.1038/nrd3975

Li, X., Hernandez, V., Rock, F. L., Choi, W., Mak, Y. S. L., Mohan, M., et al. (2017). Discovery of a potent and specific m. tuberculosis leucyl-tRNA synthetase inhibitor: (S)-3-(Aminomethyl)-4-chloro-7-(2-hydroxyethoxy)benzo[c][1,2]oxaborol-1(3H)-ol (GSK656). *J. Med. Chem.* 60 (19), 8011–8026. doi: 10.1021/acs.jmedchem.7b00631

Lilic, M., Chen, J., Boyaci, H., Braffman, N., Hubin, E. A., Herrmann, J., et al. (2020). The antibiotic sorangicin a inhibits promoter DNA unwinding in a mycobacterium tuberculosis rifampicin-resistant RNA polymerase. *Proc. Natl. Acad. Sci. U. S. A.* 117 (48), 30423–30432. doi: 10.1073/pnas.2013706117

Lin, W., Mandal, S., Degen, D., Liu, Y., Ebright, Y. W., Li, S., et al. (2017). Structural basis of mycobacterium tuberculosis transcription and transcription inhibition. *Mol. Cell.* 66 (2), 169–179.e8. doi: 10.1016/j.molcel.2017.03.001

Locher, C. P., Jones, S. M., Hanzelka, B. L., Perola, E, Shoen, CM, Cynamon, MH, et al. (2015). A novel inhibitor of gyrase b is a potent drug candidate for treatment of tuberculosis and nontuberculosis mycobacterial infections. *Antimicrob. Agents Chemother.* 59 (3), 1455–1465. doi: 10.1128/AAC.04347-14

Lohrasbi, V., Talebi, M., Bialvaei, A. Z., Fattorini, L., Drancourt, M., Heidary, M., et al. (2018). Trends in the discovery of new drugs for mycobacterium tuberculosis therapy with a glance at resistance. *Tuberculosis (Edinb).* 109, 17–27. doi: 10.1016/j.tube.2017.12.002

Maffioli, S. I., Zhang, Y., Degen, D., Carzaniga, T., Del Gatto, G., Serina, S., et al. (2017). Antibacterial nucleoside-analog inhibitor of bacterial RNA polymerase. *Cell* 169 (7), 1240–1248.e23. doi: 10.1016/j.cell.2017.05.042

Ma, S., Morrison, R., Hobbs, S. J., Soni, V., Farrow-Johnson, J., Frando, A., et al. (2021). Transcriptional regulator-induced phenotype screen reveals drug potentiators in mycobacterium tuberculosis. *Nat. Microbiol.* 6 (1), 44–50. doi: 10.1038/s41564-020-00810-x

Mann, S., and Ploux, O. (2006). 7,8-diaminoperlargonic acid aminotransferase from mycobacterium tuberculosis, a potential therapeutic target. characterization and inhibition studies. *FEBS J.* 273 (20), 4778–4789. doi: 10.1111/j.1742-4658.2006.05479.x

Mason, D. J., Dietz, A., and Smith, R. M. (1961). Actinospectacin, a new antibiotic. i. discovery and biological properties. *Antibiot. Chemother. (Northfield).* 11, 118–122.

Mazlan, M. K. N., Mohd Tazizi, M. H. D., Ahmad, R., Noh, M. A. A., Bakhtiar, A., Wahab, H. A., et al. (2021). Antituberculosis targeted drug delivery as a potential future treatment approach. *Antibiotics (Basel)* 10 (8), 908. doi: 10.3390/antibiotics10080908

Merget, B., Zilian, D., Müller, T., and Sotriffer, C. A. (2013). MycPermCheck: the mycobacterium tuberculosis permeability prediction tool for small molecules. *Bioinformatics* 29 (1), 62–68. doi: 10.1093/bioinformatics/bts641

Merrifield, R. B. (1963). Solid phase peptide synthesis. i. the synthesis of a tetrapeptide. *J. Am. Chem. Soc* 85, 2149–2154.

Miao, S., Anstee, M. R., LaMarco, K., Matthew, J., Huang, L. H. T., and Brasseur, M. M. (1997). Inhibition of bacterial RNA polymerases. peptide metabolites from the cultures of streptomyces sp. *J. Nat. Prod* 60 (8), 858–861. doi: 10.1021/np960709c

Miotto, P., Tessema, B., Tagliani, E., Chindelevitch, L., Starks, A. M., Emerson, C., et al. (2017). A standardised method for interpreting the association between mutations and phenotypic drug resistance in mycobacterium tuberculosis. *Eur. Respir. J.* 50 (6), 1701354. doi: 10.1183/13993003.01354-2017

Morreale, F. E., Kleine, S., Leodolter, J., Junker, S., Hoi, D. M., Ovchinnikov, S., et al. (2022). BacPROTACs mediate targeted protein degradation in bacteria. *Cell* 185 (13), 2338–2353.e18. doi: 10.1016/j.cell.2022.05.009

Mosaei, H., Molodtsov, V., Kepplinger, B., Harbottle, J., Moon, C. W., Jeeves, R. E., et al. (2018). An ansamycin natural product that is active against rifampicin-resistant mycobacterium tuberculosis. *Mol. Cell.* 72 (2), 263–274. doi: 10.1016/j.molcel.2018.08.028

Naran, K., Moosa, A., Barry, C. E. 3rd, Boshoff, H. I., Mizrahi, V., and Warner, D. F. (2016). Bioluminescent reporters for rapid mechanism of action assessment in tuberculosis drug discovery. *Antimicrob. Agents Chemother.* 60 (11), 6748–6757. doi: 10.1128/AAC.01178-16

Noufflard-Guy-Loé, H., and Berteaux, S. (1965). Action antituberculeuse expérimentale d'une nouvelle substance antibiotique: Le 11.072 R.P. *Rev. Tuberc. Pneumol* (Paris) 29, 301–326.

Nuermberger, E. L., Martínez-Martínez, M. S., Sanz, O., Urones, B., Esquivias, J., Soni, H., et al. (2022). GSK2556286 is a novel antitubercular drug candidate effective *In vivo* with the potential to shorten tuberculosis treatment. *Antimicrob. Agents Chemother.* 66 (6), e0013222. doi: 10.1128/aac.00132-22

Okami, Y., Kitahara, T., Hamada, M., Naganawa, H., and Kondo, S. (1974). Studies on a new amino acid antibiotic, amiclenomycin. *J. Antibiot. (Tokyo)* 27 (9), 656–664. doi: 10.7164/antibiotics.27.656

Oliveira, G. S., Costa, R. P., Gomes, P., Gomes, M. S., Silva, T., and Teixeira, C. (2021). Antimicrobial peptides as potential anti-tubercular leads: A concise review. *Pharm. (Basel)* 14 (4), 323. doi: 10.3390/ph14040323

Oliveira, C. S., Torres, M. T., Pedron, C. N., Andrade, V. B., Silva, P. I.Jr, Silva, F. D., et al. (2021). Synthetic peptide derived from scorpion venom displays minimal toxicity and anti-infective activity in an animal model. *ACS Infect. Dis.* 7 (9), 2736–2745. doi: 10.1021/acsinfecdis.1c00261

Peek, J., Xu, J., Wang, H., Suryavanshi, S., Zimmerman, M., Russo, R., et al. (2020). A semisynthetic kanglemycin shows *In vivo* efficacy against high-burden rifampicin resistant pathogens. *ACS Infect. Dis.* 6 (9), 2431–2440. doi: 10.1021/acsinfecdis.0c00223

Phanumartwiwath, A., Kesornpun, C., and Sureram, S.. (2021). Antitubercular and antibacterial activities of isoxazolines derived from natural products: Isoxazolines as inhibitors of mycobacterium tuberculosis InhA. *J. Chem. Res.* 45 (11–12), 1003–1015. doi: 10.1177/17475198211047801

Prasad, M. S., Bhole, R. P., Khedekar, P. B., and Chikhale, R. V. (2021). Mycobacterium enoyl acyl carrier protein reductase (InhA): A key target for antitubercular drug discovery. *Bioorg Chem.* 115, 105242. doi: 10.1016/j.bioorg.2021.105242

Prevet, H., Moune, M., Tanina, A., Kemmer, C., Herledan, A., Frita, R., et al. (2019). A fragment-based approach towards the discovery of n-substituted tropinones as inhibitors of mycobacterium tuberculosis transcriptional regulator EthR2. *Eur. J. Med. Chem.* 167, 426–438. doi: 10.1016/j.ejmech.2019.02.023

Ragunathan, P., Cole, M., Latka, C., Aragaw, W. W., Hegde, P., Shin, J., et al. (2021). Mycobacterium tuberculosis PanD structure-function analysis and identification of a potent pyrazinoic acid-derived enzyme inhibitor. *ACS Chem. Biol.* 16 (6), 1030–1039. doi: 10.1021/acschembio.1c00131

Ramón- García, S., Bates, R., González del Río, Rubén, Gamallo, P., Mendoza, A., Ferrer, S., et al. (2019). "Thompson And David barros. sanfetrinem, repurposing an oral beta-lactam with intracellular activity for the treatment of tuberculosis," in *Abstract book of the 50th World Conference on Lung Health of the International Union Against Tuberculosis and Lung Disease (The Union)*, 23 (Supplement 1), S591. Available at: http://https/araid.es/en/content/sanfetrinem-repurposing-oral-beta-lactam-intracellular-activity-treatment-tuberculosis

Reiche, M. A., Warner, D. F., and Mizrahi, V. (2017). Targeting DNA replication and repair for the development of novel therapeutics against tuberculosis. *Front. Mol. Biosci.* 4. doi: 10.3389/fmolb.2017.00075

Renner, M. K., Shen, Y., Cheng, X., Jensen, P. R., Frankmoelle, W., Kauffman, C. A., et al. (1999). Cyclomarins a–c, new antiinflammatory cyclic peptides produced by a marine bacterium (Streptomyces sp.). *J. Am. Chem. Soc.* 121, 11273–11276. doi: 10.1021/ja992482o

Rožman, K., Sosič, I., Fernandez, R., RJ, Y., Mendoza, A., Gobec, S., et al. (2017). A new 'golden age' for the antitubercular target InhA. *Drug Discovery Today* 22 (3), 492–502. doi: 10.1016/j.drudis.2016.09.009

Sala, A., Bordes, P., and Genevaux, P. (2014). Multiple toxin-antitoxin systems in mycobacterium tuberculosis. *Toxins (Basel)* 6 (3), 1002–1020. doi: 10.3390/toxins6031002

Salomon, J. J., Galeron, P., Schulte, N., Morow, P. R., Severynse-Stevens, D., Huwer, H., et al. (2013). Biopharmaceutical *in vitro* characterization of CPZEN-45, a drug candidate for inhalation therapy of tuberculosis. *Ther. Deliv.* 4 (8), 915–923. doi: 10.4155/tde.13.62

Sandhaus, S., Annamalai, T., Welmaker, G., Houghten, R. A., Paz, C., Garcia, P. K., et al. (2016). Small-molecule inhibitors targeting topoisomerase I as novel antituberculosis agents. *Antimicrob. Agents Chemother.* 60 (7), 4028–4036. doi: 10.1128/AAC.00288-16

Sarathy, J. P., Zuccotto, F., Hsinpin, H., Sandberg, L., Via, L. E., Marriner, G. A., et al. (2016). Prediction of drug penetration in tuberculosis lesions. *ACS Infect. Dis.* 2 (8), 552–563. doi: 10.1021/acsinfecdis.6b00051

Schmitt, E. K., Riwanto, M., Sambandamurthy, V., Roggo, S., Miault, C., Zwingelstein, C., et al. (2011). The natural product cyclomarin kills mycobacterium tuberculosis by targeting the ClpC1 subunit of the caseinolytic protease. *Angew Chem. Int. Ed Engl.* 50 (26), 5889–5891. doi: 10.1002/anie.201101740

Shetye, G. S., Franzblau, S. G., and Cho, S. (2020). New tuberculosis drug targets, their inhibitors, and potential therapeutic impact. *Transl. Res.* 220, 68–97. doi: 10.1016/j.trsl.2020.03.007

Singh, V., and Chibale, K. (2021). Strategies to combat multi-drug resistance in tuberculosis. *Acc Chem. Res.* 54 (10), 2361–2376. doi: 10.1021/acs.accounts.0c00878

Singh, K. V., Coque, T. M., and Murray, B. E. (1996). *In vitro* activity of the trinem sanfetrinem (GV104326) against gram-positive organisms. *Antimicrob. Agents Chemother.* 40 (9), 2142–2146. doi: 10.1128/AAC.40.9.2142

Singh, J., Garg, T., Rath, G., and Goyal, A. K. (2016). Advances in nanotechnology-based carrier systems for targeted delivery of bioactive drug molecules with special emphasis on immunotherapy in drug resistant tuberculosis - a critical review. *Drug Deliv.* 23 (5), 1676–1698. doi: 10.3109/10717544.2015.1074765

Smith, C. M., Baker, R. E., Proulx, M. K., Mishra, B. B., Long, J. E., Park, S. W., et al. (2022). Host-pathogen genetic interactions underlie tuberculosis susceptibility in genetically diverse mice. *Elife* 11, e74419. doi: 10.7554/eLife.74419

Smith, T. C.2nd, Pullen, K. M., Olson, M. C., McNellis, M. E., Richardson, I., Hu, S., et al. (2020). Morphological profiling of tubercle bacilli identifies drug pathways of action. *Proc. Natl. Acad. Sci. U. S. A.* 117 (31), 18744–18753. doi: 10.1073/pnas.2002738117

Stefan, M. A., Velazquez, G. M., and Garcia, G. A. (2020). High-throughput screening to discover inhibitors of the CarD·RNA polymerase protein-protein interaction in mycobacterium tuberculosis. *Sci. Rep.* 10 (1), 21309. doi: 10.1038/s41598-020-78269-3

Tangallapally, R. P., Sun, D., Rakesh,, Budha, N., Lee, R. E., Lenaerts, A. J., et al. (2007). Discovery of novel isoxazolines as anti-tuberculosis agents. *Bioorg Med. Chem. Lett.* 17 (23), 6638–6642. doi: 10.1016/j.bmcl.2007.09.048

Toyohara, M. (1987). Aspects of the antituberculous activity of 27753-RP, a new semisynthetic derivative of griselimycine. *Ann. Inst. Pasteur. Microbiol.* 138, 737–744. doi: 10.1016/0769-2609(87)90151-7

Villemagne, B., Machelart, A., Tran, N. C., Flipo, M., Moune, M., Leroux, F., et al. (2020). Fragment-based optimized EthR inhibitors with in vivo ethionamide boosting activity. *ACS Infect. Dis.* 6 (3), 366–378. doi: 10.1021/acsinfecdis.9b00277

Walker, T. M., Miotto, P., Köser, C. U., Fowler, P. W., Knaggs, J., Iqbal, Z., et al. (2022). The 2021 WHO catalogue of mycobacterium tuberculosis complex mutations associated with drug resistance: A genotypic analysis. *Lancet Microbe* 3 (4), e265–e273. doi: 10.1016/S2666-5247(21)00301-3

Wasserman, S., and Furin, J. (2020). Clarity with INHindsight: High-dose isoniazid for drug-resistant tuberculosis with inhA mutations. *Am. J. Respir. Crit. Care Med.* 201 (11), 1331–1333. doi: 10.1164/rccm.202002-0264ED

Watt, B., Edwards, J. R., Rayner, A., Grindey, A. J., and Harris, G. (1992). *In vitro* activity of meropenem and imipenem against mycobacteria: development of a daily antibiotic dosing schedule. *Tuber Lung Dis.* 73 (3), 134–136. doi: 10.1016/0962-8479(92)90145-A

Willand, N., Dirié, B., Carette, X., Bifani, P., Singhal, A., Desroses, M., et al. (2009). Synthetic EthR inhibitors boost antituberculous activity of ethionamide. *Nat. Med.* 15 (5), 537–544. doi: 10.1038/nm.1950

Working Group for New TB Drugs. Available at: https://www.newtbdrugs.org (Accessed July 15, 2022).

World Health Organization (2016) *Target regimen profiles for TB treatment: candidates: rifampicin-susceptible, rifampicin resistant and pan-TB treatment regimens* (World Health Organization). Available at: https://apps.who.int/iris/handle/10665/250044 (Accessed July 15, 2022).

Xu, J., Li, S. Y., Almeida, D. V., Tasneen, R., Barnes-Boyle, K., Converse, P. J., et al. (2019). Contribution of pretomanid to novel regimens containing bedaquiline with either linezolid or moxifloxacin and pyrazinamide in murine models of tuberculosis. *Antimicrob. Agents Chemother.* 63 (5), e00021–e00019. doi: 10.1128/AAC.00021-19

Yan, L., Gao, L., Cui, Z., Hu, Z., Lu, J., Shen, X, et al. (2015). Investigation of cross-resistance between rifampin and rifabutin in multi-drug resistant mycobacterium tuberculosis. *Med. Chem.* 5, 412–414. doi: 10.4172/2161-0444.1000293

Youmans, G. P., and Youmans, A. S. (1951). The effect of viomycin *in vitro* and *in vivo* on mycobacterium tuberculosis. *Am. Rev. Tuberculosis* 63 (1), 25–29. doi: 10.1164/art.1951.63.1.25

Zhao, W., Guo, Z., Zheng, M., Zhang, J., Wang, B., Li, P., et al. (2014). Activity of linezolid-containing regimens against multidrug-resistant tuberculosis in mice. *Int. J. Antimicrob. Agents* 43 (2), 148–153. doi: 10.1016/j.ijantimicag.2013.10.010

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 07 October 2022
DOI 10.3389/fcimb.2022.954074

OPEN ACCESS

EDITED BY
Digby Warner,
University of Cape Town, South Africa

REVIEWED BY
Daniel Alford Powell,
University of Arizona, United States
Anna Lyander,
Karolinska Institutet (KI), Sweden
Katia Peñuelas-Urquides,
Instituto Mexicano del Seguro Social, Mexico

*CORRESPONDENCE
Camus Nimmo
camus.nimmo@crick.ac.uk

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 26 May 2022
ACCEPTED 20 September 2022
PUBLISHED 07 October 2022

CITATION
Nimmo C, Millard J, Faulkner V, Monteserin J, Pugh H and Johnson E (2022) Evolution *Mycobacterium tuberculosis* drug resistance in the genomic era.
Front. Cell. Infect. Microbiol. 12:954074.
doi: 10.3389/fcimb.2022.954074

Evolution of *Mycobacterium tuberculosis* drug resistance in the genomic era

Camus Nimmo[1]*, James Millard[2], Valwynne Faulkner[1], Johana Monteserin[1], Hannah Pugh[1] and Eachan Oliver Johnson[1]

[1]Systems Chemical Biology of Infection and Resistance Laboratory, Francis Crick Institute, London, United Kingdom, [2]Institute of Infection and Global Health, University of Liverpool, Liverpool, United Kingdom

Mycobacterium tuberculosis has acquired drug resistance to all drugs that have been used against it, including those only recently introduced into clinical practice. Compared to other bacteria, it has a well conserved genome due to its role as an obligate human pathogen that has adapted to a niche over five to ten thousand years. These features facilitate reconstruction and dating of *M. tuberculosis* phylogenies, giving key insights into how resistance has been acquired and spread globally. Resistance to each new drug has occurred within five to ten years of clinical use and has occurred even more rapidly with recently introduced drugs. In most cases, resistance-conferring mutations come with a fitness cost, but this can be overcome by compensatory mutations which restore fitness to that of wild-type bacteria. It is likely that *M. tuberculosis* acquires drug resistance while maintaining limited genomic variability due the generation of low frequency within-host variation, combined with ongoing purifying selection causing loss of variants without a clear fitness advantage. However, variants that do confer an advantage, such as drug resistance, can increase in prevalence amongst all bacteria within a host and become the dominant clone. These resistant strains can then be transmitted leading to primary drug resistant infection in a new host. As many countries move towards genomic methods for diagnosis of *M. tuberculosis* infection and drug resistance, it is important to be aware of the implications for the evolution of resistance. Currently, understanding of resistance-conferring mutations is incomplete, and some targeted genetic diagnostics create their own selective pressures. We discuss an example where a rifampicin resistance-conferring mutation which was not routinely covered by standard testing became dominant. Finally, resistance to new drugs such as bedaquiline and delamanid is caused by individually rare mutations occurring across a large mutational genomic target that have been detected over a short time, and do

not provide statistical power for genotype-phenotype correlation – in contrast to longer-established drugs that form the backbone of drug-sensitive antituberculosis therapy. Therefore, we need a different approach to identify resistance-conferring mutations of new drugs before their resistance becomes widespread, abrogating their usefulness.

KEYWORDS

TB, acquired resistance, within-host evolution, clonal expansion, compensatory mutations, bedaquiline, delamanid, pretomanid

Introduction

Mycobacterium tuberculosis is an ancient bacterial pathogen that has acquired drug resistance to all drugs that have been used against it, despite lacking several key mechanisms available to other bacteria to facilitate rapid spread of resistance such as horizontal gene transfer and mobile resistance elements. In the absence of such mechanisms, all antituberculosis drug resistance is conferred by genomic mutations, mostly single nucleotide polymorphisms (SNPs), that are propagated through replication of resistant bacteria and onward transmission. In this review, we discuss how *M. tuberculosis* can develop drug resistance despite maintaining a comparatively well-conserved genome compared to other bacterial pathogens (Eldholm and Balloux, 2016) and give examples of how it has acquired resistance at the between-host and within-host levels. Finally, we assess how diagnostics may be affected by resistance and shape its emergence, and we outline the implications for identifying resistance to new drugs against tuberculosis that are entering clinical use.

Key features of the *Mycobacterium tuberculosis* genome

The whole genome sequence of H37Rv, originally isolated from a patient treated in New York in 1905 and now the most used laboratory strain of *M. tuberculosis*, was published in 1998 (Cole et al., 1998). The most recent annotation reports it as 4.4 megabases in length, making it 33% smaller than *Mycobacterium smegmatis* [also named *Mycolicibacterium smegmatis*, (Gupta et al., 2018), although the usefulness of this is contested (Tortoli et al., 2019)], and one of the smallest apart from *Mycobacterium leprae* (1.6 megabases). It contains 3906 coding genes, of which a large number are responsible for fatty acid metabolism due to the complex mycobacterial cell wall. It is very rich in guanine and cytosine residues, and unlike many other bacteria (e.g. gram-negatives) there is no evidence of recombination and no accessory genome (Eldholm and Balloux, 2016). About 10% of the genome is devoted to a characteristic set of proline (P)- and glutamate (E)-rich proteins called the PE and PPE gene families, which are heterogenous and consist of numerous tandem repeats and are hypothesised to be surface antigens that are responsible for interaction with the host immune system (Fishbein et al., 2015). They are difficult to resolve by short read sequencing and as a result have been historically excluded from many genomic analyses of *M. tuberculosis*.

M. tuberculosis has traditionally been viewed as a genetically homogenous bacterium that has evolved into a specialised human pathogen with a lower mutation rate than most other bacteria at 0.3 to 0.5 SNPs per genome per year (Eldholm and Balloux, 2016). The *M. tuberculosis* complex (MTBC) is likely to have originated from the transition of an environmental mycobacterial ancestor shared with the pathogen *M. canetti* (Soolingen et al., 1997). The transition came with a corresponding reduction in genome size and loss of the ability for genetic recombination or gene transfer, perhaps because it developed into a specialised pathogen that lives only in one ecological niche. The original divergence of the MTBC from environmental mycobacteria is likely to have happened in Africa and then been spread globally by human migration (Gagneux, 2018). Animal-adapted strains of the MTBC, including *M. bovis* (cows) and *M. caprae* (goats) are likely to have been transferred from humans as evidenced by comparative genomic studies that show loss of genes from *M. tuberculosis sensu stricto* to other members of the MTBC (Comas et al., 2013). Genetic evidence suggests that MTBC is likely to have originated around 5,000-10,000 years ago (Bos et al., 2014; Kay et al., 2015; Chiner-Oms et al., 2019), corresponding with archaeological evidence of *M. tuberculosis* DNA and lipids in skeletal remains from 9,000 years ago (Hershkovitz et al., 2008).

The modern MTBC comprises seven human-adapted lineages, which are phylogenetically distinct groups clades that have evolved separately, having diverged over a period of 500 to 3000 years (O'Neill et al., 2019) and several animal-adapted strains. Lineages 1, 2, 3, 4 and 7 are traditionally referred to as *M. tuberculosis sensu stricto*, while lineages 5 and 6 are known as *M. africanum*. Lineages 5 and 6 are restricted to West Africa and

lineage 7 to East Africa, suggesting that they may have specifically adapted to their host populations (Asante-Poku et al., 2015). Lineage 1 to 4 are globally distributed, with lineages 2 and 4 being the most prevalent worldwide (Gagneux, 2018)

Mechanisms of drug resistance

The majority of *M. tuberculosis* antibiotic resistance is conferred by genomic mutations – usually SNPs or small insertions or deletions, and occasionally larger deletions or inversions. Given the lack of horizontal gene transfer or episomal resistance genes (Boritsch et al., 2016), these generally arise spontaneously and are chromosomally encoded, with spread through replication within host and onward transmission between hosts of resistant bacteria.

In contrast to organisms which exhibit horizontal gene transfer and therefore can also acquire extrachromosomal drug-inactivating resistance genes, there are three main mechanisms through which antituberculosis drug resistance can be acquired: target-based mutations, activator mutations and modulation of efflux pumps. Target-based mutations are where the drug target itself becomes mutated, usually preventing drug binding. Many antituberculosis drugs are administered as prodrugs that require activation by bacterial enzymes to produce their active form. In these cases, mutations of drug activators can lead to resistance. Finally, efflux pumps may pump active drug out of the bacterial cell, although there are fewer examples of these. Examples of each of these mechanisms are shown in Table 1 (Silva and Palomino, 2011; Dookie et al., 2018). Some drugs have multiple mechanisms of resistance, for example isoniazid resistance can be conferred by target-based (*inhA*) or activator (*katG*) mutations. Some mutations may lead to cross-resistance, while others monoresistance. For example, *atpE* is the target for only bedaquiline. However, resistance to bedaquiline, clofazimine, and even new tuberculosis inhibitors like BRD-9327 can be conferred by efflux pump regulator mutations in *Rv0678* (Johnson et al., 2020).

Factors affecting acquisition of drug resistance

Prevalence of drug resistance varies by drug, patterns of drug usage (including the combinations of drugs it was used with), bacterial genetic background and country (Brynildsrud et al., 2018; Ektefaie et al., 2021). The ability of *M. tuberculosis* to acquire drug resistance to each drug is underpinned by the rate at which spontaneous mutants arise and survive. This is different for each drug, with pyrazinamide having a particularly high rate of resistance acquisition *in vitro* and rifampicin a lower rate (Figure 1) (McGrath et al., 2014; David, 1970; Billington et al., 1999; Werngren abd Hoffner, 2003; O'Sullivan et al., 2008; Bergval et al., 2009, 2012; Huitric et al., 2010; Kana et al., 2010; Ford et al., 2011; Stoffels et al., 2012). However, the clinical relevance of *in vitro* mutation rates is only one aspect of the likely robustness of a drug against the development of resistance against it. For example, the studies examining spontaneous development of bedaquiline-resistant mutants (Huitric et al., 2010) recorded the rate at which bacteria developed *atpE* mutations, which is the main gene determining bedaquiline resistance *in vitro* but not *in vivo*, where virtually all clinically reports mutations are in the

TABLE 1 Categories of mutation leading to *M. tuberculosis* drug resistance.

Category	Gene (drug)	Mechanism of resistance
Target-based	*rpoB* (rifampicin)	Rifampicin is unable to bind RNA polymerase, responsible for mRNA elongation (Telenti et al., 1993)
	inhA (isoniazid, ethionamide)	Both drugs unable to bind NADH-dependent enoyl-acyl carrier protein reductase responsible for mycolic acid synthesis (Banerjee et al., 1994)
	gyrA/B (fluoroquinolones)	Mutations prevent binding to DNA gyrase required for DNA replication (Takiff et al., 1994)
	rrl (aminoglycosides)	Prevent binding to 23S ribosomal RNA which prevents protein synthesis (Suzuki et al., 1998)
	atpE (bedaquiline)	Prevents binding to F1F0 proton ATP synthase, part of electron transport chain (Huitric et al., 2010)
	embB (ethambutol)	Mutations in the mycobacterial arabinosyl transferase enzyme preventing synthesis of arabinogalactan for the cell wall (Telenti et al., 1997)
Drug activator	*katG* (isoniazid)	Isoniazid is activated by the katG-encoded catalase-peroxidase enzyme. S315T mutations prevent activation while maintaining native gene function (Pym et al., 2002)
	ethA (ethionamide)	Mutations in the activating mono-oxygenase enzyme encoded by ethA, or its regulator ethR (Morlock et al., 2003)
	pncA (pyrazinamide)	Diverse range of mutations in pyrazinamide activating PZase encoded by pncA lead to resistance, including any loss of function mutation as PZase loss does not impair fitness (Scorpio and Zhang, 1996)
	fbiA/B/C, fgd1, ddn (delamanid/pretomanid)	Wide variety of mutations inactivating enzymes ddn and co-enzyme fgd1. Also mutations in synthetic pathway for F420 cofactor required for activation (fbiA/B/C) (Haver et al., 2015)
Efflux pumps	*Rv0678* (bedaquiline, clofazimine)	Mutations affecting or preventing function of the Rv0678 repressor of the MmpL5 efflux pump lead to overexpression of the pump and presumed efflux of bedaquiline and clofazimine (Hartkoorn et al., 2014)

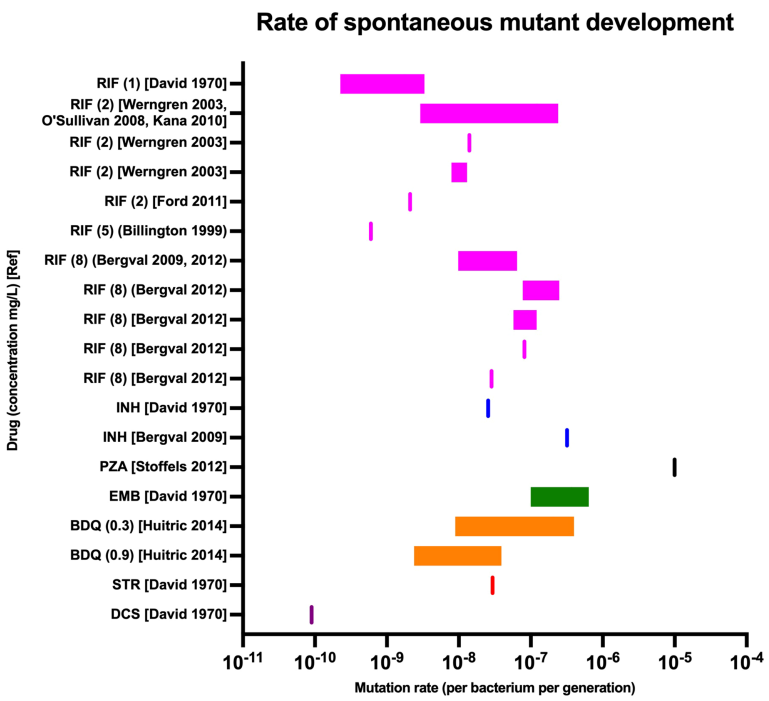


FIGURE 1
Spontaneous *in vitro* mutation rates for key antituberculous drugs (McGrath et al., 2014). Rates were measured in a variety of laboratory and clinical strains from varying bacterial lineages. Drug names abbreviated: RIF, rifampicin; INH, isoniazid; PZA, pyrazinamide; EMB, ethambutol; BDQ, bedaquiline; STR, streptomycin; DCS, D-cycloserine. Concentrations in mg/L shown in brackets followed by original reference (David, 1970; Billington et al., 1999; Werngren and Hoffner, 2003; O'Sullivan et al., 2008; Bergval et al., 2009, 2012; Huitric et al., 2010; Kana et al., 2010; Ford et al., 2011; Stoffels et al., 2012).

Rv0678 gene (Huitric et al., 2010). This is likely because *atpE* is an essential gene and mutations carry a high fitness cost *in vitro*, underlining the importance of understanding *in vivo* fitness costs of mutations.

Bacterial genetic background is also likely to affect the ability of certain strains to acquire drug resistance. The best described example of this is higher prevalence of drug resistance amongst lineage 2 strains compared to other bacterial lineages (Parwati et al., 2010; Ektefaie et al., 2021). It is likely that lineage 2 *M. tuberculosis* strains have a greater inherent ability to acquire drug resistance, and this has now been suggested by multiple studies (Ford et al., 2013; Hakamata et al., 2020; Nimmo et al., 2020; Ortiz et al., 2021), although at least one study did not find this link (Guerra-Assunção et al., 2015). While the mechanisms through which this may occur have not been fully elucidated, *in vitro* work with *M. smegmatis* (a related mycobacterium often used for laboratory studies) has shown that ribosomal mutations can lead to resistance to multiple antibiotics and enhanced bacterial survival (Gomez et al., 2017). An alternative explanation for higher rates of drug resistance amongst lineage 2 strains may be the founder effect, where lineage 2 strains that were already drug resistant clonally expanded rapidly in an area with high rates of transmission (Grandjean et al., 2015).

Finally, country-specific factors have been shown to influence the development of resistance even within a given bacterial strain. One example from a reconstructed phylogeny of the Central Asian Clade, a subgroup of lineage 2.2 (Beijing strain), showed it was in circulation in former Soviet republics in the 1960s and 1970s, before its introduction into Afghanistan in the 1980s (Eldholm et al., 2016). Many resistance mutations arose independently amongst strains that were circulating in the former Soviet republics, while very few apart from the original lineage-defining *rpoB* mutation were present in the Afghan strains, with the vast majority of these mutations arising in the years after the collapse of the Soviet Union. Another analysis of the global spread of lineage 4 found that it was likely to have been dispersed from Europe during colonial expansion, that most drug resistance conferring mutations arose and were subsequently spread within individual countries (Brynildsrud et al., 2018). Taken together, this suggests that a variety of factors that are hard to quantify, such as differing healthcare systems and political instability that are likely to impact on patterns of antimicrobial prescription, supply and usage. Additionally, country-level variation in sequencing and drug susceptibility testing is also likely to play a significant role in

the determined level of resistance in each country. For example, in 2020 94% of new TB cases in the WHO Europe Region were tested for rifampicin resistance, compared to 50% in the African Region (World Health Organization, 2022).

Emergence of resistance between hosts

Since the introduction of the first antituberculosis drug, streptomycin, in the 1950s, resistance to most new drugs has been identified as occurring within 5 to 10 years of their clinical use, with similar mutations occurring independently in different parts of the world (convergent evolution) (Cohen et al., 2015; Manson et al., 2017; Brynildsrud et al., 2018). This phenomenon has occurred even more rapidly with recently introduced drugs.

An analysis of the world's first comprehensively described extensively drug-resistant TB (XDR-TB, by historical definition of injectable and fluoroquinolone resistance) outbreak in Tugela Ferry, KwaZulu-Natal, South Africa, revealed that the drug resistance mutations carried by the strain had been acquired sequentially over 50 years. Genomic dating techniques revealed resistance developing broadly in the order in which drugs were introduced into clinical practice (Cohen et al., 2015), and a similar pattern was demonstrated in a global collection of over 1500 lineage 4 strains (Brynildsrud et al., 2018). This confirms the pattern established after the introduction of streptomycin in the mid-1940s, where clinical resistance was reported within two years (Youmans et al., 1946) (Figure 2). Two other studies of multiple global lineages have also shown the same order of resistance development, starting with isoniazid and streptomycin resistance, followed by rifampicin, fluoroquinolones and injectables (Manson et al., 2017; Ektefaie et al., 2021). Interestingly, these studies did not show a correlation between the date of drug introduction and the date of resistance emerging. This is likely to represent the fact that other variables affect the development of resistance, such as the spontaneous rate at which mutations develop, *in vivo* fitness costs associated with resistance, and the clinical combinations in which drugs tended to be used (for example, while injectable drugs have been available since the 1950s they were much less commonly used than rifampicin and isoniazid).

Intriguingly, *Rv0678* mutations likely to confer bedaquiline resistance have been identified long prior to the development of the drug van (van Dorp et al., 2020). It has been hypothesised that this could have been selected for by the use of clofazimine, which was developed for the treatment of TB in the 1950s, although due to the development of more effective TB drugs was mostly used for the treatment of leprosy until it was repurposed for MDR-TB in the 2000s. The earliest emergence was dated to the beginning of the 18th century, although interestingly this clade had an associated inactivating mutation in *mmpL5* which was likely to counteract the resistant phenotype (Sonnenkalb et al., 2021). However, later emergences in the late 19th and early 20th century still pre-date the use of any antituberculosis therapy, and may be due to other environmental stressors, including microbial antagonism in the environment before the transition to obligate pathogenicity. As one role of MmpL5 is efflux – especially of siderophores – this could include adapting to low iron availability or presence of a toxin for example. Overall, this demonstrates that, at least for bedaquiline and clofazimine, and potentially other new drugs, resistant bacteria may already exist in the environment and could be rapidly selected for as therapy is expanded.

Compensatory mutations

Many TB drugs target essential cellular processes, hence resistance-conferring mutations can come with a fitness cost, manifested as a slower growth rate in culture and reduced transmission within a population compared to wild-type strains. This has been best described for *rpoB* mutations conferring rifampicin resistance (Gagneux et al., 2006; Knight et al., 2015). The most common isoniazid resistance mutation in

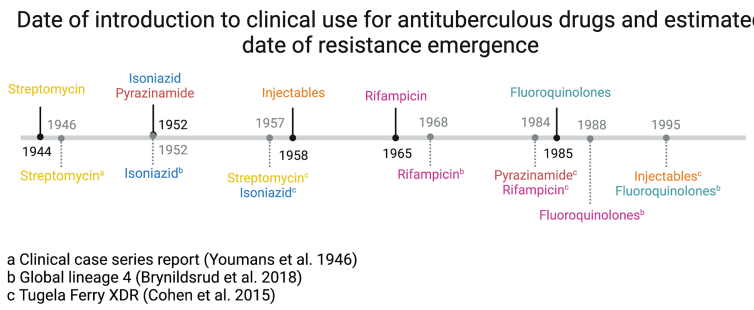


FIGURE 2
Date of introduction to clinical use for antituberculous drugs (above line, denoted by solid black line) and estimated date of resistance emergence (below line, denoted by grey dashed line) (Ektefaie et al., 2021).

katG (S315T) is thought to have only minimal fitness cost, while pyrazinamide resistance may have a fitness cost (Pečerska et al., 2021). Most fluoroquinolone resistance-conferring mutations do not affect fitness, although impaired growth has been reported for the *gyrA* G88C and G88D mutations, although these occur infrequently in clinical isolates (Emane et al., 2021).

The fitness cost imposed by *rpoB* mutations can be reversed by compensatory mutations, specifically mutations in *rpoA* and *rpoC* which encode two other subunits of the RNA polymerase enzyme (alpha and beta prime). These have been identified in *in vitro* culture experiments and additionally are seen at an increased prevalence in countries with a high burden of MDR-TB (Comas et al., 2011; Merker et al., 2018; Trauner et al., 2021), linked to high rates of transmission of MDR-TB strains in some settings (Gygli et al., 2021).

Emergence of resistance within hosts

It is likely that the ability of *M. tuberculosis* to rapidly acquire drug resistance while maintaining limited genomic variability over time is due the generation of low frequency within-host variation. This is not surprising given that *M. tuberculosis* infections typically last months to years and within-host bacterial populations may peak at over 10^9 colony forming units. Greater insight into this has been achieved through the adoption of high throughput sequencing, which has more recently enabled the identification significant within-host *M. tuberculosis* genetic diversity. Within-host diversity can in principle arise from mixed infection with multiple genetically distinct strains or within-host microevolution of a single infecting strain, or both (Figure 3) (Ford et al., 2012). At one extreme, up to 50 consensus-level SNP differences having been reported to occur over the duration of infection in patients with advanced disease when sampling from multiple body sites (Lieberman et al., 2016). However in the majority of cases of *M. tuberculosis* infection, the genetic diversity is constrained by purifying selection that leads to loss of variants without a clear fitness advantage for this specialised pathogen which is adapted to a pathogenic lifestyle in the human host (Figure 3).

Although most *M. tuberculosis* variants are lost over the course of infection, it is still possible for those that confer an advantage, such as drug resistance, to increase in prevalence and

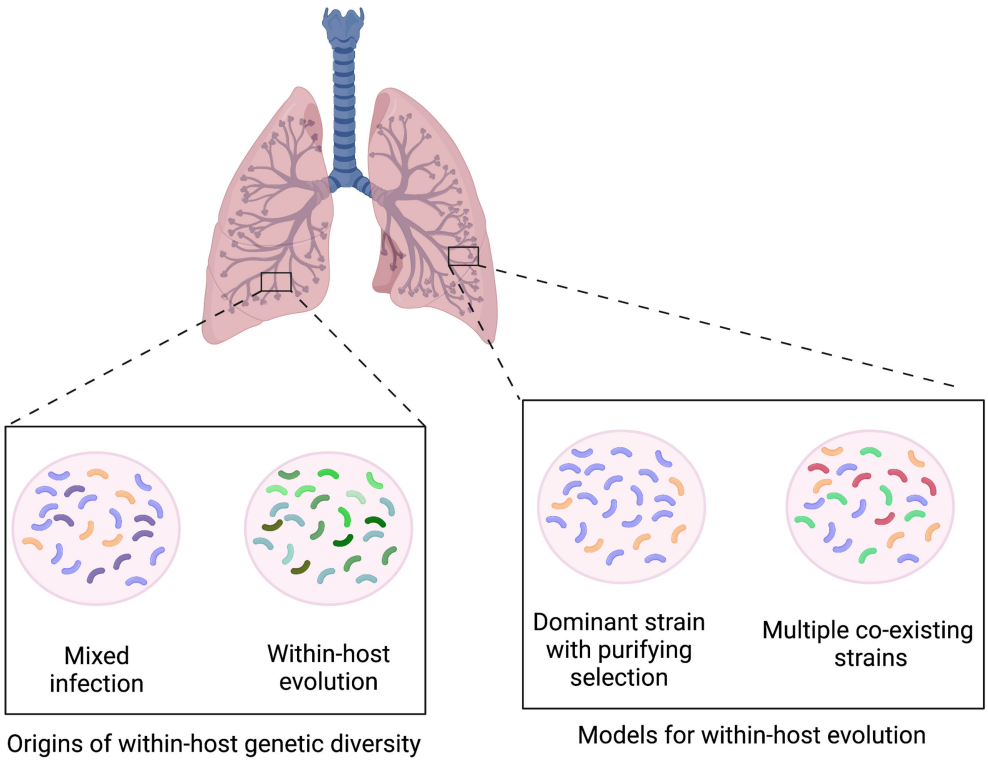


FIGURE 3
Infection model showing how within-host genetic diversity may occur through mixed infection with genetically different strains or within-host evolution of a clonal infecting strain. Model of within-host evolution showing single dominant strain, with purifying selection leading to loss of variants with reduced fitness and multiple co-existing strains within lung.

become the dominant clone over time. These resistant strains can then be transmitted leading to primary drug resistant infection in a new host. This may contrast with other bacteria where multiple variant strains may co-exist separately, as may be seen in non-specialised pathogens such as *Pseudomonas aeruginosa* (Winstanley et al., 2016) or non-tuberculous mycobacteria (Bryant et al., 2016; Shaw et al., 2019) in patients with cystic fibrosis, where there is an abnormal airway and immune environment (Figure 3).

Understanding factors affecting overall within-host *M. tuberculosis* genetic diversity may offer insights into mechanisms controlling bacterial replication and evolution. Most studies to date rely on sequencing mycobacterial DNA extracted from culture to ensure sufficient DNA for sequencing, although this is likely to introduce bias by stochastic loss and selecting for bacterial subpopulations more suited to growth in culture (Metcalfe et al., 2017). However, it has been demonstrated that culture-independent sequencing of *M. tuberculosis* directly from sputum identifies more genetic diversity than sequencing from culture (Nimmo et al., 2019; Shockey et al., 2019). As techniques for direct-from-sample sequencing improve, our understanding of within patient genetic diversity may therefore continue to develop.

From current work that had relied on sequencing from culture, one detailed study of five patients revealed that overall *M. tuberculosis* genomic diversity increased with disease severity and was particularly high in pre-mortem isolates from two patients, presumably due to high bacterial load (O'Neill et al., 2015). The most sequence-diverse genes were those involved in production of cell envelope lipids. No evidence for a decrease in diversity during treatment or any effect of *M. tuberculosis* lineage or drug resistance profile was found, while HIV statuses were not available for analysis. An analysis of 200 patients from eight publicly available studies reporting patients who failed treatment found that genes associated with antibiotic resistance displayed highest diversity, while the within-host diversity across remaining gene classes (*in vitro* essential, non-essential, PE/PPE genes and antigen genes) seemed unaffected (Vargas et al., 2021). South African cohort studies revealed greater genetic diversity in patients with cavitary disease, infection with lineage 2 strains and absence of second-line drug resistance, although no association between time to positivity in culture and diversity (Nimmo et al., 2020). This suggests that diversity may be more influenced by higher intrinsic mutation rates (as seen with lineage 2), variable drug penetration (in cavitary disease) or impaired immune control (in untreated HIV) than bacterial population size. However, there was no association between diversity and clinical outcomes at six months.

Mixed populations of wild-type alleles and resistance-associated variants (RAVs) confer heteroresistance, where populations of resistant and susceptible bacteria co-exist within the same host. This may occur as the result of differential drug penetration to spatially and pathologically distinct lung regions (Dheda et al., 2018) leading in effect to monotherapy and subsequent resistance acquisition or survival of susceptible bacteria. Baseline genetic heteroresistance appears to be particularly common for bedaquiline (up to 60%) (Nimmo et al., 2020) and fluoroquinolones (11-26%) (Operario et al., 2017; Nimmo et al., 2020).

Several case reports have identified heterozygous RAVs that have increased in frequency over the course of treatment (Sun et al., 2012; Eldholm et al., 2014; Trauner et al., 2017) leading to fixed resistance, including variants originally identified at <1% frequency (Vos et al., 2019). A retrospective deep sequencing study identified very low frequency RAVs (<1%) predating acquired phenotypic resistance (Engelthaler et al., 2019). However, due to high levels of turnover of low-frequency variants, it may be difficult to predict which heterozygous RAVs are likely to persist or become fixed and which ones will disappear. In a prospective cohort study of almost 400 patients, most with heterozygous RAVs detectable on WGS with sequential isolates available and sensitivity to detect variants above 5% frequency demonstrated RAV persistence or fixation (17/20, 85%) (Nimmo et al., 2020). However, only one case of a very low frequency RAV (<5%) expanding to cause resistance was identified. Another study showed no effect on treatment outcome amongst patients with RAVs at <1% frequency (Chen et al., 2021), while modelling from multiple cohort studies suggests that variants at ≥19% frequency predicted subsequent fixation (Vargas et al., 2021).

Taken together, the current evidence suggests that the significance of heterozygous RAVs is likely to depend on their frequency, with much greater clinical significance of those at higher frequency (>15-20%) than lower frequency (especially <5%). While heterozygous RAVs are likely to be variably identified by current diagnostics (Ng et al., 2019; Rigouts et al., 2019) with newer sequencing-based techniques offering good sensitivity even for RAVs identified at very low frequency, establishing how to interpret low frequency heterozygous RAVs is going to become an important clinical decision.

Implications for diagnostics

As many countries move towards genomic-based methods such as molecular PCR-based tools (for instance Xpert MTB/RIF), and progressively to targeted sequencing and whole genome sequencing (WGS) for diagnosis of *M. tuberculosis* infection and identification of drug resistance, it is important to be aware of the implications of the evolution of resistance. Currently, understanding of resistance-conferring mutations is inevitably limited, particularly for newer drugs, despite recent large global studies (The CRyPTIC Consortium, 2022). The clearest example of a targeted molecular tool creating its own selective pressure has been demonstrated in Eswatini where the non-canonical rifampicin resistance-conferring mutation, *rpoB* I491F, which

falls outside the rifampicin-resistance determining region became dominant (Figure 4A) (Sanchez-Padilla et al., 2015).

This was first identified following the Eswatini 2009 drug resistance survey, which revealed a surprisingly high rate of MDR-TB (7.7% in previously untreated patients and 33.8% in previously treated patients) (Sanchez-Padilla et al., 2012). Xpert MTB/RIF was implemented in 2012 to enable rapid diagnosis of MDR-TB (Sikhondze et al., 2015), but in 2015 a detailed genetic and phenotypic analysis of strains stored from the 2009 survey showed that 30% of rifampicin resistance was actually conferred by a the *rpoB* I491F mutation, which is not identified by Xpert MTB/RIF and such strains would therefore be reported as rifampicin susceptible (Sanchez-Padilla et al., 2015). Patients infected with such strains would therefore be treated with an ineffective standard drug-susceptible regimen for their rifampicin-resistant infection. By the time of the next drug resistance survey in 2017, 56% of rifampicin resistance was conferred by the I491F mutation (World Health Organization, 2020). The Eswatini National Tuberculosis Control Programme has since proposed presumptively treating all isoniazid-resistant TB as MDR-TB until phenotypic testing has been completed, as most I491F mutations are present in isoniazid resistant strains (Ardizzoni et al., 2021).

Finally, resistance to new drugs such as bedaquiline and delamanid is caused by many individually rare mutations that do not provide statistical power for genotype-phenotype correlation in the way that has been performed for most first-line drugs. For example, in the *Rv0678* gene responsible for most clinical bedaquiline resistance, mutations are spread throughout the gene with no clear resistance-conferring hotspot (Figure 4B). Additionally, there is not a clear separation of minimum inhibitory concentrations of bedaquiline between wild type and resistant isolates, which is likely to complicate attempts to categorise individual mutations as susceptible or resistant, with many likely to fall near the critical concentration and be vulnerable to technical variation. A pragmatic approach may be to use molecular or genetic methods to screen resistance-associated genes for variants, which are rare amongst susceptible isolates, followed by phenotypic evaluation of isolates containing mutants.

Conclusions

M. tuberculosis has shown a remarkable ability to develop resistance to all antituberculosis drugs that have been developed, including those brought into clinical use for DR-TB in recent years such as bedaquiline, linezolid and delamanid/pretomanid. It is important to bear in mind these drugs remain highly effective in the vast majority of patients with DR-TB and have

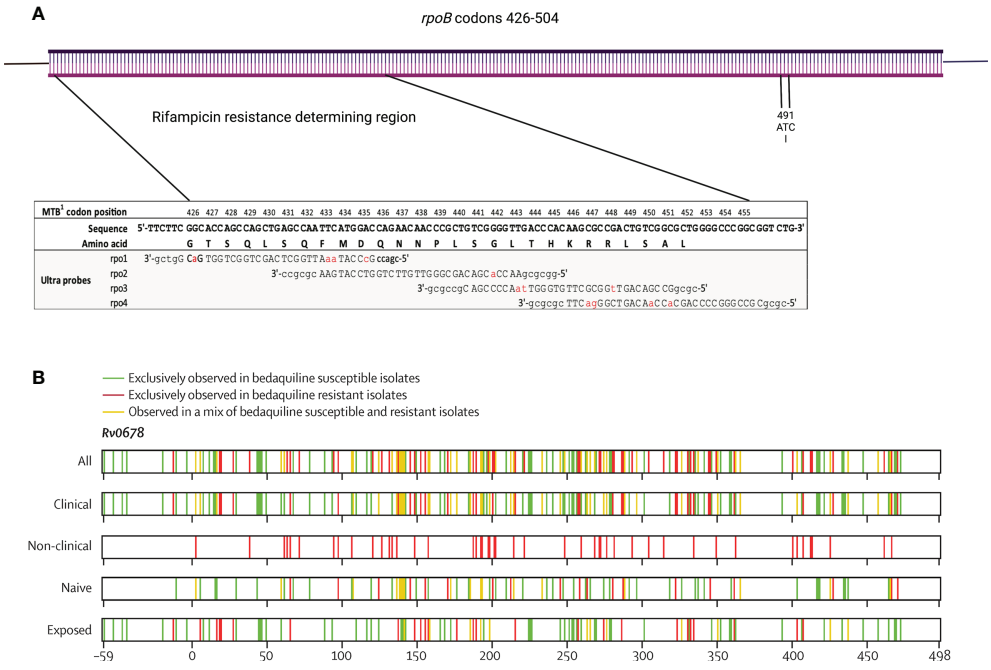


FIGURE 4
Schematic diagrams of **(A)** *rpoB* gene codons 426-504 showing rifampicin resistance determining region, coverage of Xpert MTB/RIF probes (modified from (Chakravorty et al., 2017) under CC BY 4.0 licence) and location of I491 codon, and **(B)** *Rv0678* gene showing distribution of reported gene substitutions, coloured by association with resistance (reproduced from (Ismail et al., 2021) under CC BY 4.0 licence).

undoubtedly been responsible for the major improvements in DR-TB outcomes that have been seen in the last 10 years (World Health Organization, 2022). However, examples such as the spread of XDR-TB across South Africa, first identified as the Tugela Ferry outbreak, and the rapid amplification of the *rpoB* I491F mutation amongst *M. tuberculosis* strains in Eswatini, highlight that this progress cannot be taken for granted (Gandhi et al., 2006; Sanchez-Padilla et al., 2015). Progression towards the World Health Organization's End TB targets of a 90% reduction in TB transmission between 2015 and 2035 will require strict control of the spread of DR-TB, which require highly effective drugs to be available rapidly to those infected.

To achieve this, important strategic decisions will be required. While there are now a number of exciting new drug candidates progressing through the TB drug development pipeline, their impact will remain uncertain. The effectiveness of currently available drugs depends on limiting the spread of resistance to them. It is therefore questionable whether effective drugs for DR-TB such as bedaquiline and pretomanid should be incorporated into drug-susceptible TB (DS-TB) regimens, which may increase the spread of resistance and reduce their effectiveness for DR-TB, unless their overall benefits to patients with TB and progress towards elimination is outweighed by improvements in DS-TB treatment. This needs to be accounted for when evaluating the results of trials such as SimpliciTB (ClinicalTrials.gov Identifier: NCT03338621), where bedaquiline and pretomanid are used to reduce DS-TB treatment duration from six to four months, which was already been demonstrated to be possible using rifapentine and moxifloxacin (Dorman et al., 2021), or adopting a stratified treatment approach for some patients (Imperial et al., 2018).

In addition, it is essential not to assume susceptibility to drugs and TB programmes should aim to perform susceptibility testing for all drugs that are included in treatment regimens. It is now clearly demonstrated that there is a pre-existing pool of bedaquiline resistance and it can therefore be expected to occur in patients without any clear risk factors for resistance (Beckert et al., 2020; van Dorp et al., 2020; Ismail et al., 2022) Genotypic susceptibility testing is clearly very effective in many cases, but it is important to be aware of the limitations. While the limited genotypic-phenotypic understanding for new drugs such as bedaquiline and delamanid/pretomanid will be one challenge, the spread of *rpoB* I491F shows how significant the selective pressure from diagnostics can be.

The most effective strategy is therefore going to require greater understanding of how resistance develops within patients with a view to preventing its occurrence. This will need to be backed up by preventing spread of resistance through use of up-front susceptibility testing for all drugs – using combined genotypic and phenotypic methods – along with ongoing surveillance for development of resistance and changes to the prevalence of resistance-conferring mutations, and strategic use of available medications to maximise benefit to individual patients as well as the End TB strategy.

Author contributions

Conceptualisation: CN;Writing – original draft: CN; Writing – review and editing: CN, JaM, VF, JoM, JE, HP, and EJ; Visualisation: CN, VF, JoM, and HP; Supervision: EOJ; All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Francis Crick Institute which receives its core funding from Cancer Research UK (CC2169), the UK Medical Research Council (CC2169), and the Wellcome Trust (CC2169).

Acknowledgments

Figures 2, 3 and 4 created with BioRender.com. This research was funded in part by the Wellcome Trust (CC2169). For the purpose of Open Access, the author has applied a CC BY public copyright licence to any Author Accepted Manuscript version arising from this submission. The authors would like to thank Joanna Evans (Systems Chemical Biology of Infection and Resistance Laboratory, Francis Crick Institute) for critically reading the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Ardizzoni, E., Ariza, E., Mulengwa, D., Mpala, Q., Tour, R., de, L., et al. (2021). Thin-Layer-Agar-Based direct phenotypic drug susceptibility testing on sputum in eswatini rapidly detects mycobacterium tuberculosis growth and rifampicin resistance otherwise missed by WHO-endorsed diagnostic tests. *Antimicrob. Agents Ch* 65, e02263–e02220. doi: 10.1128/aac.02263-20

Asante-Poku, A., Yeboah-Manu, D., Otchere, I. D., Aboagye, S. Y., Stucki, D., Hattendorf, J., et al. (2015). Mycobacterium africanum is associated with patient ethnicity in Ghana. *PloS Negl. Trop. D* 9, e3370. doi: 10.1371/journal.pntd.0003370

Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., et al. (1994). inhA, a gene encoding a target for isoniazid and ethionamide in mycobacterium tuberculosis. *Science* 263, 227–230. doi: 10.1126/science.8284673

Beckert, P., Sanchez-Padilla, E., Merker, M., Dreyer, V., Kohl, T. A., Utpatel, C., et al. (2020). MDR m. Tuberculosis outbreak clone in eswatini missed by xpert has elevated bedaquiline resistance dated to the pre-treatment era. *Genome Med.* 12, 104. doi: 10.1186/s13073-020-00793-8

Bergval, I., Kwok, B., Schuitema, A., Kremer, K., van Soolingen, D., Klatser, P., et al. (2012). Pre-existing isoniazid resistance, but not the genotype of mycobacterium tuberculosis drives rifampicin resistance codon preference *in vitro*. *PloS One* 7, e29108. doi: 10.1371/journal.pone.0029108

Bergval, I. L., Schuitema, A. R. J., Klatser, P. R., and Anthony, R. M. (2009). Resistant mutants of mycobacterium tuberculosis selected *in vitro* do not reflect the *in vivo* mechanism of isoniazid resistance. *J. Antimicrob. Chemoth* 64, 515–523. doi: 10.1093/jac/dkp237

Billington, O. J., McHugh, T. D., and Gillespie, S. H. (1999). Physiological cost of rifampin resistance induced *In vitro* in mycobacterium tuberculosis. *Antimicrob. Agents Ch* 43, 1866–1869. doi: 10.1128/aac.43.8.1866

Boritsch, E. C., Khanna, V., Pawlik, A., Honoré, N., Navas, V. H., Ma, L., et al. (2016). Key experimental evidence of chromosomal DNA transfer among selected tuberculosis-causing mycobacteria. *Proc. Natl. Acad. Sci.* 113, 9876–9881. doi: 10.1073/pnas.1604921113

Bos, K. I., Harkins, K. M., Herbig, A., Coscolla, M., Weber, N., Comas, I., et al. (2014). Pre-Columbian mycobacterial genomes reveal seals as a source of new world human tuberculosis. *Nature* 514, 494–497. doi: 10.1038/nature13591

Bryant, J. M., Grogono, D. M., Rodriguez-Rincon, D., Everall, I., Brown, K. P., Moreno, P., et al. (2016). Emergence and spread of a human-transmissible multidrug-resistant nontuberculous mycobacterium. *Science* 354, 751–757. doi: 10.1126/science.aaf8156

Brynildsrud, O. B., Pepperell, C. S., Suffys, P., Grandjean, L., Monteserin, J., Debech, N., et al. (2018). Global expansion of mycobacterium tuberculosis lineage 4 shaped by colonial migration and local adaptation. *Sci. Adv.* 4, eaat5869. doi: 10.1126/sciadv.aat5869

Chakravorty, S., Simmons, A. M., Rowneki, M., Parmar, H., Cao, Y., Ryan, J., et al. (2017). The new xpert MTB/RIF ultra: Improving detection of mycobacterium tuberculosis and resistance to rifampin in an assay suitable for point-of-Care testing. *Mbio* 8, e00812–e00817. doi: 10.1128/mbio.00812-17

Chen, Y., Jiang, Q., Zou, J., Yang, T., Liu, Q., Luo, G., et al. (2021). Deep whole-genome sequencing reveals no evidence for heteroresistance influencing treatment outcomes among drug-susceptible tuberculosis patients. *Tuberculosis* 130, 102120. doi: 10.1016/j.tube.2021.102120

Chiner-Oms, Á., Sánchez-Busó, L., Corander, J., Gagneux, S., Harris, S. R., Young, D., et al. (2019). Genomic determinants of speciation and spread of the mycobacterium tuberculosis complex. *Sci. Adv.* 5, eaaw3307. doi: 10.1126/sciadv.aaw3307

Cohen, K. A., Abeel, T., McGuire, A. M., Desjardins, C. A., Munsamy, V., Shea, T. P., et al. (2015). Evolution of extensively drug-resistant tuberculosis over four decades: Whole genome sequencing and dating analysis of mycobacterium tuberculosis isolates from KwaZulu-natal. *PloS Med.* 12, e1001880. doi: 10.1371/journal.pmed.1001880

Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., et al. (1998). Deciphering the biology of mycobacterium tuberculosis from the complete genome sequence. *Nature* 393, 537–544. doi: 10.1038/31159

Comas, I., Borrell, S., Roetzer, A., Rose, G., Malla, B., Kato-Maeda, M., et al. (2011). Whole-genome sequencing of rifampicin-resistant m. tuberculosis strains identifies compensatory mutations in RNA polymerase. *Nat. Genet.* 44, 106–110. doi: 10.1038/ng.1038

Comas, I., Coscolla, M., Luo, T., Borrell, S., Holt, K. E., Kato-Maeda, M., et al. (2013). Out-of-Africa migration and neolithic co-expansion of mycobacterium tuberculosis with modern humans. *Nat. Genet.* 45, 1176–1182. doi: 10.1038/ng.2744

David, H. L. (1970). Probability distribution of drug-resistant mutants in unselected populations of mycobacterium tuberculosis. *Appl. Microbiol.* 20, 810–814. doi: 10.1128/am.20.5.810-814.1970

Dheda, K., Lenders, L., Magombedze, G., Srivastava, S., Raj, P., Arning, E., et al. (2018). Drug penetration gradients associated with acquired drug resistance in tuberculosis patients. *Am. J. Resp. Crit. Care* 198, 1208–1219. doi: 10.1164/rccm.201711-2333oc

Dookie, N., Rambaran, S., Padayatchi, N., Mahomed, S., and Naidoo, K. (2018). Evolution of drug resistance in mycobacterium tuberculosis: a review on the molecular determinants of resistance and implications for personalized care. *J. Antimicrob. Chemoth* 73, 1138–1151. doi: 10.1093/jac/dkx506

Dorman, S. E., Nahid, P., Kurbatova, E. V., Phillips, P. P. J., Bryant, K., Dooley, K. E., et al. (2021). Four-month rifapentine regimens with or without moxifloxacin for tuberculosis. *New Engl. J. Med.* 384, 1705–1718. doi: 10.1056/nejmoa2033400

van Dorp, L., Nimmo, C., Ortiz, A. T., Pang, J., Acman, M., Tan, C. C. S., et al. (2020)Detection of a bedaquiline / clofazimine resistance reservoir in mycobacterium tuberculosis predating the antibiotic era. *Biorxiv*. doi: 10.1101/2020.10.06.328799

Ektefaie, Y., Dixit, A., Freschi, L., and Farhat, M. R. (2021). Globally diverse mycobacterium tuberculosis resistance acquisition: A retrospective geographical and temporal analysis of whole genome sequences. *Lancet Microbe* 2, e96–e104. doi: 10.1016/s2666-5247(20)30195-6

Eldholm, V., and Balloux, F. (2016). Antimicrobial resistance in mycobacterium tuberculosis: The odd one out. *Trends Microbiol.* 24, 637–648. doi: 10.1016/j.tim.2016.03.007

Eldholm, V., Norheim, G., Lippe, B.v. d., Kinander, W., Dahle, U. R., Caugant, D. A., et al. (2014). Evolution of extensively drug-resistant mycobacterium tuberculosis from a susceptible ancestor in a single patient. *Genome Biol.* 15, 490. doi: 10.1186/s13059-014-0490-3

Eldholm, V., Pettersson, J. H.-O., Brynildsrud, O. B., Kitchen, A., Rasmussen, E. M., Lillebaek, T., et al. (2016). Armed conflict and population displacement as drivers of the evolution and dispersal of mycobacterium tuberculosis. *Proc. Natl. Acad. Sci.* 113, 13881–13886. doi: 10.1073/pnas.1611283113

Emane, A. K. A., Guo, X., Takiff, H. E., and Liu, S. (2021). Drug resistance, fitness and compensatory mutations in mycobacterium tuberculosis. *Tuberculosis* 129, 102091. doi: 10.1016/j.tube.2021.102091

Engelthaler, D. M., Streicher, E. M., Kelley, E. J., Allender, C. J., Wiggins, K., Jimenez, D., et al. (2019). Minority mycobacterium tuberculosis genotypic populations as an indicator of subsequent phenotypic resistance. *Am. J. Resp. Cell Mol.* 61, 789–791. doi: 10.1165/rcmb.2019-0178le

Fishbein, S., Wyk, N., Warren, R. M., and Sampson, S. L. (2015). Phylogeny to function: PE/PPE protein evolution and impact on mycobacterium tuberculosis pathogenicity. *Mol. Microbiol.* 96, 901–916. doi: 10.1111/mmi.12981

Ford, C. B., Lin, P. L., Chase, M. R., Shah, R. R., Iartchouk, O., Galagan, J., et al. (2011). Use of whole genome sequencing to estimate the mutation rate of mycobacterium tuberculosis during latent infection. *Nat. Genet.* 43, 482–486. doi: 10.1038/ng.811

Ford, C. B., Shah, R. R., Maeda, M. K., Gagneux, S., Murray, M. B., Cohen, T., et al. (2013). Mycobacterium tuberculosis mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis. *Nat. Genet.* 45, 784–790. doi: 10.1038/ng.2656

Ford, C., Yusim, K., Ioerger, T., Feng, S., Chase, M., Greene, M., et al. (2012). Mycobacterium tuberculosis – heterogeneity revealed through whole genome sequencing. *Tuberculosis* 92, 194–201. doi: 10.1016/j.tube.2011.11.003

Gagneux, S. (2018). Ecology and evolution of mycobacterium tuberculosis. *Nat. Rev. Microbiol.* 16, 202–213. doi: 10.1038/nrmicro.2018.8

Gagneux, S., Long, C. D., Small, P. M., Van, T., Schoolnik, G. K., and Bohannan, B. J. M. (2006). The competitive cost of antibiotic resistance in mycobacterium tuberculosis. *Science* 312, 1944–1946. doi: 10.1126/science.1124410

Gandhi, N. R., Moll, A., Sturm, A. W., Pawinski, R., Govender, T., Lalloo, U., et al. (2006). Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of south Africa. *Lancet* 368, 1575–1580. doi: 10.1016/s0140-6736(06)69573-1

Gomez, J. E., Kaufmann-Malaga, B. B., Wivagg, C. N., Kim, P. B., Silvis, M. R., Renedo, N., et al. (2017). Ribosomal mutations promote the evolution of antibiotic resistance in a multidrug environment. *Elife* 6, e20420. doi: 10.7554/elife.20420

Grandjean, L., Iwamoto, T., Lithgow, A., Gilman, R. H., Arikawa, K., Nakanishi, N., et al. (2015). The association between mycobacterium tuberculosis genotype and drug resistance in Peru. *PloS One* 10, e0126271. doi: 10.1371/journal.pone.0126271

Guerra-Assunção, J., Crampin, A., Houben, R., Mzembe, T., Mallard, K., Coll, F., et al. (2015). Large-Scale whole genome sequencing of m. tuberculosis provides

insights into transmission in a high prevalence area. *Elife* 4, e05166. doi: 10.7554/elife.05166

Gupta, R. S., Lo, B., and Son, J. (2018). Phylogenomics and comparative genomic studies robustly support division of the genus mycobacterium into an emended genus mycobacterium and four novel genera. *Front. Microbiol.* 9. doi: 10.3389/fmicb.2018.00067

Gygli, S. M., Loiseau, C., Jugheli, L., Adamia, N., Trauner, A., Reinhard, M., et al. (2021). Prisons as ecological drivers of fitness-compensated multidrug-resistant mycobacterium tuberculosis. *Nat. Med.* 27, 1171–1177. doi: 10.1038/s41591-021-01358-x

Hakamata, M., Takihara, H., Iwamoto, T., Tamaru, A., Hashimoto, A., Tanaka, T., et al. (2020). Higher genome mutation rates of Beijing lineage of mycobacterium tuberculosis during human infection. *Sci. Rep-uk* 10, 17997. doi: 10.1038/s41598-020-75028-2

Hartkoorn, R. C., Uplekar, S., and Cole, S. T. (2014). Cross-resistance between clofazimine and bedaquiline through upregulation of MmpL5 in mycobacterium tuberculosis. *Antimicrob. Agents Ch* 58, 2979–2981. doi: 10.1128/aac.00037-14

Haver, H. L., Chua, A., Ghode, P., Lakshminarayana, S. B., Singhal, A., Mathema, B., et al. (2015). Mutations in genes for the F420 biosynthetic pathway and a nitroreductase enzyme are the primary resistance determinants in spontaneous *In vitro*-selected PA-824-Resistant mutants of mycobacterium tuberculosis. *Antimicrob. Agents Ch* 59, 5316–5323. doi: 10.1128/aac.00308-15

Hershkovitz, I., Donoghue, H. D., Minnikin, D. E., Besra, G. S., Lee, O. Y.-C., Gernaey, A. M., et al. (2008). Detection and molecular characterization of 9000-Year-Old mycobacterium tuberculosis from a neolithic settlement in the Eastern Mediterranean. *PloS One* 3, e3426. doi: 10.1371/journal.pone.0003426

Huitric, E., Verhasselt, P., Koul, A., Andries, K., Hoffner, S., and Andersson, D. I. (2010). Rates and mechanisms of resistance development in mycobacterium tuberculosis to a novel diarylquinoline ATP synthase inhibitor. *Antimicrob. Agents Ch* 54, 1022–1028. doi: 10.1128/aac.01611-09

Imperial, M. Z., Nahid, P., Phillips, P. P. J., Davies, G. R., Fielding, K., Hanna, D., et al. (2018). A patient-level pooled analysis of treatment-shortening regimens for drug-susceptible pulmonary tuberculosis. *Nat. Med.* 24, 1708–1715. doi: 10.1038/s41591-018-0224-2

Ismail, N. A., Omar, S. V., Moultrie, H., Bhyat, Z., Conradie, F., Enwerem, M., et al. (2022). Assessment of epidemiological and genetic characteristics and clinical outcomes of resistance to bedaquiline in patients treated for rifampicin-resistant tuberculosis: a cross-sectional and longitudinal study. *Lancet Infect. Dis.* 22, 496–506. doi: 10.1016/s1473-3099(21)00470-9

Ismail, N., Rivière, E., Limberis, J., Huo, S., Metcalfe, J. Z., Warren, R. M., et al. (2021). Genetic variants and their association with phenotypic resistance to bedaquiline in mycobacterium tuberculosis: a systematic review and individual isolate data analysis. *Lancet Microbe* 2, e604–e616. doi: 10.1016/s2666-5247(21)00175-0

Johnson, E. O., Office, E., Kawate, T., Orzechowski, M., and Hung, D. T. (2020). Large-Scale chemical-genetic strategy enables the design of antimicrobial combination chemotherapy in mycobacteria. *ACS Infect. Dis.* 6, 56–63. doi: 10.1021/acsinfecdis.9b00373

Kana, B. D., Abrahams, G. L., Sung, N., Warner, D. F., Gordhan, B. G., Machowski, E. E., et al. (2010). Role of the DinB homologs Rv1537 and Rv3056 in mycobacterium tuberculosis. *J. Bacteriol* 192, 2220–2227. doi: 10.1128/jb.01135-09

Kay, G. L., Sergeant, M. J., Zhou, Z., Chan, J. Z.-M., Millard, A., Quick, J., et al. (2015). Eighteenth-century genomes show that mixed infections were common at time of peak tuberculosis in Europe. *Nat. Commun.* 6, 6717. doi: 10.1038/ncomms7717

Knight, G. M., Colijn, C., Shrestha, S., Fofana, M., Cobelens, F., White, R. G., et al. (2015). The distribution of fitness costs of resistance-conferring mutations is a key determinant for the future burden of drug-resistant tuberculosis: A model-based analysis. *Clin. Infect. Dis.* 61, S147–S154. doi: 10.1093/cid/civ579

Lieberman, T. D., Wilson, D., Misra, R., Xiong, L. L., Moodley, P., Cohen, T., et al. (2016). Genomic diversity in autopsy samples reveals within-host dissemination of HIV-associated mycobacterium tuberculosis. *Nat. Med.* 22, 1470–1474. doi: 10.1038/nm.4205

Manson, A. L., Cohen, K. A., Abeel, T., Desjardins, C. A., Armstrong, D. T., Barry, C. E., et al. (2017). Genomic analysis of globally diverse mycobacterium tuberculosis strains provides insights into the emergence and spread of multidrug resistance. *Nat. Genet.* 49, 395–402. doi: 10.1038/ng.3767

McGrath, M., van Pittius, N. C. G., van Helden, P. D., Warren, R. M., and Warner, D. F. (2014). Mutation rate and the emergence of drug resistance in mycobacterium tuberculosis. *J. Antimicrob. Chemoth* 69, 292–302. doi: 10.1093/jac/dkt364

Merker, M., Barbier, M., Cox, H., Rasigade, J.-P., Feuerriegel, S., Kohl, T. A., et al. (2018). Compensatory evolution drives multidrug-resistant tuberculosis in central Asia. *Elife* 7, e38200. doi: 10.7554/elife.38200

Metcalfe, J. Z., Streicher, E., Theron, G., Colman, R. E., Penaloza, R., Allender, C., et al. (2017). Mycobacterium tuberculosis subculture results in loss of potentially clinically relevant heteroresistance. *Antimicrob. Agents Ch* 61 (11): e00888–17. doi: 10.1128/aac.00888-17

Morlock, G. P., Metchock, B., Sikes, D., Crawford, J. T., and Cooksey, R. C. (2003). ethA , inhA , and katG loci of ethionamide-resistant clinical mycobacterium tuberculosis isolates. *Antimicrob. Agents Ch* 47, 3799–3805. doi: 10.1128/aac.47.12.3799-3805.2003

Ng, K. C. S., Supply, P., Cobelens, F. G. J., Gaudin, C., Gonzalez-Martin, J., de Jong, B. C., et al. (2019). How well do routine molecular diagnostics detect rifampin heteroresistance in mycobacterium tuberculosis? *J. Clin. Microbiol.* 57, e00717–e00719. doi: 10.1128/jcm.00717-19

Nimmo, C., Brien, K., Millard, J., Grant, A. D., Padayatchi, N., Pym, A. S., et al. (2020). Dynamics of within-host mycobacterium tuberculosis diversity and heteroresistance during treatment. *Ebiomedicine* 55, 102747. doi: 10.1016/j.ebiom.2020.102747

Nimmo, C., Shaw, L. P., Doyle, R., Williams, R., Brien, K., Burgess, C., et al. (2019). Whole genome sequencing mycobacterium tuberculosis directly from sputum identifies more genetic diversity than sequencing from culture. *BMC Genomics* 20, 389. doi: 10.1186/s12864-019-5782-2

O'Neill, M. B., Mortimer, T. D., and Pepperell, C. S. (2015). Diversity of mycobacterium tuberculosis across evolutionary scales. *PloS Pathog.* 11, e1005257. doi: 10.1371/journal.ppat.1005257

O'Neill, M. B., Shockey, A., Zarley, A., Aylward, W., Eldholm, V., Kitchen, A., et al. (2019). Lineage specific histories of mycobacterium tuberculosis dispersal in Africa and Eurasia. *Mol. Ecol.* 28, 3241–3256. doi: 10.1111/mec.15120

Operario, D. J., Koeppel, A. F., Turner, S. D., Bao, Y., Pholwat, S., Banu, S., et al. (2017). Prevalence and extent of heteroresistance by next generation sequencing of multidrug-resistant tuberculosis. *PloS One* 12, e0176522. doi: 10.1371/journal.pone.0176522

Ortiz, A. T., Coronel, J., Vidal, J. R., Bonilla, C., Moore, D. A. J., Gilman, R. H., et al. (2021). Genomic signatures of pre-resistance in mycobacterium tuberculosis. *Nat. Commun.* 12, 7312. doi: 10.1038/s41467-021-27616-7

O'Sullivan, D. M., McHugh, T. D., and Gillespie, S. H. (2008). The effect of oxidative stress on the mutation rate of mycobacterium tuberculosis with impaired catalase/peroxidase function. *J. Antimicrob. Chemoth* 62, 709–712. doi: 10.1093/jac/dkn259

Parwati, I., van Crevel, R., and van Soolingen, D. (2010). Possible underlying mechanisms for successful emergence of the mycobacterium tuberculosis Beijing genotype strains. *Lancet Infect. Dis.* 10, 103–111. doi: 10.1016/s1473-3099(09)70330-5

Pečerska, J., Kühnert, D., Meehan, C. J., Coscollá, M., Jong, B. C., Gagneux, S., et al. (2021). Quantifying transmission fitness costs of multi-drug resistant tuberculosis. *Epidemics-neth* 36, 100471. doi: 10.1016/j.epidem.2021.100471

Pym, A. S., Saint-Joanis, B., and Cole, S. T. (2002). Effect of katG mutations on the virulence of mycobacterium tuberculosis and the implication for transmission in humans. *Infect. Immun.* 70, 4955–4960. doi: 10.1128/iai.70.9.4955-4960.2002

Rigouts, L., Miotto, P., Schats, M., Lempens, P., Cabibbe, A. M., Galbiati, S., et al. (2019). Fluoroquinolone heteroresistance in mycobacterium tuberculosis: detection by genotypic and phenotypic assays in experimentally mixed populations. *Sci. Rep-uk* 9, 11760. doi: 10.1038/s41598-019-48289-9

Sanchez-Padilla, E., Dlamini, T., Ascorra, A., Rüsch-Gerdes, S., Tefera, Z. D., Calain, P., et al. (2012). High prevalence of multidrug-resistant tuberculosis, swazilan –2010. *Emerg. Infect. Dis.* 18, 29–37. doi: 10.3201/eid1801.110850

Sanchez-Padilla, E., Merker, M., Beckert, P., Jochims, F., Dlamini, T., Kahn, P., et al. (2015). Detection of drug-resistant tuberculosis by xpert MTB/RIF in Swaziland. *New Engl. J. Med.* 372, 1181–1182. doi: 10.1056/nejmc1413930

Scorpio, A., and Zhang, Y. (1996). Mutations in pncA, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. *Nat. Med.* 2, 662–667. doi: 10.1038/nm0696-662

Shaw, L. P., Doyle, R. M., Kavaliunaite, E., Spencer, H., Balloux, F., Dixon, G., et al. (2019). Children with cystic fibrosis are infected with multiple subpopulations of mycobacterium abscessus with different antimicrobial resistance profiles. *Clin. Infect. Dis. Off. Publ Infect. Dis. Soc. Am.* 69, 1678–1686. doi: 10.1093/cid/ciz069

Shockey, A. C., Dabney, J., and Pepperell, C. S. (2019). Effects of host, sample, and *in vitro* culture on genomic diversity of pathogenic mycobacteria. *Front. Genet.* 10. doi: 10.3389/fgene.2019.00477

Sikhondze, W., Dlamini, T., Khumalo, D., Maphalala, G., Dlamini, S., Zikalala, T., et al. (2015). Countrywide roll-out of xpert® MTB/RIF in Swaziland: the first three years of implementation. *Public Heal Action* 5, 140–146. doi: 10.5588/pha.15.0001

Silva, P. E. A. D., and Palomino, J. C. (2011). Molecular basis and mechanisms of drug resistance in mycobacterium tuberculosis: classical and new drugs. *J. Antimicrob. Chemoth* 66, 1417–1430. doi: 10.1093/jac/dkr173

Sonnenkalb, L., Carter, J., Spitaleri, A., Iqbal, Z., Hunt, M., Malone, K., et al. (2021). Deciphering bedaquiline and clofazimine resistance in tuberculosis: An evolutionary medicine approach. *Biorxiv*. doi: 10.1101/2021.03.19.436148 Available at: https://www.biorxiv.org/content/10.1101/2021.03.19.436148v4

Soolingen, D. V., Hoogenboezem, T., Haas, P. E. W. D., Hermans, P. W. M., Koedam, M. A., Teppema, K. S., et al. (1997). A novel pathogenic taxon of the mycobacterium tuberculosis complex, canetti: Characterization of an exceptional isolate from Africa. *Int. J. Syst. Evol. Micr* 47, 1236–1245. doi: 10.1099/00207713-47-4-1236

Stoffels, K., Mathys, V., Fauville-Dufaux, M., Wintjens, R., and Bifani, P. (2012). Systematic analysis of pyrazinamide-resistant spontaneous mutants and clinical isolates of mycobacterium tuberculosis. *Antimicrob. Agents Ch* 56, 5186–5193. doi: 10.1128/aac.05385-11

Sun, G., Luo, T., Yang, C., Dong, X., Li, J., Zhu, Y., et al. (2012). Dynamic population changes in mycobacterium tuberculosis during acquisition and fixation of drug resistance in patients. *J. Infect. Dis.* 206, 1724–1733. doi: 10.1093/infdis/jis601

Suzuki, Y., Katsukawa, C., Tamaru, A., Abe, C., Makino, M., Mizuguchi, Y., et al. (1998). Detection of kanamycin-resistant mycobacterium tuberculosis by identifying mutations in the 16S rRNA gene. *J. Clin. Microbiol.* 36, 1220–1225. doi: 10.1128/jcm.36.5.1220-1225.1998

Takiff, H. E., Salazar, L., Guerrero, C., Philipp, W., Huang, W. M., Kreiswirth, B., et al. (1994). Cloning and nucleotide sequence of mycobacterium tuberculosis gyrA and gyrB genes and detection of quinolone resistance mutations. *Antimicrob. Agents Ch* 38, 773–780. doi: 10.1128/aac.38.4.773

Telenti, A., Imboden, P., Marchesi, F., Matter, L., Schopfer, K., Bodmer, T., et al. (1993). Detection of rifampicin-resistance mutations in mycobacterium tuberculosis. *Lancet* 341, 647–651. doi: 10.1016/0140-6736(93)90417-f

Telenti, A., Philipp, W. J., Sreevatsan, S., Bernasconi, C., Stockbauer, K. E., Wieles, B., et al. (1997). The emb operon, a gene cluster of mycobacterium tuberculosis involved in resistance to ethambutol. *Nat. Med.* 3, 567–570. doi: 10.1038/nm0597-567

The CRyPTIC Consortium (2022). A data compendium associating the genomes of 12,289 mycobacterium tuberculosis isolates with quantitative resistance phenotypes to 13 antibiotics. *PloS Biol.* 20, e3001721. doi: 10.1371/journal.pbio.3001721

Tortoli, E., Brown-Elliott, B. A., Chalmers, J. D., Cirillo, D. M., Daley, C. L., Emler, S., et al. (2019). Same meat, different gravy: ignore the new names of mycobacteria. *Eur. Respir. J.* 54, 1900795. doi: 10.1183/13993003.00795-2019

Trauner, A., Banaei-Esfahani, A., Gygli, S. M., Warmer, P., Feldmann, J., Zampieri, M., et al. (2021). Expression dysregulation as a mediator of fitness costs in antibiotic resistance. *Antimicrob. Agents Ch* 65, e00504–e00521. doi: 10.1128/aac.00504-21

Trauner, A., Liu, Q., Via, L. E., Liu, X., Ruan, X., Liang, L., et al. (2017). The within-host population dynamics of mycobacterium tuberculosis vary with treatment efficacy. *Genome Biol.* 18, 71. doi: 10.1186/s13059-017-1196-0

Vargas, R., Freschi, L., Marin, M., Epperson, L. E., Smith, M., Oussenko, I., et al. (2021). In-host population dynamics of mycobacterium tuberculosis complex during active disease. *Elife* 10, e61805. doi: 10.7554/elife.61805

Vos, M., Ley, S. D., Wiggins, K. B., Derendinger, B., Dippenaar, A., Grobbelaar, M., et al. (2019). Bedaquiline micro-heteroresistance after tuberculosis treatment cessation. *New Engl. J. Med.* 380, 2178–2180. doi: 10.1056/nejmc1815121

Werngren, J., and Hoffner, S. E. (2003). Drug-susceptible mycobacterium tuberculosis Beijing genotype does not develop mutation-conferred resistance to rifampin at an elevated rate. *J. Clin. Microbiol.* 41, 1520–1524. doi: 10.1128/jcm.41.4.1520-1524.2003

Winstanley, C., O'Brien, S., and Brockhurst, M. A. (2016). Pseudomonas aeruginosa evolutionary adaptation and diversification in cystic fibrosis chronic lung infections. *Trends Microbiol.* 24, 327–337. doi: 10.1016/j.tim.2016.01.008

World Health Organization (2020) *Global tuberculosis report 2019*. Available at: https://www.who.int/publications/i/item/9789241565714 (Accessed April 27, 2022).

World Health Organization (2022) *Global tuberculosis programme: tuberculosis data*. Available at: https://www.who.int/teams/global-tuberculosis-programme/data (Accessed April 27, 2022).

Youmans, G. P., Williston, E. H., Feldman, W. H., and Hinshaw, H. C. (1946). Increase in resistance of tubercle bacilli to streptomycin; a preliminary report. *Proc. Staff Meet Mayo Clin.* 21, 126.

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 17 October 2022
DOI 10.3389/fcimb.2022.997283

OPEN ACCESS

EDITED BY
Ben Gold,
Weill Cornell Medicine, United States

REVIEWED BY
Thomas Dick,
Hackensack Meridian Health, United States
Sangeeta Tiwari,
The University of Texas at El Paso, United States
Christopher Ealand,
University of the Witwatersrand, South Africa

*CORRESPONDENCE
Jeremy M. Rock
rock@rockefeller.edu

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 18 July 2022
ACCEPTED 30 September 2022
PUBLISHED 17 October 2022

CITATION
Poulton NC and Rock JM (2022) Unraveling the mechanisms of intrinsic drug resistance in *Mycobacterium tuberculosis*. *Front. Cell. Infect. Microbiol.* 12:997283. doi: 10.3389/fcimb.2022.997283

Unraveling the mechanisms of intrinsic drug resistance in *Mycobacterium tuberculosis*

Nicholas C. Poulton and Jeremy M. Rock*

Laboratory of Host-Pathogen Biology, The Rockefeller University, New York, NY, United States

Tuberculosis (TB) is among the most difficult infections to treat, requiring several months of multidrug therapy to produce a durable cure. The reasons necessitating long treatment times are complex and multifactorial. However, one major difficulty of treating TB is the resistance of the infecting bacterium, *Mycobacterium tuberculosis* (Mtb), to many distinct classes of antimicrobials. This review will focus on the major gaps in our understanding of intrinsic drug resistance in Mtb and how functional and chemical-genetics can help close those gaps. A better understanding of intrinsic drug resistance will help lay the foundation for strategies to disarm and circumvent these mechanisms to develop more potent antitubercular therapies.

KEYWORDS

tuberculosis, intrinsic resistance, chemical genetics, drug repurposing, drug discovery

Introduction

Mycobacterium tuberculosis (Mtb) infection is notoriously difficult to treat. Standard treatment regimens for drug sensitive tuberculosis (TB) typically last for 6 months and involve combination therapy with 2-4 antibiotics, depending on the stage of treatment (Dorman et al., 2021; WHO, 2021). Even with 6 months of chemotherapy, 5-10% of patients may experience disease relapse (Lambert et al., 2003; Merle et al., 2014; Colangeli et al., 2018). The difficulty of treating TB can be attributed to a multitude of factors including variable drug penetration into infected lesions (Dartois, 2014; Lenaerts et al., 2015) and treatment lapses due to toxic drug side effects (Tostmann et al., 2008; Seddon et al., 2012; Si et al., 2018; Conradie et al., 2020). However, a major contributor to the difficulty of treating TB is the problem of bacterial drug resistance, which can broadly be classified into two main categories: intrinsic drug resistance and acquired drug resistance (Walker et al., 2015; Xu et al., 2017; Batt et al., 2020). Bacterial drug resistance is phenotypically distinct from drug tolerance and persistence (Brauner et al., 2016; Balaban et al., 2019), which will not be reviewed here.

Intrinsic resistance refers to an innate property of a bacterial species that renders an antibacterial, or group of antibacterials, less effective (Blair et al., 2015;

Peterson and Kaur, 2018). Importantly, intrinsic resistance mechanisms are usually present in all (or almost all) members of a bacterial species. In some cases, genes imparting intrinsic resistance appear to have evolved specifically for protection against antibacterial compounds (Madsen et al., 2005; Liu et al., 2019). For example, Mtb encodes *erm(37)*, a 23S rRNA methyltransferase that protects the ribosome from macrolide, lincosamide, and streptogramin binding (Madsen et al., 2005). *erm(37)* does not have a known role in bacterial growth, virulence, or stress tolerance and likely evolved to protect ancestral, soil-dwelling actinobacteria against ribosome-targeting natural products produced by themselves or their neighbors (Morris et al., 2005). In other cases, genes essential for microbial growth and virulence can contribute to intrinsic drug resistance (Tan et al., 2012; Batt et al., 2020; Dulberger et al., 2020). For example, many essential genes in Mtb are involved in cell envelope biosynthesis and regulation. The Mtb cell envelope protects Mtb from host immune pressure and serves as a selective barrier to antibiotic penetration (Johnson et al., 2019; Peterson et al., 2021).

Acquired drug resistance refers to antibiotic resistance that evolves through specific chromosomal mutations or horizontal gene transfer (Peterson and Kaur, 2018; Evans et al., 2020). In Mtb all acquired drug resistance arises as a result of mutation since there is no evidence for recent horizontal gene transfer in Mtb (Boritsch et al., 2016). Many of the mutations that confer high-level acquired drug resistance in Mtb have been well studied and characterized (Walker et al., 2015; CRyPTIC Consortium, 2018; Hunt et al., 2019). For example, partial loss-of-function mutations in the isoniazid (INH)-activating enzyme *katG* are the primary mechanism by which INH resistance emerges (Zhang et al., 1992). Rifampicin resistance emerges primarily through point mutations in the rifampicin resistance determining region on the beta subunit of RNA polymerase (*rpoB*) (Yamada et al., 1985; Telenti et al., 1993). Although many resistance-conferring mutations have been identified over the years, there is a growing appreciation for drug resistance mutations that fall outside the drug activator or target and which typically confer low-to-intermediate resistance (Wong et al., 2011; Colangeli et al., 2018; Hicks et al., 2020). Such low-to-intermediate resistance is clinically relevant (Colangeli et al., 2018) but much more poorly understood. While there remains much to be explored regarding acquired drug resistance in Mtb, this is a topic covered extensively in other reviews (including reviews in this series) and will not be a major focus here.

This review will first briefly outline existing methods used to define intrinsic drug resistance mechanisms in Mtb. We will then review our current understanding and knowledge gaps of intrinsic drug resistance in Mtb and highlight how functional and chemical-genetics (Sassetti et al., 2001; Kim et al., 2013; Bosch et al., 2021) can help close those gaps. We end with a brief discussion on how existing genetic approaches could be expanded to further intrinsic drug resistance research.

Chemical-genetic approaches to define intrinsic drug resistance mechanisms in Mtb

Chemical-genetic studies have been a pillar of biology for decades. This vast body of literature covers studies of species from all three domains of life and serves as a rich resource for understanding basic biology as well as informing drug discovery efforts (Parsons et al., 2006; Lehár et al., 2008; Nichols et al., 2011; Brown et al., 2014; Cacace et al., 2017; Antonova-Koch et al., 2018). Broadly speaking, chemical-genetics is the study of how genetic alterations influence the activity of a chemical compound. The simplest form of chemical-genetics relies on spontaneous mutagenesis to study the relationships between genes and drugs. In a given population of bacteria, drug resistance mutations can arise spontaneously at a low frequency and can be isolated by plating on a selective antibiotic concentration (Luria and Delbrück, 1943; Jin and Gross, 1988). Genomes of drug-resistant clones can then be sequenced to determine the mutations causing drug resistance. This simple yet elegant approach has been used for decades to identify some of the most common mechanisms of antibiotic resistance.

Other applications of chemical-genetics in Mtb rely on active disruption of target genes (Figure 1). One such technique, transposon mutagenesis, involves phage-mediated transduction and integration of a mariner transposon at random TA dinucleotide sequences in the Mtb genome (Sassetti et al., 2001; Dejesus et al., 2017). As typically used in Mtb, this approach results in the irreversible inactivation of target genes and is thus restricted to the analysis of *in vitro* non-essential genes as mutants for *in vitro* essential genes are lost during library construction. Despite the strong GC bias in the Mtb genome, the overwhelming majority of Mtb genes are sufficiently susceptible to transposition for this technique to work efficiently at genome scale (Dejesus et al., 2017). Transposon sequencing (TnSeq) has been used to study chemical-genetic interactions in axenic culture (Xu et al., 2017; Furió et al., 2021; Thiede et al., 2022) as well as in macrophage and mouse models of infection (Bellerose et al., 2020; Kreutzfeldt et al., 2022).

More recently, genetic techniques have been developed that are more applicable to the study of *in vitro* essential Mtb genes. One such technique relies on a regulated proteolysis system, wherein the protein of interest is tagged with a C-terminal "degron" that is recognized by a tetracycline-regulated proteolytic adapter. Upon

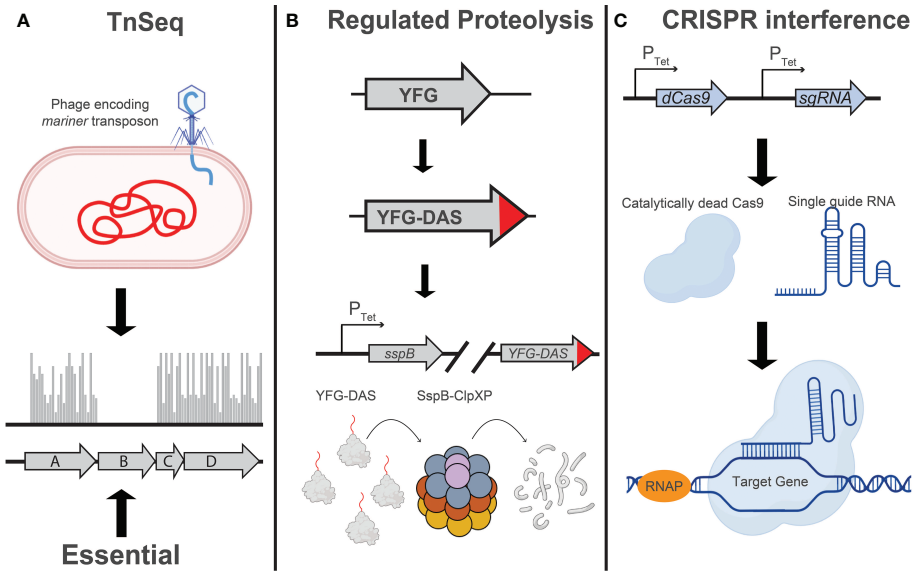


FIGURE 1
Techniques for large-scale genetic studies in Mtb: Illustration depicting the principles of **(A)** TnSeq, **(B)** regulated proteolysis, and **(C)** CRISPR interference as implemented in mycobacteria. **(A)** Transposon sequencing (TnSeq) involves transduction of Mtb with a phage encoding a mariner transposon, which will randomly insert at TA dinucleotides in the Mtb genome (Sassetti et al., 2001; Dejesus et al., 2017). The absence of transposon insertion events, as determined by next generation sequencing, indicates the essentiality of that gene under the conditions assayed. **(B)** Regulated proteolysis involves the addition of a C-terminal "DAS" tag to an endogenous Mtb gene (Kim et al., 2013; Johnson et al., 2019). Addition or removal of tetracycline, depending on the variant of TetR being used, will induce expression of the proteolytic adaptor, SspB, and facilitate ClpXP-mediated degradation of the DAS-tagged protein. The level of target knockdown can be modulated by using different Tet-regulated promoters to drive *sspB* expression. **(C)** CRISPR interference (CRISPRi) utilizes a single guide RNA (sgRNA) to localize a nuclease-dead Cas9 enzyme to a specific target sequence in the Mtb genome, sterically blocking transcription initiation or elongation. Modulation of target knockdown level can be achieved by designing an sgRNA targeting a divergent protospacer adjacent motif (PAM) sequence, which is directly adjacent to the sgRNA target sequence (Rock et al., 2017), or modulating the extent of complementarity between the sgRNA and the DNA target (Qi et al., 2013; Vigouroux et al., 2018; Hawkins et al., 2020; Bosch et al., 2021; Mathis et al., 2021). Figure graphics were generated using Biorender software.

addition or removal of tetracycline (depending on the variant of the TetR used in the study), the *sspB* adapter is expressed and the corresponding protein is degraded (Kim et al., 2013). In a tour de force, Johnson et al. used a barcoded library of Mtb degron mutants coupled with next generation sequencing to profile over 50,000 compounds to identify target-compound chemical-genetic interactions. The authors identify the putative molecular target for over 40 of these compounds, some of which are active against novel therapeutic targets such as the essential efflux pump *efpA* (Johnson et al., 2019). Throughout the review we will use the term "degron libraries" to refer to this regulated proteolysis technique.

Blending some of the attractive capabilities of both TnSeq and the degron approach, CRISPR interference (CRISPRi) has been used by several labs, including our own, to perform targeted transcriptional inhibition of essential and non-essential genes (Choudhary et al., 2015; de Wet et al., 2020; Bosch et al., 2021; McNeil et al., 2021). This technique leverages the targeting specificity of CRISPR-Cas systems to localize a catalytically dead Cas9 protein to a gene of interest, serving as a steric block to transcription (Qi et al., 2013; Peters et al., 2016). Recently, we have used this system at genome-scale to profile a select group of antitubercular drugs (Li et al., 2022).

The mycobacterial envelope as a first line of intrinsic antibiotic resistance

It has long been appreciated that mycobacteria have a high level of intrinsic resistance to a diverse set of antibiotics (Jarlier and Nikaido, 1994; Gygli et al., 2017; Xu et al., 2017; Batt et al., 2020; Dulberger et al., 2020). This phenotype has generally been attributed to the relative impermeability of the mycobacterial envelope, which is distinct from those of classic Gram-negative or Gram-positive bacterial species. The Mtb envelope is a complex network composed primarily of peptidoglycan, arabinogalactan, and mycolic acids, which we will refer to as the mAGP complex. At the innermost layer closest to the plasma membrane is the peptidoglycan (PG), which is itself covalently linked to a network of arabinogalactan (AG) polymers (Dulberger et al., 2020). Connected to the AG by esterification is a thick layer of long-chain fatty acids called mycolic acids which form a pseudo-outer membrane bilayer known as the mycobacterial outer membrane (MOM) or mycomembrane (Jankute et al., 2015). Interspersed in the mycolic acids are a

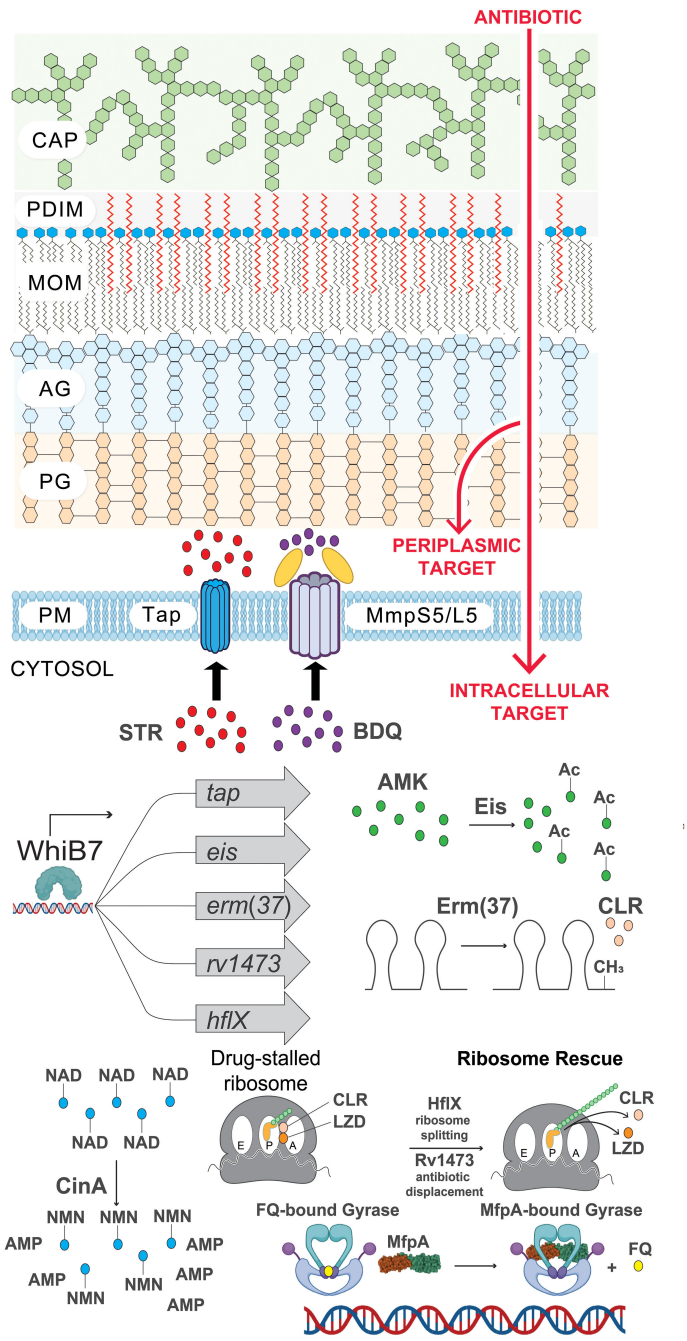


FIGURE 2
The many layers of intrinsic antibiotic resistance in Mtb. Illustration of intrinsic resistance factors at the Mtb cell surface and inside the cytosol. CAP, capsule; PDIM, phthiocerol dimycocerosates; MOM, mycobacterial outer membrane; AG, arabinogalactan; PG, peptidoglycan; PM, plasma membrane; STR, streptomycin; BDQ, bedaquiline; AMK, amikacin; Ac, acetylation modification (CH_3CO); CLR, clarithromycin; CH_3, methylation of ribosomal RNA; LZD, linezolid; FQ, fluoroquinolone; NAD, nicotinamide adenine dinucleotide (depicted as a drug adduct); NMN, nicotinamide mononucleotide; AMP, adenosine monophosphate. Figure graphics were generated using Biorender software.

select group of proteins including porin-like proteins (Siroy et al., 2008; Wang et al., 2020) and secretion systems (Ates et al., 2015; Tiwari et al., 2020), as well as virulence associated glycolipids such as phthiocerol dimycocerosates (PDIMs) (Rens et al., 2021). Outside of the MOM is a mycobacterial capsule which is composed primarily of complex carbohydrates such as α-D-glucan and D-arabino-D-mannan, but also a select group of lipids and proteins (Stokes et al., 2004; Kalscheuer et al., 2019).

Due to the typical growth conditions used in mycobacterial media, the capsule is generally stripped from the Mtb cell surface and is not studied during axenic growth (Stokes et al., 2004). The implications of this fact will be discussed later in this review.

The intrinsic resistance of Mtb to many different classes of antibiotics is often attributed to the impermeability of the MOM (Jarlier and Nikaido, 1994; Batt et al., 2020). Hydrophilic solutes are unable to traverse the mycolic acids and are thought to rely on protein-mediated translocation *via* porin-like proteins (Jarlier and Nikaido, 1994; Ates et al., 2015; Batt et al., 2020; Wang et al., 2020). Hydrophobic compounds are thought to get stuck in a poorly fluid mycolic acid sink and fail to efficiently traverse the MOM. There are substantial data to support this model using both chemical and genetic disruption of the mycolic acid network to potentiate antibiotic uptake and activity (Liu and Nikaido, 1999; Larrouy-Maumus et al., 2016; Xu et al., 2017). The clinical implications of this phenomenon can be seen by the synergistic interaction between rifampicin, which inhibits RNA polymerase (Campbell et al., 2001), and ethambutol, which inhibits arabinogalactan and lipoarabinomannan (LAM) biosynthesis (Goude et al., 2009; McNeil et al., 2019; Zhang et al., 2020). Because arabinogalactan serves as an anchor for the mycolic acid layer, AG inhibitors like ethambutol also perturb the MOM (Kilburn and Takayama, 1981; Mikusová et al., 1995; Dulberger et al., 2020). Despite its relatively modest *in vitro* and *in vivo* activity, ethambutol is included as part of the first-line RIPE (**r**ifampicin, **i**soniazid, **p**yrazinamide, **e**thambutol) regimen for drug sensitive TB. It has previously been suggested that ethambutol mainly serves as a "safety net" to prevent the emergence of rifampicin and isoniazid resistant TB (Dubé et al., 1997). More recently, the clinical importance of ethambutol has been attributed to the efficient distribution of this drug throughout TB lung lesions (Zimmerman et al., 2017). In addition to these roles, ethambutol's clinical success may be due to its synergistic interaction with rifampicin (Cokol et al., 2017; McNeil et al., 2019). Rifampicin is a hydrophobic, high molecular weight compound for which the mycobacterial envelope serves as a permeability barrier (Xu et al., 2017; McNeil et al., 2019; Li et al., 2022). By disrupting proper formation of arabinogalactan, ethambutol promotes more efficient uptake of rifampicin to exert its bactericidal effect (Cokol et al., 2017; McNeil et al., 2019).

Despite the long-standing appreciation that bacterial surface structures can impede antibiotic uptake, the physiochemical details of this phenomenon are not fully understood in mycobacteria. Chemical-genetic studies have shown that mAGP-related mutants in Mtb are hypersusceptible to certain antibiotics but not others, suggesting that the cell envelope is a relevant barrier for certain drugs such as rifampicin and bedaquiline, but not other drugs like linezolid (Davis et al., 2014; Larrouy-Maumus et al., 2016; Li et al., 2022). In the latter scenario it is unclear whether there are specific importers for these drugs (Rempel et al., 2020; Li et al., 2022) or whether drug diffusion is unaffected by the envelope. While compound size certainly seems to negatively affect uptake beyond a certain threshold (Davis et al., 2014; Li et al., 2022), the physiochemical properties that allow some compounds but not others to easily traverse the Mtb envelope are not fully established. Future chemical-genetic studies could be focused on profiling mAGP-associated mutants against a physiochemically diverse set of antitubercular compounds, or in practice any compounds for which uptake can be quantitatively monitored, to identify which chemical scaffolds are efficiently blocked by the Mtb envelope (Davis et al., 2014; Zgurskaya and Rybenkov, 2019). This approach can be achieved using existing genome-scale CRISPRi libraries (Bosch et al., 2021). Alternatively, more targeted libraries (degron or CRISPRi) can be generated to specifically target mAGP-associated genes and profile the susceptibilities of each mutant. These screens could help reveal which physiochemical properties are associated with the ability or inability to traverse the mycobacterial envelope and help to define the "rules" of drug uptake in mycobacteria (Davis et al., 2014; Larrouy-Maumus et al., 2016; Zgurskaya and Rybenkov, 2019; Zhao et al., 2020).

At the same time, such screens could also reveal how different molecular structures within the envelope serve as a barrier to antibiotic uptake. Although often viewed as a unitary structure, the mAGP network is remarkably complex and disrupting different components of this structure may differentially sensitize Mtb to particular compounds. For example, knockdown of many arabinogalactan and mycolic acid biosynthetic enzymes seems to potentiate the activity of bedaquiline (Lupien et al., 2018; Li et al., 2022). However, for reasons that remain unclear, this effect is not observed as strongly with disruption of peptidoglycan biosynthetic enzymes (Li et al., 2022). Are these differential phenotypes simply a result of genetic redundancy in peptidoglycan biosynthesis, or do they reflect some degree of barrier specificity for different envelope structures?

While chemical-genetic interactions can help inform which physiochemical properties and potentially which envelope structures are most important for intrinsic drug resistance, there are several limitations. For example, many antitubercular compounds target envelope biosynthesis either directly (Banerjee et al., 1994; Goude et al., 2009) or indirectly (Stover et al., 2000; Thiede et al., 2022). Let's assume that a CRISPRi knockdown strain against the essential arabinogalactan biosynthetic enzyme *dprE1* renders Mtb more sensitive to a given compound. There are several potential explanations to explain this interaction. First, DprE1 or another target involved in arabinogalactan biosynthesis may be the direct target of the screened compound (Kumar et al., 2018). Second, lack of *dprE1* activity may weaken the arabinogalactan layer sufficiently to increase envelope permeability and compound uptake. Third, the chemical-genetic interaction may be independent of compound uptake and reflect a more mechanism-specific

collateral vulnerability associated with *dprE1* inhibition and arabinogalactan biosynthesis perturbation (Wang et al., 2019). Therefore, care should be exercised when interpreting chemical-genetic interactions and such studies should be coupled with mass spectrometry drug-uptake quantification to differentiate between these various possibilities (Davis et al., 2014; Planck and Rhee, 2021). Further, structural and/or biochemical approaches can be used to identify the target of a particular compound, helping to differentiate between a direct or an indirect mechanism for a specific chemical-genetic interaction (Pellecchia et al., 2002; Zhang et al., 2020; Ottavi et al., 2022).

Moreover, growth of Mtb in axenic culture often ignores two key components of the mycobacterial cell surface. PDIMs are a family of lipids involved in Mtb virulence, with over 1% of the Mtb genome dedicated to PDIM biosynthetic genes (Trivedi et al., 2005; Domenech and Reed, 2009; Rens et al., 2021). Because of the metabolic costs associated with synthesizing PDIMs and the fact that these lipids are not only dispensable in standard axenic culture but can restrict permeability of culture carbon sources (Ates et al., 2015; Wang et al., 2020), lab-grown Mtb frequently sustains loss of function mutations in PDIM biosynthetic enzymes (Domenech and Reed, 2009). The lack of PDIMs has been associated with increased sensitivity to drugs and altered nutrient uptake, consistent with PDIMs being a relevant permeability barrier in Mtb (Soetaert et al., 2015; Wang et al., 2020). Therefore, care should be taken to assess the role of PDIM in compound uptake. Lastly, mycobacteria are frequently cultured in the presence of detergent to prevent cell clumping. Detergents act by stripping the mycobacterial capsule, which may influence Mtb's small molecule permeability (Stokes et al., 2004; Kalscheuer et al., 2019). Therefore, as with PDIM, confirmation of relevant chemical-genetic interactions should be performed under conditions in which the Mtb capsule is intact (e.g. infection models, detergent-free plates, etc.).

The Mtb envelope is not a static structure and is influenced by the growth environment of the bacteria (Sarathy et al., 2013; Larrouy-Maumus et al., 2016; Koh et al., 2022). For example, Sarathy et al. demonstrated that non-replicating and nutrient-starved bacteria display greatly reduced drug uptake, likely through a cell wall remodeling process that is not entirely understood (Cunningham and Spreadbury, 1998; Wu et al., 2016). Perturbing envelope integrity may play a greater role in increasing compound uptake in Mtb grown under these conditions than standard replicating conditions (Sarathy et al., 2013). Further, Koh et al. showed that rifampicin is less effective when Mtb is grown on the *in vivo*-relevant carbon source cholesterol as a result of modifications to the Mtb envelope (Koh et al., 2022). This effect could be specifically reversed through selective cell envelope disruption. These studies highlight the importance of performing chemical-genetic screens in host-relevant carbon sources and stress conditions. This will help identify environments in which successful cell envelope disruption will facilitate antibiotic entry.

Having a more complete understanding of the mycobacterial envelope as a barrier to antibiotic uptake will pave the way for several important applications. First, this knowledge can be used to better predict synergistic drug combinations and inform the preclinical testing of new combination therapies. For example, several studies have shown that bedaquiline can be potentiated by inhibiting proper mAGP synthesis (Lechartier et al., 2012; Lupien et al., 2018; Li et al., 2022). Part of the success of the bedaquiline, pretomanid, and linezolid (BPaL) combination (Conradie et al., 2020) may be due to the disruption of mycolic acids by pretomanid (Stover et al., 2000; Manjunatha et al., 2009), resulting in increased bedaquiline uptake. This synergy would likely extend to pre-clinical DprE1 inhibitors (Lechartier et al., 2012; Lupien et al., 2018). Second, knowledge of the genetic regulation of cell envelope synthesis will allow for the rational prioritization of target-based drug discovery candidates. Ideally, these studies will identify targets for which inhibition not only leads to bacterial death but also potentiates the uptake and activity of other drugs. Third, as mentioned above, knowledge of the physiochemical "rules" that allow compounds to traverse the mAGP will help direct medicinal chemistry efforts to improve compound uptake. Lastly, this knowledge can be used to identify mechanism-specific synergies that target different components of the mAGP complex. Isoniazid and ethambutol have been used together in first-line TB therapy for decades but have been shown to be slightly antagonistic or additive at best (Cokol et al., 2017; Larkins-Ford et al., 2021). Chemical-genetic profiling of cell envelope-targeting compounds may help to reveal other targets, either in the same pathway or parallel pathways, that will act synergistically, thus optimizing the therapeutic potential of this highly vulnerable chemical complex.

Efflux pumps as the next line of defense against antibiotics

The selective permeability of the mycobacterial envelope collaborates with additional mechanisms to promote intrinsic drug resistance (Nikaido, 1994). Once a chemical compound traverses the mAGP, it can encounter another line of defense in the form of drug efflux pumps (Piddock et al., 2000; Davis et al., 2014; Laws et al., 2022). Efflux pumps are transmembrane proteins that facilitate the transport of small molecules out of the periplasm and/or the cytosol (Blair et al., 2015; Venter et al., 2015; Boyer et al., 2022). Mtb encodes several dozen putative and validated drug efflux pumps, which have been comprehensively reviewed by Laws et al. (Laws et al., 2022). Some efflux pumps, such as those of the ATP Binding Cassette (ABC) family, are regulated by ATP hydrolysis (Braibant et al., 2000; Pasca et al., 2004), whereas major facilitator superfamily (MFS) efflux pumps are regulated by proton-induced conformational changes (Li

et al., 2017; Laws et al., 2022). Other pumps, such as those of the resistance-nodulation-cell-division (RND) superfamily rely on a drug-proton antiporter mechanism (Venter et al., 2015; Li et al., 2017; Laws et al., 2022). The efflux pumps of Mtb vary greatly in their compound specificity with some having a single validated transported substrate and others having many substrates (Liu et al., 2019).

The clinical importance of drug efflux in Mtb has been well established. For example, the MmpS5/L5 efflux pump (RND superfamily) has been shown to be active against several drugs including bedaquiline and clofazimine (Hartkoorn et al., 2014; Briffotaux et al., 2017). Expression of the *mmpS5/L5* operon is negatively regulated by the transcriptional repressor MmpR (Rv0678) (Briffotaux et al., 2017). Loss of function mutations in *rv0678* result in constitutive MmpS5/L5 expression and confer acquired drug resistance to bedaquiline, representing a significant complication to the long-term success of this new TB drug (de Vos et al., 2019; Nimmo et al., 2020). Interestingly, for reasons that remain unclear, some clinical Mtb strains harbor loss-of-function *rv0678* mutations that pre-date the clinical use of bedaquiline (Villellas et al., 2017). The presence of these mutations could reflect earlier clinical exposure to clofazimine or other drugs. Another example is Rv1258c (Tap), an MFS efflux pump active against several antituberculars including streptomycin and rifampicin (Adams et al., 2011; Adams et al., 2019; Liu et al., 2019). Tap expression is activated by the transcription factor *whiB7*. WhiB7 or genes involved in its regulation can in turn sustain mutations that result in constitutive activation of the WhiB7 regulon, including Tap, to promote acquired drug resistance (Reeves et al., 2013; Schrader et al., 2021; Li et al., 2022). Thus, efflux pumps like MmpS5/L5 and Tap promote intrinsic resistance in Mtb and can further be augmented by mutation to promote acquired drug resistance.

Most validated or putative efflux pumps in Mtb are poorly characterized, but it is likely that efflux pumps beyond MmpS5/L5 and Tap contribute to intrinsic drug resistance in Mtb (Remm et al., 2022). An in-depth characterization of these under-studied efflux pumps is much needed (Szumowski et al., 2012). To facilitate this characterization, one could systematically generate underexpression and overexpression strains for all predicted Mtb efflux pumps. For example, a small, targeted CRISPRi library (Bosch et al., 2021) could be generated that contains knockdown strains for all validated and predicted efflux pumps. This library could be treated with a wide range of antitubercular compounds to determine which mutants display reduced fitness under which drug treatment conditions. Some efflux pumps may overlap in the types of compounds transported (Smith and Blair, 2014). To address this possibility, combinatorial libraries in which multiple efflux pump genes are simultaneously silenced (Wong and Rock, 2021; Li et al., 2022) may help to identify functional redundancies between efflux pumps. Further, because some efflux pumps may not be highly expressed under standard lab conditions (Gupta et al., 2010; Adams et al., 2011), knocking down the corresponding gene may not produce a phenotype. To overcome this, a parallel pool of barcoded overexpression strains for each efflux pump could be generated and screened against the same panel of antitubercular compounds (Hicks et al., 2018). Lastly, as in the case for MmpS5/L5 and Tap, one could interrogate the increasing amount of Mtb clinical strain genome sequencing available to identify predicted efflux pumps or their regulators under positive selection. Should such evidence exist, it seems reasonable to predict that the relevant selective pressure is antibiotics, although other mechanisms cannot be ruled out.

In designing these experiments, it will be important to carefully curate the list of predicted efflux pumps. For example, several ABC proteins have been annotated as efflux pumps even though these proteins lack transmembrane helices (Duan et al., 2019; Laws et al., 2022). Two such previously annotated efflux pumps, Rv1473 and EttA, turn out to indeed influence drug activity in Mtb but have nothing to do with efflux and rather are ATP-dependent regulators of the ribosome (Sharkey et al., 2016; Cui et al., 2022; Li et al., 2022). Furthermore, genetic studies of efflux pumps should be validated with biochemical approaches to unambiguously demonstrate drug efflux activity.

Given the role of drug efflux in intrinsic and acquired Mtb drug resistance, there has been considerable interest in developing efflux pump inhibitors (EPIs) to potentiate TB drug regimens. Numerous small molecule EPIs, including both natural products and synthetic compounds, have been described (Szumowski et al., 2012; Machado et al., 2016; Laws et al., 2022). Many of these EPIs show broad activity against numerous efflux pumps and appear to act in a relatively non-specific manner by disrupting membrane energetics (Adams et al., 2014; Ruth et al., 2020; Laws et al., 2022). Indeed, many EPIs have antimycobacterial activity as single agents, which could reflect synthetic lethality of multi-efflux pump inhibition or an efflux pump independent mode of action, e.g. disruption of membrane energetics (Chen et al., 2018; Remm et al., 2022). The pleiotropic consequence of disrupting membrane energetics may confound the interpretation how EPIs potentiate the activity of other TB drugs (Amaral and Viveiros, 2017; Chen et al., 2018). Despite their unclear mode of action, some EPIs may have potential for use in TB therapy. For example, there are several studies showing that the antipsychotic drug thioridazine has direct antitubercular activity (Amaral et al., 2001; Abbate et al., 2012; Pieroni et al., 2015). Thioridazine also displays synergy with other drugs, possibly by altering membrane potential and reducing drug efflux (Coelho et al., 2015; Machado et al., 2016; Ruth et al., 2020). Although there is no evidence of direct efflux pump inhibition by thioridazine, its ability to disrupt membrane potential may lower cellular ATP levels, thereby limiting the activity of ATP-dependent efflux pumps. Alternatively, or in addition, thioridazine-mediated disruption of proton gradients may alter the ability of MFS efflux pumps to undergo proton-

induced conformational changes or of RND family efflux pumps to carry out drug-proton antiport exchange.

Ultimately, while non-specific EPIs may have clinical value, specific and selective EPIs could help augment TB treatment. However, substantial advances in our understanding of efflux pump specificity and structure may be required to identify such compounds. Until then, more generic EPIs, especially those that have stand-alone antitubercular activity, may be of utility for TB treatment (Abbate et al., 2012; Rodrigues et al., 2020).

Beyond drug uptake and efflux: Cytosolic mechanisms of intrinsic antibiotic resistance

An intrepid compound has traversed the mycobacterial envelope, avoided efflux, and is ready to engage its target. What next? Once again, Mtb is well-equipped with numerous cytosolic mechanisms of intrinsic drug resistance. Once again, Mtb is well-equipped with numerous cytosolic mechanisms of intrinsic drug resistance (Figure 2). (Blair et al., 2015). Not surprisingly, these processes tend to be more drug-specific than selective envelope permeability and efflux, and based on current knowledge are most frequently seen with the antituberculars which target the ribosome (Wilson, 2014). Some of the most well-studied mechanisms of cytosolic intrinsic drug resistance in Mtb are listed in Table 1.

The different layers of intrinsic cytosolic resistance can all be seen within the *whiB7* pathway. WhiB7 is a transcription factor that senses translational stalling which can be triggered by ribosome stress during drug treatment, host-derived stressors, and poorly characterized metabolic changes (Morris et al., 2005; Burian et al., 2013). During unstressed conditions, *whiB7* expression is low due to upstream ORF (uORF)-mediated transcription attenuation (Lee et al., 2022). Translation of the uORF in the *whiB7* 5' leader fails to prevent formation of a Rho-independent terminator, resulting in transcription termination prior to transcription of the *whiB7* ORF. However, stalled translation of the uORF promotes formation of an antiterminator, resulting in high-level transcription of the *whiB7* ORF. This subsequently further activates transcription from the *whiB7* promoter and those of the WhiB7 regulon genes. Among the WhiB7 regulon is *tap*, the multidrug efflux pump described in the previous section. Furthermore, WhiB7 promotes the transcription of several cytosolic resistance factors including *eis*, an aminoglycoside acetyltransferase that can chemically modify and inactivate amikacin and kanamycin (drug modification) (Zaunbrecher et al., 2009). Another WhiB7 regulon gene is *erm(37)*, a ribosomal RNA methyltransferase which modifies the macrolide binding site on the 23S rRNA to prevent drug binding (target modification) (Madsen et al., 2005). Moreover, WhiB7 promotes transcription of *hflX*, a ribosome recycling factor that can help to rescue stalled ribosomes (target rescue) (Rudra et al., 2020). This WhiB7 pathway presumably evolved in an ancestral soil-dwelling actinobacterium that encountered ribosome-targeting antibiotics in its environment.

Expanding our knowledge of cytosolic intrinsic resistance factors & how to overcome them

Compared to the hundreds of genes which contribute to intrinsic resistance by regulating cell envelope processes (Xu et al., 2017; Li et al., 2022), there are many fewer known instances of resistance factors in the Mtb cytosol. This can likely be explained by the fact that cell envelope-associated intrinsic resistance factors are likely to be pleiotropic by preventing the uptake of many diverse compounds, whereas cytosolic resistance factors are likely to be specific to a particular drug or class of drugs. The relative paucity of known cytosolic resistance factors may also be explained by the limited scope of the drugs that have been screened in chemical-genetic studies. Future chemical-genetic screening efforts could focus on

TABLE 1 Cytosolic intrinsic drug resistance factors of Mtb.

Resistance Gene	Protection specificity	Mechanism	Reference
whiB7 (*rv3197A*)	Ribosome-targeting antibiotics	Transcription of other resistance factors	(Morris et al., 2005)
erm(37) (*rv1988*)	Macrolides, lincosamides, streptogramin B antibiotics	Methylation of the 23S rRNA drug binding site	(Madsen et al., 2005)
eis (*rv2416c*)	Amikacin and kanamycin	Aminoglycoisde acetylation and inactivation	(Zaunbrecher et al., 2009)
hflX (*rv2725c*)	Macrolides, lincosamides	Rescue of stalled ribosomes	(Rudra et al., 2020)
ocrA (rv1473)	Oxazolidinones and phenicols	Drug displacement from ribosome	(Sharkey et al., 2016; Antonelli et al., 2018; Li et al., 2022)
smpB/ssr (*rv3100c/ssr*)	Oxazolidinones, phenicols, clarithromycin	Rescue of stalled ribosomes	(Li et al., 2022)
mfpAB (*rv3361c/rv3362c*)	Fluoroquinolones	DNA mimicry, protection of DNA gyrase from FQs	(Hegde et al., 2005; Tao et al., 2013)
cinA (*rv1901*)	Isoniazid, ethionamide, nitroimidazoles	Cleavage of drug-NAD adducts	(Wang et al., 2011; Kreutzfeldt et al., 2022)

comprehensively defining the intrinsic "resistome" for a larger panel of antitubercular drugs.

We posit that there is merit to performing chemical-genetic profiling on FDA approved drugs with detectable but limited antitubercular activity. Although in some cases this lack of potency may be explained by poor drug uptake (which could potentially be improved by mAGP disruption) or alteration of a specific molecular target, in some cases it may be the result of a specific intrinsic resistance factor. This is the case with macrolides which are ineffective against Mtb due to *whiB7*-mediated expression of *erm(37)* (Morris et al., 2005). In instances where *whiB7* has been mutationally inactivated, clarithromycin displays potent activity against Mtb (Warit et al., 2015; Li et al., 2022). Using the macrolide paradigm, where *whiB7* is central to Mtb's intrinsic resistance, there may be parallel cases where one or several factors are responsible for limiting the activity of a given drug. Ultimately, by defining the intrinsic "resistome" for these compounds it may allow for three potential follow up strategies to advance their potential use in the clinic.

One such strategy involves leveraging "acquired drug sensitivities." Here, clinically prevalent mutations in intrinsic resistance factors may present therapeutic opportunities for drugs that are already approved for clinical uses outside of TB treatment. As described above, there are cases where Mtb clinical isolates sustain loss-of-function mutations in intrinsic resistance genes. In addition to the loss-of-function mutations in *whiB7*, there are several documented loss-of-function mutations in the *mmpS5/L5* efflux pump (Merker et al., 2020; Li et al., 2022). These mutations render Mtb hypersusceptible to bedaquiline and clofazimine. As the use of whole genome sequencing is expanded in clinical labs, we may be able to predict unique drug susceptibilities based on the genome sequence of the infecting strain (CRyPTIC Consortium, 2018; Doyle et al., 2018). This may be particularly useful for multidrug-resistant and extensively-drug resistant TB cases with limited treatment options. Until "personalized" TB treatment is more widely available, geographically concentrated sublineages may be targeted on the basis of their unique vulnerability to particular drugs (Phelan et al., 2019; Li et al., 2022).

Another strategy to potentiate antibiotic activity against Mtb is to specifically inhibit intrinsic resistance pathways. Developing small molecule inhibitors of intrinsic resistance factors may synergize with the drug of interest (Hugonnet et al., 2009; Kurz et al., 2016). The classic example of this can be seen with the use of beta lactamase inhibitors to prevent the degradation of beta lactam antibiotics. In addition to the Mtb beta lactamase, BlaC, two other well-characterized examples which modify drugs or drug adducts are Eis and CinA (Zaunbrecher et al., 2009; Kreutzfeldt et al., 2022). Many more drug-modifying enzymes likely remain to be discovered (Zaunbrecher et al., 2009; Kreutzfeldt et al., 2022). Small molecule discovery efforts could focus on identifying inhibitors of particular drug modifying enzymes. As such, inhibitors of Eis would likely potentiate aminoglycoside activity whereas inhibition of CinA would likely potentiate isoniazid and pretomanid activity. However, both *eis* and *cinA* are non-essential for Mtb growth under standard conditions and inhibitors of these enzymes may face difficulties in preclinical development because in monotherapy, they would be unlikely to have any antimycobacterial activity. Therefore, genetic strategies focusing on essential genes are ideal (degron libraries, CRISPRi) since these represent some of the most attractive drug targets. Essential genes that impart intrinsic drug resistance could be prioritized for target-based drug discovery in order to form synergistic drug combinations where both compounds have individual activity but work more efficiently in combination. One such target is the mycobacterial superoxide dismutase (*sodA*), an essential oxygen radical quenching enzyme. Our previous work identified that knockdown of *sodA* sensitizes Mtb to several different classes of drugs (Li et al., 2022). Inhibitors of *sodA* would likely synergize with other drugs while also producing a direct, antimycobacterial effect.

Finally, a comprehensive understanding of the Mtb intrinsic resistome may allow for the rational design of drug analogs that avoid specific intrinsic resistance mechanisms. There are several examples of this concept, where derivatives of a particular drug are recalcitrant to the resistance mechanisms that target the parent compound. For example, 3rd generation tetracyclines avoid the drug-displacing activity of TetM in certain Gram-positive species (Jenner et al., 2013). Similarly, ketolides are a family of macrolide derivatives that have been engineered to bind Erm-methylated bacterial ribosomes (Capobianco et al., 2000). The ketolide drug telithromycin is approved for the treatment of erythromycin-resistant *S. pneumoniae* infections (Lonks and Goldmann, 2005). This concept is further exemplified by the aminoglycosides in Mtb. Despite being structurally and chemically similar, the aminoglycoside acetyltransferase Eis seems to have activity against kanamycin and amikacin but not streptomycin (Zaunbrecher et al., 2009). Conversely, the drug efflux pump Tap is active against streptomycin but not amikacin (Liu et al., 2019; Li et al., 2022). Another example from Mtb is seen with the beta lactamase BlaC. BlaC efficiently degrades penicillins and cephalosporins, which likely explains the poor activity of these beta lactams against Mtb (Hugonnet et al., 2009; Kurz et al., 2016). However, carbapenem antibiotics such as meropenem are relatively recalcitrant to BlaC activity. Accordingly, meropenem has strong activity against Mtb and has shown promising results in early clinical trials (Diacon et al., 2016). Although meropenem is still paired with a beta lactamase inhibitor, the poor activity of BlaC in degrading meropenem likely explains its superior activity relative to other beta lactams. Although there are only a handful of published drug modifying enzymes in Mtb, this phenomenon is likely to be more common than is currently appreciated (Warrier et al., 2016; Luthra et al., 2018). More expansive metabolomic studies may help identify the modifications made to antibiotics within the Mtb periplasm and

cytosol. A better understanding of intrinsic resistance mechanisms coupled with advances in structural biology and docking algorithms (Pagadala et al., 2017; Jumper et al., 2021) may facilitate the design highly specific drug analogs that circumvent the activity of particular intrinsic resistance proteins.

Although a more chemically comprehensive screening effort may reveal unique and novel mechanisms of intrinsic drug resistance, there are some potential pitfalls to this approach. This chemical-genetics strategy would almost certainly fail for a drug like fosfomycin, a non-TB antibiotic which targets the peptidoglycan synthetic enzyme MurA. The lack of fosfomycin activity against Mtb is due to lack of conservation in the drug binding site, not the presence of a specific intrinsic resistance factor (de Smet et al., 1999). Compounds chosen to undergo chemical-genetic profiling should be rationally selected on the basis of target conservation if that information is available. Finally, we believe this strategy of chemical-genetic profiling is important for compounds early in pre-clinical development, especially those with poorly understood mechanisms. Defining the intrinsic resistome may provide insights regarding the molecular target of the compound and also its mode of action (i.e., downstream effects of activity). Following the logic described above, lead compounds may be optimized further through careful pairing in synergistic drug combinations and engineering to avoid the activity of specific resistance factors.

New genetic tools for chemical-genetic studies

So far we have primarily discussed chemical-genetic studies employing three main genetic techniques: transposon mutagenesis, regulated proteolysis, and CRISPRi. High-density transposon mutagenesis was first applied to Mtb almost two decades ago and it continues to be a rich genetic resource. Regulated proteolysis systems and CRISPRi are relatively new genetic tools for Mtb and present powerful strategies to investigate the role of essential genes. However, all three strategies have technical limitations. Continued innovation in mycobacterial genetics will be important to address some of the gaps in our knowledge of Mtb biology, especially intrinsic drug resistance. As mentioned above, many Mtb genes are not expressed at high levels during standard laboratory culture and thus loss-of-function genetic approaches may be insufficient to reveal a phenotype (Schnappinger et al., 2003; Adams et al., 2011). For example, genetic disruption of a macrophage-induced efflux pump may not sensitize Mtb to antibiotics in broth since it has a low level of expression under those conditions. However, a drug sensitivity phenotype would likely be observed in drug-treated macrophages. These sorts of chemical-genetic interactions could potentially be captured by screening drugs in complex environments that best mimic host-relevant conditions.

Alternatively, overexpression of that efflux pump in broth conditions would likely confer drug resistance. Gain-of-function genetics represents a complementary tool to capture chemical-genetic phenotypes that would be difficult to observe with loss-of-function techniques. However, high throughput mechanisms of gene activation do not yet exist in Mtb. Such techniques have been employed quite successfully in mammalian systems (Ho et al., 2020) and, with a lesser degree of success in other bacterial species (Ho et al., 2020; Kiattisewee et al., 2021). CRISPR activation (CRISPRa) is one such strategy in which a catalytically dead Cas9 is fused to a transcriptional activator (Ho et al., 2020). A specific guide RNA can be used to locate the transcription activating Cas9 to the promoter region of a specific gene or operon. Another option for systematic gene activation in bacteria is to use a transposon carrying a strong, outward-facing promoter (Coe et al., 2019). This system is less specific than CRISPRa and is confounded in many cases by simultaneous activation of one gene and disruption of an adjacent gene. Alternatively, with advances in DNA synthesis, barcoded overexpression plasmids could be built for individual Mtb genes or operons to systematically overexpress Mtb genes. If any of these techniques were employed successfully in Mtb, it may allow for the identification of chemical-genetic phenotypes missed by loss-of-function genetics, particularly for lowly expressed genes.

Lastly, new genetic techniques will be useful for chemical-genetic screens that rely on bacterial outgrowth. For example, Kreutzfeldt et al. (Kreutzfeldt et al., 2022) performed a TnSeq screen to identify mutants with reduced survival in isoniazid-treated macrophages, which involved outgrowth of the surviving bacteria on agar plates. Transposon mutagenesis is well suited for assays that involve bacterial outgrowth since it generally results in irreversible target gene disruption and does not require an "off-switch." However, both CRISPRi and regulated proteolysis will likely have residual target knockdown after the removal of tetracycline, which may prevent or delay viable mutants for essential genes from successfully resuming growth (Qi et al., 2013). As such, these techniques may not be ideally suited, at least as currently implemented, for these types of screens. Therefore, next generation derivatives of both of these strategies that allow for an efficient "off-switch" will be paramount to screens relying on outgrowth such as those seeking to identify mutants with impaired survival during drug treatment.

Exploring intrinsic resistance heterogeneity across diverse Mtb strains

Mycobacterium tuberculosis displays a remarkable degree of genetic conservation across its major lineages and sublineages (Kremer et al., 1999). This is likely due to the recent evolutionary emergence of Mtb as well as the lack of horizontal gene transfer (Boritsch et al., 2016). However, there is a growing appreciation that

the genetic differences that do exist between Mtb lineages, sublineages, and strains can have profound impacts on bacterial physiology and can influence virulence, immunogenicity, and drug resistance (Portevin et al., 2011; Carey et al., 2018). When measuring drug susceptibility profiles of clinical Mtb isolates, many groups have reported that clinical strains can have a wide range of minimum inhibitory concentrations (MIC) for some drugs (CRyPTIC Consortium, 2018; Farhat et al., 2019). Even strains that fall below the critical breakpoint for resistance can display a wide range of MIC values. Some of this MIC heterogeneity may be attributable to low-level acquired drug resistance mutations which are generally poorly understood (Wong et al., 2011; Colangeli et al., 2018; Hicks et al., 2020). However, MIC heterogeneity is also observed for new drugs with novel mechanisms of action and which have been used little (if at all) in the clinic (Bateson et al., 2022). Accordingly, barring unknown mechanisms of cross-resistance, there has been little or no selective pressure for the evolution of resistance towards these drugs.

There are at least two obvious explanations for MIC heterogeneity for new drugs. First, these differences could be explained by inter-strain differences in drug target vulnerability (Carey et al., 2018; Bosch et al., 2021). Using transposon sequencing, Carey et al. found differences in genetic essentiality between different Mtb isolates, with lineage 2 strains displaying a reduced reliance on the glyoxylate shunt (Carey et al., 2018). The authors could recapitulate this finding using a chemical inhibitor of malate synthase (GlcB), a key enzyme in this pathway. Further demonstrating this concept, recent work by Bosch et al. showed that the cytochrome C reductase gene, *qcrB*, displays enhanced genetic vulnerability in the lineage 2 strain, HN878, compared to the lineage 4 strain H37Rv (Bosch et al., 2021). Accordingly, HN878 is more susceptible to the QcrB inhibitor, Q203, than is H37Rv.

Alternatively, MIC heterogeneity may reflect differences in the levels of intrinsic drug resistance between Mtb strains. In cases where a particular strain is lacking an intrinsic resistance factor (i.e. *whiB7 or mmpL5*) or sustained mutations that result in its hyperactivitity, there can be a pronounced change in drug sensitivity with a clear genetic basis (Warit et al., 2015; Villellas et al., 2017; Merker et al., 2020; Li et al., 2022). However, in most cases it is difficult to pinpoint a genetic basis for the differences in intrinsic resistance levels between strains. Future studies aiming to map the genetic basis of intrinsic drug resistance could seek to define these mechanisms in representative Mtb clinical strains, beyond the most common lineage 4 reference strains H37Rv, Erdman, and CDC1551 (Borrell et al., 2019).

Conclusions

TB remains one of the most difficult infectious diseases to treat. Even strains that are classified as drug sensitive display a remarkably high level of intrinsic resistance to many categories of drugs. Effective strategies must be developed not only to treat drug-resistant TB, but also to treat drug-sensitive TB in a shorter amount of time and with lower relapse rates. To do so, we must identify mechanisms of intrinsic drug resistance in Mtb and find ways around them. The cell envelope remains the most well-characterized and, perhaps, most important contributor to intrinsic drug resistance in Mtb. Successful disruption of the mAGP complex is an established method of disarming intrinsic resistance and sensitizing Mtb to antibiotics. In addition to the envelope, Mtb also encodes many other factors that can block antibiotic action once a drug has entered the cell. Future chemical-genetic studies will be paramount in furthering our understanding of the many layers of intrinsic drug resistance that Mtb has against a diverse set of antibiotics. A thorough genetic dissection of intrinsic resistance in Mtb will hopefully pave the way for more prioritized target-based drug discovery and medicinal chemistry efforts to develop faster-acting TB treatment regimens.

Author contributions

Literature review: NP and JR, Writing and editing of manuscript: NP and JR. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Robertson Therapeutic Development Fund (JR), an NIH/NIAID New Innovator Award (1DP2AI14485001, JR), and a shared NIH TB research unit grant (U19AI162584, JR)

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Abbate, E., Vescovo, M., Natiello, M., Cufré, M., García, A., Montaner, P. G., et al. (2012). Successful alternative treatment of extensively drug-resistant tuberculosis in Argentina with a combination of linezolid, moxifloxacin and thioridazine. *J. Antimicrobial. Chemother.* 67, 473–477. doi: 10.1093/jac/dkr500

Adams, K. N., Szumowski, J. D., and Ramakrishnan, L. (2014). Verapamil, and its metabolite norverapamil, inhibit macrophage-induced, bacterial efflux pump-mediated tolerance to multiple anti-tubercular drugs. *J. Infect. Dis.* 210, 456–466. doi: 10.1093/infdis/jiu095

Adams, K. N., Takaki, K., Connolly, L. E., Wiedenhoft, H., Winglee, K., Humbert, O., et al. (2011). Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell* 145, 39–53. doi: 10.1016/j.cell.2011.02.022

Adams, K. N., Verma, A. K., Gopalaswamy, R., Adikesavalu, H., Singhal, D. K., Tripathy, S., et al. (2019). Diverse clinical isolates of *Mycobacterium tuberculosis* develop macrophage-induced rifampin tolerance. *J. Infect. Dis.* 219, 1554–1558. doi: 10.1093/infdis/jiy710

Amaral, L., Kristiansen, J. E., Viveiros, M., and Atouguia, J. (2001). Activity of phenothiazines against antibiotic-resistant *Mycobacterium tuberculosis*: a review supporting further studies that may elucidate the potential use of thioridazine as anti-tuberculosis therapy. *J. Antimicrob. Chemother.* 47, 505–511. doi: 10.1093/jac/47.5.505

Amaral, L., and Viveiros, M. (2017). Thioridazine: A non-antibiotic drug highly effective, in combination with first line anti-tuberculosis drugs, against any form of antibiotic resistance of *Mycobacterium tuberculosis* due to its multi-mechanisms of action. *Antibiotics* 6. doi: 10.3390/antibiotics6010003

Antonelli, A., D'Andrea, M. M., Brenciani, A., Galeotti, C. L., Morroni, G., Pollini, S., et al. (2018). Characterization of poxtA, a novel phenicol-oxazolidinone-tetracycline resistance gene from an MRSA of clinical origin. *J. Antimicrobial. Chemother.* 73, 1763–1769. doi: 10.1093/jac/dky088

Antonova-Koch, Y., Meister, S., Abraham, M., Luth, M. R., Ottilie, S., Lukens, A. K., et al. (2018). Open-source discovery of chemical leads for next-generation chemoprotective antimalarials. *Science* 1979) 362, 1129. doi: 10.1126/science.aat9446

Ates, L. S., Ummels, R., Commandeur, S., van de Weerd, R., van der Weerd, R., Sparrius, M., et al. (2015). Essential role of the ESX-5 secretion system in outer membrane permeability of pathogenic mycobacteria. *PloS Genet.* 11, e1005190. doi: 10.1371/journal.pgen.1005190

Balaban, N. Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D. I., et al. (2019). Definitions and guidelines for research on antibiotic persistence. *Nat. Rev. Microbiol.* 17, 441–448. doi: 10.1038/s41579-019-0196-3

Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., et al. (1994). inhA, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science* 263, 227–230. doi: 10.1126/science.8284673

Bateson, A., Ortiz Canseco, J., McHugh, T. D., Witney, A. A., Feuerriegel, S., Merker, M., et al. (2022). Ancient and recent differences in the intrinsic susceptibility of *Mycobacterium tuberculosis* complex to pretomanid. *J. Antimicrob. Chemother.* 77, 1685–1693. doi: 10.1093/jac/dkac070

Batt, S. M., Minnikin, D. E., and Besra, G. S. (2020). The thick waxy coat of mycobacteria, a protective layer against antibiotics and the host's immune system. *Biochem. J.* 477, 1983–2006. doi: 10.1042/BCJ20200194

Bellerose, M. M., Proulx, M. K., Smith, C. M., Baker, R. E., Ioerger, T. R., and Sassetti, C. M. (2020). Distinct bacterial pathways influence the efficacy of antibiotics against *Mycobacterium tuberculosis*. *mSystems* 5, 1–18. doi: 10.1128/mSystems.00396-20

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O., and Piddock, L. (2015). Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* 13, 42–51. doi: 10.1038/nrmicro3380

Boritsch, E. C., Khanna, V., Pawlik, A., Honoré, N., Navas, V. H., Ma, L., et al. (2016). Key experimental evidence of chromosomal DNA transfer among selected tuberculosis-causing mycobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 113, 9876–9881. doi: 10.1073/pnas.1604921113

Borrell, S., Trauner, A., Brites, D., Rigouts, L., Loiseau, C., Coscolla, M., et al. (2019). Reference set of *Mycobacterium tuberculosis* clinical strains: A tool for research and product development. *PloS One* 14, 1–12. doi: 10.1371/journal.pone.0214088

Bosch, B., DeJesus, M. A., Poulton, N. C., Zhang, W., Engelhart, C. A., Zaveri, A., et al. (2021). Genome-wide gene expression tuning reveals diverse vulnerabilities of *M. tuberculosis*. *Cell* 184 (17), 4579–4592. doi: 10.1016/j.cell.2021.06.033

Boyer, E., Dessolin, J., Lustig, M., Decossas, M., Phan, G., Cece, Q., et al. (2022). Molecular determinants for OMF selectivity in tripartite RND multidrug efflux systems. *Antibiot. (Basel).* 11. doi: 10.3390/antibiotics11020126

Braibant, M., Gilot, P., and Content, J. (2000). The ATP binding cassette (ABC) transport systems of *Mycobacterium tuberculosis*. *FEMS Microbiol. Rev.* 24, 449–467. doi: 10.1016/S0168-6445(00)00034-6

Brauner, A., Fridman, O., Gefen, O., and Balaban, N. Q. (2016). Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat. Rev. Microbiol.* 14, 320–330. doi: 10.1038/nrmicro.2016.34

Briffotaux, J., Huang, W., Wang, X., and Gicquel, B. (2017). MmpS5/MmpL5 as an efflux pump in *Mycobacterium* species. *Tuberculosis. (Edinb).* 107, 13–19. doi: 10.1016/j.tube.2017.08.001

Brown, J. C. S., Nelson, J., Vandersluis, B., Deshpande, R., Butts, A., Kagan, S., et al. (2014). Unraveling the biology of a fungal meningitis pathogen using chemical genetics. *Cell* 159, 1168–1187. doi: 10.1016/j.cell.2014.10.044

Burian, J., Yim, G., Hsing, M., Axerio-Cilies, P., Cherkasov, A., Spiegelman, G. B., et al. (2013). The mycobacterial antibiotic resistance determinant WhiB7 acts as a transcriptional activator by binding the primary sigma factor SigA (RpoV). *Nucleic Acids Res.* 41, 10062–10076. doi: 10.1093/nar/gkt751

Cacace, E., Kritikos, G., and Typas, A. (2017). Chemical genetics in drug discovery. *Curr. Opin. Syst. Biol.* 4, 35–42. doi: 10.1016/j.coisb.2017.05.020

Campbell, E. A., Korzheva, N., Mustaev, A., Murakami, K., Nair, S., Goldfarb, A., et al. (2001). Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell* 104, 901–912. doi: 10.1016/S0092-8674(01)00286-0

Capobianco, J. O., Cao, Z., Shortridge, V. D., Ma, Z., Flamm, R. K., and Zhong, P. (2000). Studies of the novel ketolide ABT-773: Transport, binding to ribosomes, and inhibition of protein synthesis in *Streptococcus pneumoniae*. *Antimicrob. Agents Chemother.* 44, 1562–1567. doi: 10.1128/AAC.44.6.1562-1567.2000

Carey, A. F., Rock, J. M., Krieger, I., Chase, M. R., Fernandez-Suarez, M., Gagneux, S., et al. (2018). TnSeq of *Mycobacterium tuberculosis* clinical isolates reveals strain-specific antibiotic liabilities. *PloS Pathog.* 14, e1006939. doi: 10.1371/journal.ppat.1006939

Chen, C., Gardete, S., Jansen, R. S., Shetty, A., Dick, T., Rhee, K. Y., et al. (2018). Verapamil targets membrane energetics in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 62, e02107-17. doi: 10.1128/AAC.02107-17

Choudhary, E., Thakur, P., Pareek, M., and Agarwal, N. (2015). Gene silencing by CRISPR interference in mycobacteria. *Nat. Commun.* 6. doi: 10.1038/ncomms7267

Coe, K. A., Lee, W., Stone, M. C., Komazin-Meredith, G., Meredith, T. C., Grad, Y. H., et al. (2019). Multi-strain tn-seq reveals common daptomycin resistance determinants in *Staphylococcus aureus*. *PloS Pathog.* 15, 1–26. doi: 10.1371/journal.ppat.1007862

Coelho, T., Machado, D., Couto, I., Maschmann, R., Ramos, D., von Groll, A., et al. (2015). Enhancement of antibiotic activity by efflux inhibitors against multidrug resistant *Mycobacterium tuberculosis* clinical isolates from Brazil. *Front. Microbiol.* 6. doi: 10.3389/fmicb.2015.00330

Cokol, M., Kuru, N., Bicak, E., Larkins-Ford, J., and Aldridge, B. B. (2017). Efficient measurement and factorization of high-order drug interactions in *Mycobacterium tuberculosis*. *Sci. Adv.* 3, e170188. doi: 10.1126/sciadv.1701881

Colangeli, R., Jedrey, H., Kim, S., Connell, R., Ma, S., Chippada Venkata, U. D., et al. (2018). Bacterial factors that predict relapse after tuberculosis therapy. *N. Engl. J. Med.* 379, 823–833. doi: 10.1056/NEJMoa1715849

Conradie, F., Diacon, A. H., Ngubane, N., Howell, P., Everitt, D., Crook, A. M., et al. (2020). Treatment of highly drug-resistant pulmonary tuberculosis. *N. Engl. J. Med.* 382, 893–902. doi: 10.1056/NEJMoa1901814

CRyPTIC Consortium (2018). Prediction of susceptibility to first-line tuberculosis drugs by DNA sequencing. *New Engl. J. Med.* 379, 1403–1415. doi: 10.1056/nejmoa1800474

Cui, Z., Li, X., Shin, J., Gamper, H., Hou, Y.-M., Sacchettini, J. C., et al. (2022). Interplay between an ATP-binding cassette f protein and the ribosome from *Mycobacterium tuberculosis*. *Nat. Commun.* 13, 1–12. doi: 10.1038/s41467-022-28078-1

Cunningham, A. F., and Spreadbury, C. L. (1998). Mycobacterial stationary phase induced by low oxygen tension: cell wall thickening and localization of the 16-kilodalton alpha-crystallin homolog. *J. Bacteriol.* 180, 801–808. doi: 10.1128/JB.180.4.801-808.1998

Dartois, V. (2014). The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells. *Nat. Rev. Microbiol.* 12, 159–167. doi: 10.1038/nrmicro3200

Davis, T. D., Gerry, C. J., and Tan, D. S. (2014). General platform for systematic quantitative evaluation of small-molecule permeability in bacteria. *ACS Chem. Biol.* 9, 2535–2544. doi: 10.1021/cb5003015

Dejesus, M. A., Gerrick, E. R., Xu, W., Park, S. W., Long, J. E., Boutte, C. C., et al. (2017). Comprehensive essentiality analysis of the *Mycobacterium tuberculosis*

genome *via* saturating transposon mutagenesis. *mBio* 8, 1–17. doi: 10.1128/mBio.02133-16

de Smet, K. A. L., Kempsell, K. E., Gallagher, A., Duncan, K., and Young, D. B. (1999). Alteration of a single amino acid residue reverses fosfomycin resistance of recombinant MurA from *Mycobacterium tuberculosis*. *Microbiol. (Reading).* 145, 3177–3184. doi: 10.1099/00221287-145-11-3177

de Vos, M., Ley, S. D., Wiggins, K. B., Derendinger, B., Dippenaar, A., Grobbelaar, M., et al. (2019). Bedaquiline microheteroresistance after cessation of tuberculosis treatment. *New Engl. J. Med.* 380, 2178–2180. doi: 10.1056/nejmc1815121

de Wet, T. J., Winkler, K. R., Mhlanga, M., Mizrahi, V., and Warner, D. F. (2020). Arrayed CRISPRi and quantitative imaging describe the morphotypic landscape of essential mycobacterial genes. *Elife* 9, 1–36. doi: 10.7554/eLife.60083

Diacon, A. H., van der Merwe, L., Barnard, M., von Groote-Bidlingmaier, F., Lange, C., García-Basteiro, A. L., et al. (2016). β-lactams against tuberculosis–new trick for an old dog? *N. Engl. J. Med.* 375, 393–394. doi: 10.1056/NEJMc1513236

Domenech, P., and Reed, M. B. (2009). Rapid and spontaneous loss of phthiocerol dimycocerosate (PDIM) from *Mycobacterium tuberculosis* grown *in vitro*: implications for virulence studies. *Microbiol. (Reading).* 155, 3532–3543. doi: 10.1099/mic.0.029199-0

Dorman, S. E., Nahid, P., Kurbatova, E. V., Phillips, P. P. J., Bryant, K., Dooley, K. E., et al. (2021). Four-month rifapentine regimens with or without moxifloxacin for tuberculosis. *N. Engl. J. Med.* 384, 1705–1718. doi: 10.1056/NEJMoa2033400

Doyle, R. M., Burgess, C., Williams, R., Gorton, R., Booth, H., Brown, J., et al. (2018). Direct whole-genome sequencing of sputum accurately identifies drug-resistant *Mycobacterium tuberculosis* faster than MGIT culture sequencing. *J. Clin. Microbiol.* 56, 1–11. doi: 10.1128/JCM.00666-18

Duan, W., Li, X., Ge, Y., Yu, Z., Li, P., Li, J., et al. (2019). *Mycobacterium tuberculosis* Rv1473 is a novel macrolides ABC efflux pump regulated by WhiB7. *Future Microbiol.* 14, 47–59. doi: 10.2217/fmb-2018-0207

Dubé, M. P., Sattler, F. R., Torriani, F. J., See, D., Havlir, D., Kemper, C. A., et al. (1997). A randomized evaluation of ethambutol for prevention of relapse and drug resistance during treatment of *Mycobacterium avium* complex bacteremia with clarithromycin-based combination therapy. *J. Infect. Dis.* 176, 1225–1232. doi: 10.1086/514116

Dulberger, C. L., Rubin, E. J., and Boutte, C. C. (2020). The mycobacterial cell envelope - a moving target. *Nat. Rev. Microbiol.* 18, 47–59. doi: 10.1038/s41579-019-0273-7

Evans, D. R., Griffith, M. P., Sundermann, A. J., Shutt, K. A., Saul, M. I., Mustapha, M. M., et al. (2020). Systematic detection of horizontal gene transfer across genera among multidrug-resistant bacteria in a single hospital. *Elife* 9, 1–20. doi: 10.7554/eLife.53886

Farhat, M. R., Freschi, L., Calderon, R., Ioerger, T., Snyder, M., Meehan, C. J., et al. (2019). GWAS for quantitative resistance phenotypes in *Mycobacterium tuberculosis* reveals resistance genes and regulatory regions. *Nat. Commun.* 10, 2128. doi: 10.1038/s41467-019-10110-6

Furió, V., Moreno-Molina, M., Chiner-Oms, Á., Villamayor, L. M., Torres-Puente, M., and Comas, I. (2021). An evolutionary functional genomics approach identifies novel candidate regions involved in isoniazid resistance in *Mycobacterium tuberculosis*. *Commun. Biol.* 4, 1–12. doi: 10.1038/s42003-021-02846-z

Goude, R., Amin, A. G., Chatterjee, D., and Parish, T. (2009). The arabinosyltransferase EmbC is inhibited by ethambutol in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 53, 4138–4146. doi: 10.1128/AAC.00162-09

Gupta, A. K., Katoch, V. M., Chauhan, D. S., Sharma, R., Singh, M., Venkatesan, K., et al. (2010). Microarray analysis of efflux pump genes in multidrug-resistant *Mycobacterium tuberculosis* during stress induced by common anti-tuberculous drugs. *Microb. Drug Resist.* 16, 21–28. doi: 10.1089/mdr.2009.0054

Gygli, S. M., Borrell, S., Trauner, A., and Gagneux, S. (2017). Antimicrobial resistance in *Mycobacterium tuberculosis*: Mechanistic and evolutionary perspectives. *FEMS Microbiol. Rev.* 41, 354–373. doi: 10.1093/femsre/fux011

Hartkoorn, R. C., Uplekar, S., and Cole, S. T. (2014). Cross-resistance between clofazimine and bedaquiline through upregulation of mmpl5 in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 58, 2979–2981. doi: 10.1128/AAC.00037-14

Hawkins, J. S., Silvis, M. R., Koo, B.-M., Peters, J. M., Osadnik, H., Jost, M., et al. (2020). Mismatch-CRISPRi reveals the Co-varying expression-fitness relationships of essential genes in *Escherichia coli* and *Bacillus subtilis*. *Cell Syst.* 11, 523–535.e9. doi: 10.1016/j.cels.2020.09.009

Hegde, S. S., Vetting, M. W., Roderick, S. L., Mitchenall, L. A., Maxwell, A., Takiff, H. E., et al. (2005). A fluoroquinolone resistance protein from *Mycobacterium tuberculosis* that mimics DNA. *Science* 308, 1480–1483. doi: 10.1126/science.1110699

Hicks, N. D., Giffen, S. R., Culviner, P. H., Chao, M. C., Dulberger, C. L., Liu, Q., et al. (2020). Mutations in dnaA and a cryptic interaction site increase drug resistance in *Mycobacterium tuberculosis*. *PloS Pathog.* 16, 1–28. doi: 10.1371/journal.ppat.1009063

Hicks, N. D., Yang, J., Zhang, X., Zhao, B., Grad, Y. H., Liu, L., et al. (2018). Clinically prevalent mutations in *Mycobacterium tuberculosis* alter propionate metabolism and mediate multidrug tolerance. *Nat. Microbiol.* 3, 1032–1042. doi: 10.1038/s41564-018-0218-3

Ho, H., Fang, J. R., Cheung, J., and Wang, H. H. (2020). Programmable CRISPR-cas transcriptional activation in bacteria. *Mol. Syst. Biol.* 16, 1–12. doi: 10.15252/msb.20199427

Hugonnet, J. E., Tremblay, L. W., Boshoff, H. I., Barry, C. E., and Blanchard, J. S. (2009). Meropenem-clavulanate is effective against extensively drug-resistant *Mycobacterium tuberculosis*. *Science* 323, 1215–1218. doi: 10.1126/science.1167498

Hunt, M., Bradley, P., Lapierre, S. G., Heys, S., Thomsit, M., Hall, M. B., et al. (2019). Antibiotic resistance prediction for *Mycobacterium tuberculosis* from genome sequence data with mykrobe. *Wellcome. Open Res.* 4, 191. doi: 10.12688/wellcomeopenres.15603.1

Jankute, M., Cox, J. A. G., Harrison, J., and Besra, G. S. (2015). Assembly of the mycobacterial cell wall. *Annu. Rev. Microbiol.* 69, 405–423. doi: 10.1146/annurev-micro-091014-104121

Jarlier, V., and Nikaido, H. (1994). Mycobacterial cell wall: Structure and role in natural resistance to antibiotics. *FEMS Microbiol. Lett.* 123, 11–18. doi: 10.1111/j.1574-6968.1994.tb07194.x

Jenner, L., Starosta, A. L., Terry, D. S., Mikolajka, A., Filonava, L., Yusupov, M., et al. (2013). Structural basis for potent inhibitory activity of the antibiotic tigecycline during protein synthesis. *Proc. Natl. Acad. Sci. U.S.A.* 110, 3812–3816. doi: 10.1073/pnas.1216691110

Jin, D. J., and Gross, C. A. (1988). Mapping and sequencing of mutations in the *Escherichia coli* rpoB gene that lead to rifampicin resistance. *J. Mol. Biol.* 202, 45–58. doi: 10.1016/0022-2836(88)90517-7

Johnson, E. O., LaVerriere, E., Office, E., Stanley, M., Meyer, E., Kawate, T., et al. (2019). Large-Scale chemical-genetics yields new *M. tuberculosis* inhibitor classes. *Nature* 571, 72–78. doi: 10.1038/s41586-019-1315-z

Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., et al. (2021). Highly accurate protein structure prediction with AlphaFold. *Nature* 596, 583–589. doi: 10.1038/s41586-021-03819-2

Kalscheuer, R., Palacios, A., Anso, I., Cifuente, J., Anguita, J., Jacobs, W. R., et al. (2019). The *Mycobacterium tuberculosis* capsule: a cell structure with key implications in pathogenesis. *Biochem. J.* 476, 1995–2016. doi: 10.1042/BCJ20190324

Kiattisewee, C., Dong, C., Fontana, J., Sugianto, W., Peralta-Yahya, P., Carothers, J. M., et al. (2021). Portable bacterial CRISPR transcriptional activation enables metabolic engineering in *Pseudomonas putida*. *Metab. Eng.* 66, 283–295. doi: 10.1016/j.ymben.2021.04.002

Kilburn, J. O., and Takayama, K. (1981). Effects of ethambutol on accumulation and secretion of trehalose mycolates and free mycolic acid in *Mycobacterium smegmatis*. *Antimicrob. Agents Chemother.* 20, 401–404. doi: 10.1128/AAC.20.3.401

Kim, J. H., O'Brien, K. M., Sharma, R., Boshoff, H. I. M., Rehren, G., Chakraborty, S., et al. (2013). A genetic strategy to identify targets for the development of drugs that prevent bacterial persistence. *Proc. Natl. Acad. Sci. U.S.A.* 110, 19095–19100. doi: 10.1073/pnas.1315860110

Koh, E., Oluoch, P. O., Ruecker, N., Proulx, M. K., Soni, V., Murphy, K. C., et al. (2022). Chemical-genetic interaction mapping links carbon metabolism and cell wall structure to tuberculosis drug efficacy. *Proc. Natl. Acad. Sci. U.S.A.* 119, e2201632119. doi: 10.1073/pnas.2201632119

Kremer, K., van Soolingen, D., Frothingham, R., Haas, W. H., Hermans, P. W., Martín, C., et al. (1999). Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility. *J. Clin. Microbiol.* 37, 2607–2618. doi: 10.1128/JCM.37.8.2607-2618.1999

Kreutzfeldt, K. M., Jansen, R. S., Hartman, T. E., Gouzy, A., Wang, R., Krieger, I. V., et al. (2022). CinA mediates multidrug tolerance in *Mycobacterium tuberculosis*. *Nat. Commun.* 13, 1–11. doi: 10.1038/s41467-022-29832-1

Kumar, P., Capodagli, G. C., Awasthi, D., Shrestha, R., Maharaja, K., Sukheja, P., et al. (2018). Synergistic lethality of a binary inhibitor of *Mycobacterium tuberculosis* KasA. *mBio* 9, e02101-17. doi: 10.1128/mBio.02101-17

Kurz, S. G., Hazra, S., Bethel, C. R., Romagnoli, C., Caselli, E., Prati, F., et al. (2016). Inhibiting the β-lactamase of *Mycobacterium tuberculosis* (Mtb) with novel boronic acid transition-state inhibitors (BATSIs). *ACS Infect. Dis.* 1, 234–242. doi: 10.1021/acsinfecdis.5b00003

Lambert, M.-L., Hasker, E., van Deun, A., Roberfroid, D., Boelaert, M., and van der Stuyft, P. (2003). Recurrence in tuberculosis: relapse or reinfection? *Lancet Infect. Dis.* 3, 282–287. doi: 10.1016/s1473-3099(03)00607-8

Larkins-Ford, J., Greenstein, T., Van, N., Degefu, Y. N., Olson, M. C., Sokolov, A., et al. (2021). Systematic measurement of combination drug landscapes to predict *in vivo* treatment outcomes for tuberculosis. *bioRxiv*. doi: 10.1101/2021.02.03.429579. 2021.02.03.429579.

Larrouy-Maumus, G., Marino, L. B., Madduri, A. V. R., Ragan, T. J., Hunt, D. M., Bassano, L., et al. (2016). Cell-envelope remodeling as a determinant of phenotypic antibacterial tolerance in *Mycobacterium tuberculosis*. *ACS Infect. Dis.* 2, 352–360. doi: 10.1021/acsinfecdis.5b00148

Laws, M., Jin, P., and Rahman, K. M. (2022). Efflux pumps in *Mycobacterium tuberculosis* and their inhibition to tackle antimicrobial resistance. *Trends Microbiol.* 30, 57–68. doi: 10.1016/j.tim.2021.05.001

Lechartier, B., Hartkoorn, R. C., and Cole, S. T. (2012). *In vitro* combination studies of benzothiazinone lead compound BTZ043 against *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 56, 5790–5793. doi: 10.1128/AAC.01476-12

Lee, J. H., Lee, E. J., and Roe, J. H. (2022). uORF-mediated riboregulation controls transcription of whiB7/wblC antibiotic resistance gene. *Mol. Microbiol.* 117, 179–192. doi: 10.1111/mmi.14834

Lehár, J., Stockwell, B. R., Giaever, G., and Nislow, C. (2008). Combination chemical genetics. *Nat. Chem. Biol.* 4, 674–681. doi: 10.1038/nchembio.120

Lenaerts, A., Barry, C. E., and Dartois, V. (2015). Heterogeneity in tuberculosis pathology, microenvironments and therapeutic responses. *Immunol. Rev.* 264, 288–307. doi: 10.1111/imr.12252

Li, P., Gu, Y., Li, J., Xie, L., Li, X., and Xie, J. (2017). *Mycobacterium tuberculosis* major facilitator superfamily transporters. *J. Membrane. Biol.* 250, 573–585. doi: 10.1007/s00232-017-9982-x

Li, S., Poulton, N. C., Chang, J. S., Azadian, Z. A., DeJesus, M. A., Ruecker, N., et al. (2022). CRISPRi chemical genetics and comparative genomics identify genes mediating drug potency in *Mycobacterium tuberculosis*. *Nat. Microbiol.* 2021, 11.27.469863. doi: 10.1038/s41564-022-01130-y

Liu, J., and Nikaido, H. (1999). A mutant of *Mycobacterium smegmatis* defective in the biosynthesis of mycolic acids accumulates meromycolates. *Proc. Natl. Acad. Sci. U.S.A.* 96, 4011–4016. doi: 10.1073/pnas.96.7.4011

Liu, J., Shi, W., Zhang, S., Hao, X., Maslov, D. A., Shur, K. V., et al. (2019). Mutations in efflux pump Rv1258c (Tap) cause resistance to pyrazinamide, isoniazid, and streptomycin in *M. tuberculosis*. *Front. Microbiol.* 10. doi: 10.3389/fmicb.2019.00216

Lonks, J. R., and Goldmann, D. A. (2005). Telithromycin: A ketolide antibiotic for treatment of respiratory tract infections. *Clin. Infect. Dis.* 40, 1657–1664. doi: 10.1086/430067

Lupien, A., Vocat, A., Foo, C. S.-Y., Blattes, E., Gillon, J.-Y., Makarov, V., et al. (2018). Optimized background regimen for treatment of active tuberculosis with the next-generation benzothiazinone macozinone (PBTZ169). *Antimicrob. Agents Chemother.* 62, e00840-18. doi: 10.1128/AAC.00840-18

Luria, S. E., and Delbrück, M. (1943). Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* 28, 491–511. doi: 10.1093/genetics/28.6.491

Luthra, S., Rominski, A., and Sander, P. (2018). The role of antibiotic-Target-Modifying and antibiotic-modifying enzymes in *Mycobacterium abscessus* drug resistance. *Front. Microbiol.* 9. doi: 10.3389/fmicb.2018.02179

Machado, D., Pires, D., Perdigão, J., Couto, I., Portugal, I., Martins, M., et al. (2016). Ion channel blockers as antimicrobial agents, efflux inhibitors, and enhancers of macrophage killing activity against drug resistant *Mycobacterium tuberculosis*. *PloS One* 11, 1–28. doi: 10.1371/journal.pone.0149326

Madsen, C. T., Jakobsen, L., Buriánková, K., Doucet-Populaire, F., Pernodet, J.-L., and Douthwaite, S. (2005). Methyltransferase Erm(37) slips on rRNA to confer atypical resistance in *Mycobacterium tuberculosis*. *J. Biol. Chem.* 280, 38942–38947. doi: 10.1074/jbc.M505727200

Manjunatha, U., Boshoff, H. I., and Barry, C. E. (2009). The mechanism of action of PA-824: Novel insights from transcriptional profiling. *Commun. Integr. Biol.* 2, 215–218. doi: 10.4161/cib.2.3.7926

Mathis, A. D., Otto, R. M., and Reynolds, K. A. (2021). A simplified strategy for titrating gene expression reveals new relationships between genotype, environment, and bacterial growth. *Nucleic Acids Res.* 49, e6. doi: 10.1093/nar/gkaa1073

McNeil, M. B., Chettiar, S., Awasthi, D., and Parish, T. (2019). Cell wall inhibitors increase the accumulation of rifampicin in *Mycobacterium tuberculosis*. *Access Microbiol.* 1, e000006. doi: 10.1099/acmi.0.000006

McNeil, M. B., Keighley, L. M., Cook, J. R., Cheung, C.-Y., and Cook, G. M. (2021). CRISPR interference identifies vulnerable cellular pathways with bactericidal phenotypes in *Mycobacterium tuberculosis*. *Mol. Microbiol.* 116, 1033–1043. doi: 10.1111/mmi.14790

Merker, M., Kohl, T. A., Barilar, I., Andres, S., Fowler, P. W., Chryssanthou, E., et al. (2020). Phylogenetically informative mutations in genes implicated in antibiotic resistance in *Mycobacterium tuberculosis* complex. *Genome Med.* 12, 1–8. doi: 10.1186/s13073-020-00726-5

Merle, C. S., Fielding, K., Sow, O. B., Gninafon, M., Lo, M. B., Mthiyane, T., et al. (2014). A four-month gatifloxacin-containing regimen for treating tuberculosis. *N. Engl. J. Med.* 371, 1588–1598. doi: 10.1056/NEJMoa1315817

Mikusová, K., Slayden, R. A., Besra, G. S., and Brennan, P. J. (1995). Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. *Antimicrob. Agents Chemother.* 39, 2484–2489. doi: 10.1128/AAC.39.11.2484

Morris, R. P., Nguyen, L., Gatfield, J., Visconti, K., Nguyen, K., Schnappinger, D., et al. (2005). Ancestral antibiotic resistance in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12200–12205. doi: 10.1073/pnas.0505446102

Nichols, R. J., Sen, S., Choo, Y. J., Beltrao, P., Zietek, M., Chaba, R., et al. (2011). Phenotypic landscape of a bacterial cell. *Cell* 144, 143–156. doi: 10.1016/j.cell.2010.11.052

Nikaido, H. (1994). Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* 264, 382–388. doi: 10.1126/science.8153625

Nimmo, C., Millard, J., van Dorp, L., Brien, K., Moodley, S., Wolf, A., et al. (2020). Population-level emergence of bedaquiline and clofazimine resistance-associated variants among patients with drug-resistant tuberculosis in southern Africa: a phenotypic and phylogenetic analysis. *Lancet Microbe* 1, e165–e174. doi: 10.1016/S2666-5247(20)30031-8

Ottavi, S., Scarry, S. M., Mosior, J., Ling, Y., Roberts, J., Singh, A., et al. (2022). *In vitro* and *In vivo* inhibition of the *Mycobacterium tuberculosis* phosphopantetheinyl transferase PptT by amidinoureas. *J. Med. Chem.* 65, 1996–2022. doi: 10.1021/acs.jmedchem.1c01565

Pagadala, N. S., Syed, K., and Tuszynski, J. (2017). Software for molecular docking: a review. *Biophys. Rev.* 9, 91–102. doi: 10.1007/s12551-016-0247-1

Parsons, A. B., Lopez, A., Givoni, I. E., Williams, D. E., Gray, C. A., Porter, J., et al. (2006). Exploring the mode-of-Action of bioactive compounds by chemical-genetic profiling in yeast. *Cell* 126, 611–625. doi: 10.1016/j.cell.2006.06.040

Pasca, M. R., Guglierame, P., Arcesi, F., Bellinzoni, M., de Rossi, E., and Riccardi, G. (2004). Rv2686c-Rv2687c-Rv2688c, an ABC fluoroquinolone efflux pump in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 48, 3175–3178. doi: 10.1128/AAC.48.8.3175-3178.2004

Pellecchia, M., Sem, D. S., and Wüthrich, K. (2002). NMR in drug discovery. *Nat. Rev. Drug Discovery* 1, 211–219. doi: 10.1038/nrd748

Peters, J. M., Colavin, A., Shi, H., Czarny, T. L., Larson, M. H., Wong, S., et al. (2016). A comprehensive, CRISPR-based functional analysis of essential genes in bacteria. *Cell* 165, 1493–1506. doi: 10.1016/j.cell.2016.05.003

Peterson, E. J., Brooks, A. N., Reiss, D. J., Kaur, A., Wu, W.-J., Srinivas, V., et al. (2021). MtrA regulation of essential peptidoglycan cleavage in *Mycobacterium tuberculosis* during infection 1. *bioRxiv*. doi: 10.1101/2021.02.25.432019. 2021.02.25.432019.

Peterson, E., and Kaur, P. (2018). Antibiotic resistance mechanisms in bacteria: Relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. *Front. Microbiol.* 9. doi: 10.3389/fmicb.2018.02928

Phelan, J. E., Lim, D. R., Mitarai, S., de Sessions, P. F., Tujan, M. A. A., Reyes, L. T., et al. (2019). *Mycobacterium tuberculosis* whole genome sequencing provides insights into the Manila strain and drug-resistance mutations in the Philippines. *Sci. Rep.* 9, 1–6. doi: 10.1038/s41598-019-45566-5

Piddock, L. J., Williams, K. J., and Ricci, V. (2000). Accumulation of rifampicin by *Mycobacterium aurum*, *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. *J. Antimicrob. Chemother.* 45, 159–165. doi: 10.1093/jac/45.2.159

Pieroni, M., Machado, D., Azzali, E., Santos Costa, S., Couto, I., Costantino, G., et al. (2015). Rational design and synthesis of thioridazine analogues as enhancers of the antituberculosis therapy. *J. Med. Chem.* 58, 5842–5853. doi: 10.1021/acs.jmedchem.5b00428

Planck, K. A., and Rhee, K. (2021). Metabolomics of *Mycobacterium tuberculosis*. *Methods Mol. Biol.* 2314, 579–593. doi: 10.1007/978-1-0716-1460-0_25

Portevin, D., Gagneux, S., Comas, I., and Young, D. (2011). Human macrophage responses to clinical isolates from the *Mycobacterium tuberculosis* complex discriminate between ancient and modern lineages. *PloS Pathog.* 7, e1001307. doi: 10.1371/journal.ppat.1001307

Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., et al. (2013). Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152, 1173–1183. doi: 10.1016/j.cell.2013.02.022

Reeves, A. Z., Campbell, P. J., Sultana, R., Malik, S., Murray, M., Plikaytis, B. B., et al. (2013). Aminoglycoside cross-resistance in *Mycobacterium tuberculosis* due to mutations in the 5' untranslated region of whiB7. *Antimicrob. Agents Chemother.* 57, 1857–1865. doi: 10.1128/AAC.02191-12

Remm, S., Earp, J. C., Dick, T., Dartois, V., and Seeger, M. A. (2022). Critical discussion on drug efflux in *Mycobacterium tuberculosis*. *FEMS Microbiol. Rev.* 46, 1–15. doi: 10.1093/femsre/fuab050

Rempel, S., Gati, C., Nijland, M., Thangaratnarajah, C., Karyolaimos, A., de Gier, J. W., et al. (2020). A mycobacterial ABC transporter mediates the uptake of hydrophilic compounds. *Nature* 580, 409–412. doi: 10.1038/s41586-020-2072-8

Rens, C., Chao, J. D., Sexton, D. L., Tocheva, E. I., and Av-Gay, Y. (2021). Roles for phthiocerol dimycocerosate lipids in *Mycobacterium tuberculosis* pathogenesis. *Microbiol. (Reading).* 167, 001042. doi: 10.1099/mic.0.001042

Rock, J. M., Hopkins, F. F., Chavez, A., Diallo, M., Chase, M. R., Gerrick, E. R., et al. (2017). Programmable transcriptional repression in mycobacteria using an orthogonal CRISPR interference platform. *Nat. Microbiol.* 2. doi: 10.1038/nmicrobiol.2016.274

Rodrigues, L., Cravo, P., and Viveiros, M. (2020). Efflux pump inhibitors as a promising adjunct therapy against drug resistant tuberculosis: a new strategy to revisit mycobacterial targets and repurpose old drugs. *Expert Rev. Anti Infect. Ther.* 18, 741–757. doi: 10.1080/14787210.2020.1760845

Rudra, P., Hurst-Hess, K. R., Cotten, K. L., Partida-Miranda, A., and Ghosh, P. (2020). Mycobacterial HflX is a ribosome splitting factor that mediates antibiotic resistance. *Proc. Natl. Acad. Sci. U.S.A.* 117, 629–634. doi: 10.1073/pnas.1906748117

Ruth, M. M., Pennings, L. J., Koeken, V. A. C. M., Schildkraut, J. A., Hashemi, A., Wertheim, H. F. L., et al. (2020). Thioridazine is an efflux pump inhibitor in *Mycobacterium avium* complex but of limited clinical relevance. *Antimicrob. Agents Chemother.* 64, 1–5. doi: 10.1128/AAC.00181-20

Sarathy, J., Dartois, V., Dick, T., and Gengenbacher, M. (2013). Reduced drug uptake in phenotypically resistant nutrient-starved nonreplicating *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 57, 1648–1653. doi: 10.1128/AAC.02202-12

Sassetti, C. M., Boyd, D. H., and Rubin, E. J. (2001). Comprehensive identification of conditionally essential genes in mycobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 98, 12712–12717. doi: 10.1073/pnas.231275498

Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., et al. (2003). Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: Insights into the phagosomal environment. *J. Exp. Med.* 198, 693–704. doi: 10.1084/jem.20030846

Schrader, S. M., Botella, H., Jansen, R., Ehrt, S., Rhee, K., Nathan, C., et al. (2021). Multiform antimicrobial resistance from a metabolic mutation. *Sci. Adv.* 7, 1–18. doi: 10.1126/sciadv.abh2037

Seddon, J. A., Godfrey-Faussett, P., Jacobs, K., Ebrahim, A., Hesseling, A. C., and Schaaf, H. S. (2012). Hearing loss in patients on treatment for drug-resistant tuberculosis. *Eur. Respir. J.* 40, 1277–1286. doi: 10.1183/09031936.00044812

Sharkey, L. K. R., Edwards, T. A., and O'Neill, A. J. (2016). ABC-F proteins mediate antibiotic resistance through ribosomal protection. *mBio* 7, e01975-15. doi: 10.1128/mBio.01975-15

Si, M., Li, H., Chen, Y., and Peng, H. (2018). Ethambutol and isoniazid induced severe neurotoxicity in a patient undergoing continuous ambulatory peritoneal dialysis. *BMJ Case Rep.* 2018, 1–5. doi: 10.1136/bcr-2017-223187

Siroy, A., Mailaender, C., Harder, D., Koerber, S., Wolschendorf, F., Danilchanka, O., et al. (2008). Rv1698 of *Mycobacterium tuberculosis* represents a new class of channel-forming outer membrane proteins. *J. Biol. Chem.* 283, 17827–17837. doi: 10.1074/jbc.M800866200

Smith, H. E., and Blair, J. M. A. (2014). Redundancy in the periplasmic adaptor proteins AcrA and AcrE provides resilience and an ability to export substrates of multidrug efflux. *J. Antimicrobial. Chemother.* 69, 982–987. doi: 10.1093/jac/dkt481

Soetaert, K., Rens, C., Wang, X. M., de Bruyn, J., Lanéelle, M. A., Laval, F., et al. (2015). Increased vancomycin susceptibility in mycobacteria: A new approach to identify synergistic activity against multidrug-resistant mycobacteria. *Antimicrob. Agents Chemother.* 59, 5057–5060. doi: 10.1128/AAC.04856-14

Stokes, R. W., Norris-Jones, R., Brooks, D. E., Beveridge, T. J., Doxsee, D., and Thorson, L. M. (2004). The glycan-rich outer layer of the cell wall of *Mycobacterium tuberculosis* acts as an antiphagocytic capsule limiting the association of the bacterium with macrophages. *Infect. Immun.* 72, 5676–5686. doi: 10.1128/IAI.72.10.5676-5686.2004

Stover, C. K., Warrener, P., VanDevanter, D. R., Sherman, D. R., Arain, T. M., Langhorne, M. H., et al. (2000). A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. *Nature* 405, 962–966. doi: 10.1038/35016103

Szumowski, J. D., Adams, K. N., Edelstein, P. H., and Ramakrishnan, L. (2012). *Antimicrobial efflux pumps and mycobacterium tuberculosis drug tolerance: Evolutionary considerations* (Berlin, Heidelberg: Springer), 81–108. doi: 10.1007/82_2012_300

Tan, C. M., Therien, A. G., Lu, J., Lee, S. H., Caron, A., Gill, C. J., et al. (2012). Restoring methicillin-resistant *Staphylococcus aureus* susceptibility to β-lactam antibiotics. *Sci. Transl. Med.* 4, 126ra35. doi: 10.1126/scitranslmed.3003592

Tao, J., Han, J., Wu, H., Hu, X., Deng, J., Fleming, J., et al. (2013). *Mycobacterium* fluoroquinolone resistance protein b, a novel small GTPase, is involved in the regulation of DNA gyrase and drug resistance. *Nucleic Acids Res.* 41, 2370–2381. doi: 10.1093/nar/gks1351

Telenti, A., Imboden, P., Marchesi, F., Matter, L., Schopfer, K., Bodmer, T., et al. (1993). Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet* 341, 647–651. doi: 10.1016/0140-6736(93)90417-F

Thiede, J. M., Dillon, N. A., Howe, M. D., Aflakpui, R., Modlin, S. J., Hoffner, S. E., et al. (2022). Pyrazinamide susceptibility is driven by activation of the SigE-dependent cell envelope stress response in *Mycobacterium tuberculosis*. *mBio*. 13 (1), e0043921. doi: 10.1128/mbio.00439-21

Tiwari, S., Casey, R., Goulding, C. W., Hingley-Wilson, S., and Jacobs, W. R. (2020). Infect and inject: How *Mycobacterium tuberculosis* exploits its major virulence-associated type VII secretion system, ESX-1. *Bacteria. Intracellularity.* 1, 113–126. doi: 10.1128/9781683670261.ch8

Tostmann, A., Boeree, M. J., Aarnoutse, R. E., de Lange, W. C. M., van der Ven, A. J. A. M., and Dekhuijzen, R. (2008). Antituberculosis drug-induced hepatotoxicity: concise up-to-date review. *J. Gastroenterol. Hepatol.* 23, 192–202. doi: 10.1111/j.1440-1746.2007.05207.x

Trivedi, O. A., Arora, P., Vats, A., Ansari, M. Z., Tickoo, R., Sridharan, V., et al. (2005). Dissecting the mechanism and assembly of a complex virulence mycobacterial lipid. *Mol. Cell* 17, 631–643. doi: 10.1016/j.molcel.2005.02.009

Venter, H., Mowla, R., Ohene-Agyei, T., and Ma, S. (2015). RND-type drug efflux pumps from gram-negative bacteria: Molecular mechanism and inhibition. *Front. Microbiol.* 6. doi: 10.3389/fmicb.2015.00377

Vigouroux, A., Oldewurtel, E., Cui, L., Bikard, D., and van Teeffelen, S. (2018). Tuning dCas9's ability to block transcription enables robust, noiseless knockdown of bacterial genes. *Mol. Syst. Biol.* 14, e7899. doi: 10.15252/msb.20177899

Villellas, C., Coeck, N., Meehan, C. J., Lounis, N., de Jong, B., Rigouts, L., et al. (2017). Unexpected high prevalence of resistance-associated Rv0678 variants in MDR-TB patients without documented prior use of clofazimine or bedaquiline. *J. Antimicrob. Chemother.* 72, 684–690. doi: 10.1093/jac/dkw502

Walker, T. M., Kohl, T. A., Omar, S. V., Hedge, J., del Ojo Elias, C., Bradley, P., et al. (2015). Whole-genome sequencing for prediction of *Mycobacterium tuberculosis* drug susceptibility and resistance: a retrospective cohort study. *Lancet Infect. Dis.* 15, 1193–1202. doi: 10.1016/S1473-3099(15)00062-6

Wang, Q., Boshoff, H. I. M., Harrison, J. R., Ray, P. C., Green, S. R., Wyatt, P. G., et al. (2020). PE/PPE proteins mediate nutrient transport across the outer membrane of mycobacterium tuberculosis. *Science* 367, 1147–1151. doi: 10.1126/science.aav5912

Wang, X., Gu, J., Wang, T., Bi, L. J., Zhang, Z. P., Cui, Z. Q., et al. (2011). Comparative analysis of mycobacterial NADH pyrophosphatase isoforms reveals a novel mechanism for isoniazid and ethionamide inactivation. *Mol. Microbiol.* 82, 1375–1391. doi: 10.1111/j.1365-2958.2011.07892.x

Wang, Z., Soni, V., Marriner, G., Kaneko, T., Boshoff, H. I. M., Barry, C. E., et al. (2019). Mode-of-action profiling reveals glutamine synthetase as a collateral metabolic vulnerability of *M. tuberculosis* to bedaquiline. *Proc. Natl. Acad. Sci. U.S.A.* 116, 19646–19651. doi: 10.1073/pnas.1907946116

Warit, S., Phunpruch, S., Jityam, C., Jaitrong, S., Billamas, P., Chaiprasert, A., et al. (2015). Genetic characterisation of a whiB7 mutant of a *Mycobacterium tuberculosis* clinical strain. *J. Glob. Antimicrob. Resist.* 3, 262–266. doi: 10.1016/j.jgar.2015.07.004

Warrier, T., Kapilashrami, K., Argyrou, A., Ioerger, T. R., Little, D., Murphye, K. C., et al. (2016). N-methylation of a bactericidal compound as a resistance mechanism in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 113, E4523–E4530. doi: 10.1073/pnas.1606590113

WHO (2021). *Global tuberculosis report*. Geneva, Switzerland: World Health Organization.

Wilson, D. N. (2014). Ribosome-targeting antibiotics and mechanisms of bacterial resistance. *Nat. Rev. Microbiol.* 12, 35–48. doi: 10.1038/nrmicro3155

Wong, S. Y., Lee, J. S., Kwak, H. K., Via, L. E., Boshoff, H. I. M., and Barry, C. E. (2011). Mutations in gidB confer low-level streptomycin resistance in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 55, 2515–2522. doi: 10.1128/AAC.01814-10

Wong, A. I., and Rock, J. M. (2021). CRISPR interference (CRISPRi) for targeted gene silencing in mycobacteria. *Methods Mol. Biol.* 2314, 343–364. doi: 10.1007/978-1-0716-1460-0_16

Wu, M.-L., Gengenbacher, M., and Dick, T. (2016). Mild nutrient starvation triggers the development of a small-cell survival morphotype in mycobacteria. *Front. Microbiol.* 7. doi: 10.3389/fmicb.2016.00947

Xu, W., DeJesus, M. A., Rücker, N., Engelhart, C. A., Wright, M. G., Healy, C., et al. (2017). Chemical genetic interaction profiling reveals determinants of intrinsic antibiotic resistance in *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 61, 1–15. doi: 10.1128/AAC.01334-17

Yamada, T., Nagata, A., Ono, Y., Suzuki, Y., and Yamanouchi, T. (1985). Alteration of ribosomes and RNA polymerase in drug-resistant clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother.* 27, 921–924. doi: 10.1128/AAC.27.6.921

Zaunbrecher, M. A., Sikes, R. D., Metchock, B., Shinnick, T. M., and Posey, J. E. (2009). Overexpression of the chromosomally encoded aminoglycoside acetyltransferase eis confers kanamycin resistance in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20004–20009. doi: 10.1073/pnas.0907925106

Zgurskaya, H. I., and Rybenkov, V. V. (2019). Permeability barriers of gram-negative pathogens. *Ann. N. Y. Acad. Sci.* 1459, 5–18. doi: 10.1111/nyas.14134

Zhang, Y., Heym, B., Allen, B., Young, D., and Cole, S. (1992). The catalase - peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature* 358, 591–593. doi: 10.1038/358591a0

Zhang, L., Zhao, Y., Gao, Y., Wu, L., Gao, R., Zhang, Q., et al. (2020). Structures of cell wall arabinosyltransferases with the anti-tuberculosis drug ethambutol. *Science* 1979) 368, 1211–1219. doi: 10.1126/science.aba9102

Zhao, S., Adamiak, J. W., Bonifay, V., Mehla, J., Zgurskaya, H. I., and Tan, D. S. (2020). Defining new chemical space for drug penetration into gram-negative bacteria. *Nat. Chem. Biol.* 16, 1293–1302. doi: 10.1038/s41589-020-00674-6

Zimmerman, M., Lestner, J., Prideaux, B., O'Brien, P., Dias-Freedman, I., Chen, C., et al. (2017). Ethambutol partitioning in tuberculous pulmonary lesions explains its clinical efficacy. *Antimicrob. Agents Chemother.* 61, e00924-17. doi: 10.1128/AAC.00924-17

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 10 November 2022
DOI 10.3389/fcimb.2022.1029111

OPEN ACCESS

EDITED BY
Helena Ingrid Boshoff,
National Institutes of Health (NIH),
United States

REVIEWED BY
Bavesh Kana,
University of the Witwatersrand, South Africa
Amit Singh,
Indian Institute of Science (IISc), India
Giulia Manina,
Institut Pasteur, France
Chanchal Sur Chowdhury,
Washington University in St. Louis,
United States

*CORRESPONDENCE
Kohta Saito
kos9010@med.cornell.edu

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 26 August 2022
ACCEPTED 20 October 2022
PUBLISHED 10 November 2022

CITATION
Mishra S and Saito K (2022) Clinically encountered growth phenotypes of tuberculosis-causing bacilli and their *in vitro* study: A review.
Front. Cell. Infect. Microbiol.
12:1029111.
doi: 10.3389/fcimb.2022.1029111

Clinically encountered growth phenotypes of tuberculosis-causing bacilli and their *in vitro* study: A review

Saurabh Mishra[1] and Kohta Saito[2*]

[1]Department of Microbiology and Immunology, Weill Cornell Medicine, New York, NY, United States, [2]Department of Medicine, Weill Cornell Medicine, New York, NY, United States

The clinical manifestations of tuberculosis (TB) vary widely in severity, site of infection, and outcomes of treatment—leading to simultaneous efforts to individualize therapy safely and to search for shorter regimens that can be successfully used across the clinical spectrum. In these endeavors, clinicians and researchers alike employ mycobacterial culture in rich media. However, even within the same patient, individual bacilli among the population can exhibit substantial variability in their culturability. Bacilli *in vitro* also demonstrate substantial heterogeneity in replication rate and cultivation requirements, as well as susceptibility to killing by antimicrobials. Understanding parallels in clinical, *ex vivo* and *in vitro* growth phenotype diversity may be key to identifying those phenotypes responsible for treatment failure, relapse, and the reactivation of bacilli that progresses TB infection to disease. This review briefly summarizes the current role of mycobacterial culture in the care of patients with TB and the *ex vivo* evidence of variability in TB culturability. We then discuss current advances in *in vitro* models that study heterogenous subpopulations within a genetically identical bulk culture, with an emphasis on the effect of oxidative stress on bacillary cultivation requirements. The review highlights the complexity that heterogeneity in mycobacterial growth brings to the interpretation of culture in clinical settings and research. It also underscores the intricacies present in the interplay between growth phenotypes and antimicrobial susceptibility. Better understanding of population dynamics and growth requirements over time and space promises to aid both the attempts to individualize TB treatment and to find uniformly effective therapies.

KEYWORDS

tuberculosis, mycobacteria, persisters, VBNC, oxidative stress, heterogeneity, differentially detectable

Introduction

When a human host encounters a member of the *Mycobacterium tuberculosis* complex (MTBC), the potential outcomes range the entire breadth of clinical possibilities—from immediate sterilization, to asymptomatic infection with later progression to active disease, to fulminant tuberculosis (TB) disease. The necessary duration of therapy for relapse-free cure also varies greatly, although months of multi-drug therapy is standard. At the most simplistic level, this is a matter of whether the bacillus can replicate in its local microenvironment and, if it is unable to replicate, whether it can subsequently regain that ability when stresses are removed or altered. If disease state heterogeneity in the human population reflects the heterogeneity of *in vivo* organism replication, then it is tantalizing to use *ex vivo* and *in vitro* culturability—and resuscitation of culturability—to draw clinically relevant conclusions regarding drug efficacy and treatment outcomes. In this review, we briefly examine the role of mycobacterial culture in clinical care and clinical research, and then explore how *ex vivo* work connects these clinical observations. We then survey the conditions and cellular mechanisms driving heterogeneity in growth *in vitro*, emphasizing the role of oxidative stress. In so doing, we endeavor to show that while it would be ideal to make culture obsolete in the clinic, there is still much to be gained at the bedside from better understanding of the wide variability in growth speed and replication requirements of stressed MTBC organisms at the bench.

Clinical use of mycobacterial culture

Figure 1 provides an overview of the uses and methods for assessment of mycobacterial replication. The MTBC is a group of genetically related mycobacterium species that can cause TB, and includes *Mycobacterium tuberculosis* (Mtb), *Mycobacterium africanum*, and *Mycobacterium bovis (M. bovis)*, among others. In the clinical setting, cultivation of MTBC bacilli serves three main purposes: diagnosis of TB disease, monitoring response to therapy, and phenotypic drug susceptibility testing. Culture positivity and conversion to negativity are often used by clinicians to help personalize TB treatment, and culture-based endpoints are used in clinical trials as surrogate markers of durable cure or treatment failure. Here we will briefly describe the vital but imperfect role of culture in the clinic and in clinical research.

Diagnosis and selection of treatment

Mycobacterial culture is currently the laboratory gold standard for the microbiologic diagnosis of TB disease despite

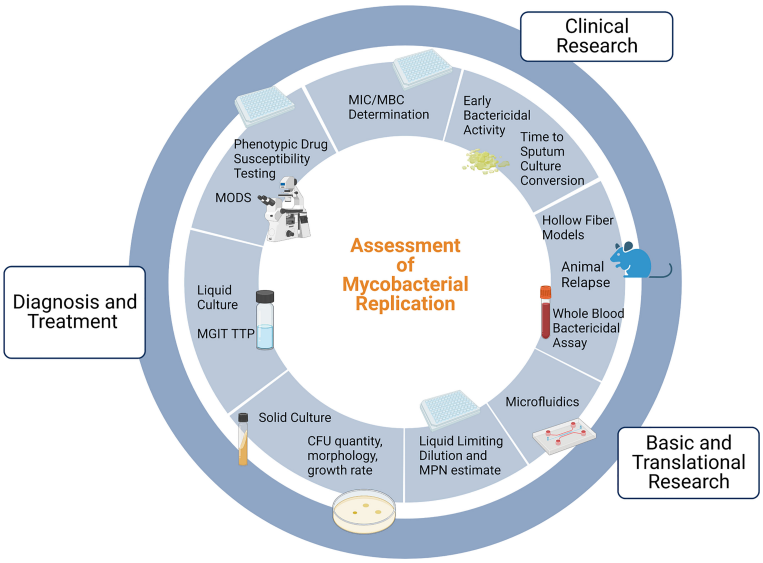


FIGURE 1
Uses of and methods for assessment of mycobacterial replication. Note that drug susceptibility testing incorporates clinical and *in vivo* factors in addition to MIC testing and is thus distinct. MGIT: Mycobacterial Growth Indicator Tube; TTP: time to positivity; MODS: Microscopic Observation Drug Susceptibility; MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; MPN: Most Probable Number; CFU: Colony Forming Units. Adapted from "Gut-Brain Axis Regulators", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

improvements in molecular methods of mycobacterial detection (Lewinsohn et al., 2017). Growth of Mtb or other MTBC bacilli from a patient produced sample can secure the diagnosis, however, culture yields can be highly variable and affected by bacillary load, specimen type, and specimen processing. As an example, MTBC organisms are isolated from less than 75% of infants and 50% of children with pulmonary TB as diagnosed by clinical criteria—the numbers are likely even lower for many extrapulmonary forms of TB (Graham et al., 2015; DiNardo et al., 2016; Thomas, 2017; Baker, 2020). Liquid culture methods have generally been more sensitive than solid culture methods—88-90% vs 76% in one meta-analysis—and have a shorter time to detection. The same meta-analysis demonstrated a drop in sensitivity of liquid culture in AFB sputum that were negative for acid fast bacilli (AFB) on smear to ≤ 80% (Cruciani et al., 2004). Innovations in liquid culture readout, such as the Mycobacteria Growth Indicator Tube (MGIT) that contains an oxygen-quenched fluorochrome which fluoresces when growing mycobacteria consume oxygen, among others, facilitate diagnosis and can be implemented in a simple, safe, and automated or semi-automated fashion (Palaci et al., 1996; Pfyffer et al., 1997; Heifets et al., 2000). The use of this fluorescence indicator mitigates the downsides of previously used radiometric readouts for liquid culture that are expensive and produce radioactive waste, while maintaining similar sensitivity for Mtb detection (Pfyffer et al., 1997; Cruciani et al., 2004). However, liquid cultures are more prone to contamination with other bacteria; therefore, current guidelines endorsed by the CDC recommend using both solid and liquid culture systems (Ichiyama et al., 1993; Lewinsohn et al., 2017). Liquid culture systems require addition of an antimicrobial cocktail to reduce contamination rates, although rates still ranged in the meta-analysis from 3.8 to 16.6% for MGIT (Cruciani et al., 2004). The MGIT system includes polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) in the media. This cannot, however, mitigate contamination with species or isolates that are resistant to the cocktail (Cornfield et al., 1997). MGIT also supports growth of many non-tuberculous mycobacteria. Given these known variabilities in culture yield, physicians must ultimately rely on clinical judgement if there is a high suspicion of active TB in a patient, even with negative culture results.

To maximize yield, current CDC guidelines recommend three sputum smears for assessment of AFB by microscopy as well as sending all collected sputum for mycobacterial culture (Lewinsohn et al., 2017). This is in line with data showing that obtaining multiple cultures improves sensitivity, with the largest improvement occurring with a second specimen and smaller incremental gains thereafter (Blair et al., 1976; Levy et al., 1989; Cascina et al., 2000; Harvell et al., 2000) It is important to note that given the laboratory requirements and costs of performing mycobacterial culture, it is not always feasible to obtain culture for TB diagnosis, and the WHO has reviewed and endorsed several rapid molecular methods as initial diagnostic tests for TB rather than smear microscopy and culture (2021).

Each patient merits careful consideration when being evaluated for TB, as host characteristics can profoundly affect the diagnostic approach. For example, in people with HIV, sputum that is smear-negative but culture-positive is common—one study out of Thailand and Vietnam found that three MGIT cultures missed only 3 of 126 patients with pulmonary TB, whereas three sputum smears missed 79 (Monkongdee et al., 2009). Interestingly, that study also found that a single liquid culture yielded a TB diagnosis in a similar number of patients as three sputa on solid media. As noted in CDC guidelines, in HIV-related TB, enlarged lymph nodes can often yield a diagnosis when aspirated, and in severely immunocompromised patients' urine and blood cultures can also yield mycobacteria (Panel on Guidelines, 2022). In children, as previously noted, microbiologic confirmation of TB is difficult, and even in pulmonary TB expectorated sputum can be difficult or impossible for young children to produce. Again, culture of other specimens is considered and has been studied, including sampling of gastric fluid, nasopharyngeal aspirates, stool, and urine. One recent study of children under 5 who had symptoms of TB found that combinations of tests and repetition of tests led to increased yield—and found that some combinations of non-invasive tests could perform similarly to more invasive procedures such as gastric aspiration or induced sputum (Song et al., 2021). In that study, MGIT was more sensitive than the rapid molecular diagnostic test Xpert, but Xpert identified some children who remained MGIT negative. However, the overall rate of microbiologic diagnosis was still low at 10%, and it is unknown how many children actually had TB given the information provided in the report (Starke and Cruz, 2021). Thus, despite current culture techniques remaining the laboratory gold standard, better and scalable diagnostics, whether or not they are culture-based, are desperately needed.

If TB exposure is diagnosed by non-culture methods, negative culture results can help define where along the clinical spectrum an individual lies. This is clinically relevant, as treatment selection can be directly impacted. For example, in considering treatment for TB infection, some patients first undergo sputum cultures to assess for TB disease—lest a patient inappropriately receive monotherapy for TB disease that would select for drug resistance. For pulmonary TB disease, growing evidence suggests that some patients who are diagnosed clinically but cannot be confirmed microbiologically can achieve durable cure with therapy shorter than the current 6-month standard. In adults, the Infectious Diseases Society of America guidelines suggest that certain patients with culture-negative pulmonary tuberculosis can be safely treated with a 4-month regimen based on a systematic review of studies out of Hong Kong, Arkansas, and Singapore (Sotgiu et al., 2016). As alluded to above, many children belong to this kind of 'paucibacillary' group, and the recent SHINE trial

demonstrated non-inferiority of a 4-month treatment regimen in drug-susceptible, smear-negative, non-cavitary TB. Of 1204 patients in that trial, only 146 had a positive Mtb culture (Turkova et al., 2022). Less clear is how to approach patients in the converse situation: with minimal-to-no symptoms but found to have microbiologic confirmation of TB disease in sputum. Such subclinical TB is increasingly recognized by studies using culture and molecular diagnostics, but questions remain regarding the clinical significance of this finding—including rates of transmission from this population, the true proportion of patients who progress to more severe disease, and what, if any, therapy is required (Frascella et al., 2021; Kendall et al., 2021; Wong, 2021).

Phenotypic drug susceptibility testing (DST) can be performed when an isolate is recovered *via* culture. This remains the reference standard DST for many TB drugs, especially as new or repurposed drugs are developed for which resistance mechanisms are unknown or not fully defined. However, this method takes resources and time, and efforts have been made to make more accessible culture-based drug susceptibility tests. For example, the Microscopic Observation Drug Susceptibility (MODS) assay takes advantage of the faster growth of MTBC organisms in liquid culture and uses an inverted microscope to rapidly detect growth. The cording pattern of mycobacterial growth allows for relatively facile visualization. If the bacilli grow in the presence of drug, resistance is detected. While not FDA approved in the US, studies have found it may have utility as a rapid, inexpensive, and reliable alternative to other methods of drug susceptibility testing, despite the need for appropriate training and specific supplies (Caviedes et al., 2000; Moore et al., 2004; Moore et al., 2006; Fitzwater et al., 2010; Minion et al., 2010; Shah et al., 2011; Alcántara et al., 2020). Very rapid molecular methods of detecting drug resistance, not reviewed here, are quickly improving and expanding available tests (Lewinsohn et al., 2017; WHO, 2021). Still, the advantages of phenotypic DST in the changeable landscape of drug resistance and therapy make the development of widely-accessible, fast, and clinically-applicable phenotypic DST necessary. Of note, phenotypic DST can be designed to assess raw minimum inhibitory concentrations (MIC) and not simply whether an Mtb isolate is susceptible based on a single breakpoint value. Intriguingly, in one study, higher pretreatment MIC values for isoniazid (INH) or rifampin that were still below the resistance breakpoint were associated with a greater risk of relapse than lower values, even after adjusting for other risk factors (Colangeli et al., 2018; Rubin, 2018). These kinds of findings may eventually allow for better personalization of care—or provide strategies for globally reducing treatment failure and relapse.

Monitoring during therapy

In TB disease, care often needs to be individualized based on clinical response and the tolerability of drug regimens. Bacillary load and response to TB chemotherapy has been monitored for decades by serially quantifying the number of colony forming units (CFU) of Mtb that grow on a solid medium from sputum. While CFU ideally arise from a single bacillus for accurate quantification, the clumpy nature of these organisms ensures at least some clumps even in well dispersed cultures (Fenner et al., 1949; Stewart et al., 1957; Diacon and Donald, 2014). Growth of a colony simultaneously allows counting *via* the naked eye as well as proof of viability of the progenitor(s) of the colony. However, as we will explore further, the CFU may frequently undercount the number of viable mycobacteria or be falsely reassuring when negative, especially during therapy.

In current guidelines, treatment failure in drug susceptible TB is defined as Mtb growth in any form of culture after 4 months (or 5 months in WHO guidelines) of appropriate therapy (Sotgiu et al., 2016). Sputum cultures are checked monthly at a minimum until two consecutive cultures are negative (Sotgiu et al., 2016). Earlier indicators of treatment failure and relapse are few. Checking whether sputum cultures grow Mtb at 2 months (the usual end of the intensive phase of treatment) is recommended, and in current CDC guidelines growth of Mtb at 2 months is one criterion for which to consider extending the continuation phase of therapy by several months (Sotgiu et al., 2016; WHO, 2022). However, while persistent positive cultures are a risk factor for failure, the sensitivity of this test in detecting relapse is overall poor—one systematic review and meta-analysis found that the pooled sensitivity for 2-month sputum culture to predict relapse was 40% (Horne et al., 2010). The WHO does not currently endorse extension of either the intensive or continuation phase based on growth at 2 months due to the modest benefit in relapse reduction (Horne et al., 2010; Phillips et al., 2016; Romanowski et al., 2019; WHO, 2022). Culture conversion at 2 months also does not allow for shortening of current standard therapy below 6 months without a significant increase in relapse rates (Johnson et al., 2009). Of note, the original studies correlating 2-month culture status with risk of relapse were performed using traditional solid media, which as previously noted are generally less sensitive than liquid cultures (Johnson et al., 2009). Given these known limitations, culture maintains an important role in treatment monitoring, but the interpretation of results must be individualized and reflect nuanced understanding of the test characteristics.

Time to positivity (TTP) of growth in liquid culture detection systems, also reported as time to detection (TTD) in the literature, has been studied as a marker of risk of relapse both before and during treatment. A short TTP correlates with higher bacillary burden by CFU, and lack of an increase in TTP with therapy may reflect a poor response. TTP is likely affected in individual samples by factors aside from pure bacillary burden, and interestingly one of the early studies found several patients with TTP < 20 hours but whose sputum AFB smears were negative (Epstein et al., 1998; Pheiffer et al., 2008; Hesseling et al., 2010; Bark et al., 2012; Olaru et al., 2014). During TB treatment,

the relationship between TTP and CFU is more complex and changes over time—it has been demonstrated that even at the same CFU, TTP becomes longer if the sample is derived from later in treatment. The authors of that study hypothesize this is due to a subpopulation of bacilli that grows and is detected in the liquid culture but is not recovered as CFU (Bowness et al., 2015). This phenotypic state of mycobacteria is reviewed in depth further below.

For drug-resistant TB, using culture in treatment monitoring and in detecting treatment failure or development of drug resistance remains a key recommendation (Nahid et al., 2019; WHO, 2019). While guidelines have been in rapid flux due to the availability of new drugs and regimens in MDR/XDR-TB, the duration of both the intensive and continuation phases of the regimen is often personalized and can be anchored to the time of sputum conversion or the persistence of positive cultures (Nahid et al., 2019). Even in standardized, shorter treatments like the bedaquiline, pretomanid, and linezolid (BPaL) regimen, sputum culture conversion is monitored and re-assessment of phenotypic drug resistance performed in those with delayed response. It should be noted that in most non-tuberculous mycobacterial pulmonary infections, treatment durations are tied to when sputum cultures convert to consistently negative (Daley et al., 2020).

The relationship between persistence of positive culture and infectivity is another debated topic that may have a profound clinical impact when it comes to isolation and public health requirements. While it has been demonstrated that patients with smear-negative but culture-positive sputa can transmit TB to some degree, and that patients can remain culture positive for weeks to months into treatment, the degree to which treated, culture-positive patients can transmit is not clear (Behr et al., 1999; Hernandez-Garduno et al., 2004; Tostmann et al., 2008; Fitzwater et al., 2010; Asadi et al., 2022). Using the TTP in liquid culture has also been proposed as a potential correlate of infectiousness to guide isolation and contact tracing requirements, and one cohort study found a TTD < 9 days in an index case was associated with an increased transmission risk (Ritchie et al., 2007; O'Shea et al., 2014). Infectiousness likely depends on several other factors beyond culturability, such as mycobacterial fitness and relative ability to generate infectious aerosols, and the public health response requires careful consideration of further variables such as the costs of isolation and exhaustive contact tracing.

Clinical evaluation of new treatment regimens

Quantifying early change in CFU counts was recognized as a way of evaluating new drugs or regimens since the 1950s, and was more formalized in a 1980 study in which 27 TB drugs and regimens were compared for their effect on sputum Mtb CFU numbers over a 2 to 14 day period (Jindani et al., 1980). The methodology is meant to characterize the early bactericidal activity (EBA) of drugs and regimens, and has been described in guidance from both the U.S. Food and Drug Administration and European Medicines Agency as endpoints during early development of new TB therapies (FDA, 2013; Agency, 2017). EBA studies can evaluate the short-term ability of a single agent to kill mycobacteria during active TB, as well as allow for dose ranging and monitoring for short-term toxicities (Diacon and Donald, 2014). The importance of such trials should not be overlooked. However, there are critical limitations. Some clinically proven drugs have modest to no EBA in the first 2 days, such as rifampin, pyrazinamide, and bedaquiline (Jindani et al., 2003; Sirgel et al., 2005; Rustomjee et al., 2008). EBA results may be discordant with the relative ability to sterilize lesions; even dramatic decreases in actively replicating mycobacteria may not accurately predict a drug's effect on non-replicating or hypometabolic mycobacteria or on the risk of clinical relapse. Interestingly, one EBA trial that used the first-line TB drug INH to optimize early EBA methodology noted a 2-fold greater rate of decline in CFU than the rate of decline in bacterial load as quantified by AFB smear microscopy, thought due to continued visualization of dead organisms (Hafner et al., 1997). While many of these bacilli may truly be dead, some proportion may be viable but not culturable on standard solid media—this phenomenon is discussed in depth in the next section.

Other culture-based, surrogate assessments of the efficacy of therapies have also been used. Time to sputum culture conversion to no growth, especially at the 2-month mark, has been used both clinically and for evaluation of new TB treatments (Wallis et al., 2009; Wallis et al., 2010). Like its clinical utility in predicting relapse, the information that time to sputum culture conversion provides for new regimens is nuanced and must be interpreted carefully (Wallis et al., 2013; Lanoix et al., 2015). TTP in liquid culture has also been studied as a surrogate measure of EBA and in other clinical trials (Diacon et al., 2010; Weiner et al., 2010). All of these culture-based measures may be influenced by geographic or regional differences across sites as well (Sirgel et al., 2001; Mac Kenzie et al., 2011; Bark et al., 2014).

The fluoroquinolones provide a stark reminder of the potential pitfalls in using EBA and time to sputum culture conversion as surrogate measures of treatment efficacy. ReMOX TB, OFLOTUB, and RIFAQUIN were three randomized controlled trials designed to test the hypothesis that inclusion of fluoroquinolones could reduce treatment duration to 4 months instead of the standard 6 months for drug-susceptible TB, and were supported by earlier data that sputum culture conversion to negative at 2 months was improved by fluoroquinolones as well as strong EBA activity (Gillespie et al., 2014; Jindani et al., 2014; Merle et al., 2014; Lanoix et al., 2015). While all three trials confirmed some improvement in time to culture conversion, none could show

clinical noninferiority to 6 months of standard treatment. A more recent study using a regimen combining a fluoroquinolone with rifapentine (instead of rifampin) was able to safely shorten treatment to 4 months in addition to demonstrating the reduction in time to culture conversion as compared to standard therapy (Dorman et al., 2021). However, a separate 4-month rifapentine arm without a fluoroquinolone similarly reduced the time to culture conversion but was clinically inferior to the 6-month treatment arm. That trial simultaneously demonstrated that "there is no magic with 6 months of therapy" and that time to sputum culture conversion can be an inadequate early biomarker in predicting relapse-free cure of new regimens (Rubin and Mizrahi, 2021). The limitations of EBA trials and time to culture conversion as surrogates for durable cure have led to the search for other, non-culture-based early markers to speed evaluation of experimental drug treatments. For example, a recent study utilized PET/CT lung imaging at day 14 of treatment and demonstrated that the ReMOX trial regimen was no better than standard therapy in reducing lesion size or inflammation, which was consistent with the subsequent failure of the trial regimen in shortening treatment duration. This method, though expensive, integrates complex host data and shows promise as a potential way of evaluating early drug or drug combination efficacy (Xie et al., 2021). This, and other methods of treatment monitoring and outcome measures, including those interrogating host characteristics and non-culture, molecular methods of mycobacterial load assessment have been recently reviewed (Heyckendorf et al., 2022).

Ex vivo heterogeneity in culturability

What accounts for the variable utility of mycobacterial culture in diagnosis, treatment monitoring, and as surrogate endpoints for treatment success? Although a myriad of host factors, such as ability to cough, relative achievable TB drug levels, adherence to treatment and baseline immune status, play a role in the limitations of detecting Mtb in culture, if we restrict our view to the perspective and experience of the bacillus, there are at least three contributing and interrelated factors: from where in the body we are sampling, the mycobacterial load in that area, and how we culture. In this section, we review the evidence of heterogeneity in growth phenotypes present within populations of mycobacteria taken from patients and argue that the limitations of sputum culture in understanding TB are due in part to a current inability for standard sputum culture to appreciate this diversity.

What we are sampling

The human body exposed to Mtb can be a sterilizing environment, a medium that allows for survival but minimal-to-no growth, or a growth permissive space, and this property can change over time and space within the same person. Since before the mid-1900s, the variability both among TB lesions and in the ability to grow Mtb from the varied lesions has been well described. In the introduction to his summary and review of the histopathology and microbiology of human lung tissue from patients with TB in the 1940s, Georges Canetti writes:

> "Consider the bacillus in the lesion, experiencing such different fates in various foci of the same patient, and the same fate in widely different patients; destroyed in a certain histologic reaction and thriving in another nearby; swarming not by virtue of some mysterious force but simply because it is situated at a site from which swarming is possible (on the surface of a canalicular system); growing rapidly in certain necrotic areas and poorly in others; finding a proper environment only in certain tissues…"

He goes on to review many forms of lesions and the ability to cultivate mycobacteria from them—for example, he summarizes that 57 of 115 caseous or partially calcified lesions are sterile on egg media, whereas four-fifths of calcified lesions are sterile, and 115 of 134 completely sclerotic lesions did not recover mycobacteria on culture (Canetti, 1955). Later work studying resected lung tissue of patients with TB demonstrated a relationship between the type of lung lesion, drug susceptibility of bacilli recovered from the lesion, and the time required for cultivation of those bacilli ex vivo. Of lesions in communication with a bronchus, 85% yielded tubercle bacilli and all but one grew within 8 weeks of incubation. 70% demonstrated drug resistance. In contrast, only 44% of the cavities closed off from bronchi produced positive cultures and required 3-10 *months* of incubation. Only one of these cultures (17%) demonstrated *ex vivo* drug resistance. After growth in culture, these bacilli appeared morphologically normal, grew normally, and produced disease in animals (Loring et al., 1955; Bloom and McKinney, 1999). In other words, even within the same patient, the local microenvironment is extremely diverse and has a profound impact on a bacterium's ability to survive the immune response and antibiotics as well as its replication rate *in vivo* and its cultivation requirements *ex vivo*.

Much progress has since been made in understanding highly complex diversity in environmental landscape and bacterial phenotype. For example, the caseous core of a necrotic granuloma has been studied with respect to available carbon sources, relative oxygenation, pH, iron availability, variances in distribution of chemotherapy, and host cell types present—and the relative effect on Mtb growth and phenotypic tolerance to antibiotics (Lenaerts et al., 2015; Sarathy and Dartois, 2020). A link between growth rate and drug susceptibility has long been observed, and lack of replication has been used to explain the proportion of bacilli that are able to survive antibiotic exposure

when their genetically identical brethren are killed. The true relationship between replication and drug susceptibility is more complex, nuanced, and specific to the drug and the organism, as evidenced by observed dissociations between drug survival and growth rate or replication *in vitro* (Balaban et al., 2013; Wakamoto et al., 2013; Manina et al., 2015; Zhu et al., 2018). This is explored in further detail when we discuss *in vitro* studies in the manuscript. The population of bacteria that survive antibiotics for prolonged periods without classic genetically encoded resistance are called persisters, but the term thus encompasses a diverse spectrum of phenotypes with respect to metabolic activity and replicative capacity (Gold and Nathan, 2017). The phenomenon of relatively prolonged survival in the face of antibiotics—and the evolving terms used to describe forms of this phenomena—are critical to framing research questions and priorities and have been reviewed elsewhere (Balaban et al., 2019; Schrader et al., 2020). Rapid killing of the diverse array of persisters can likely reduce relapse risk with shorter therapies as well as more efficiently treat latent TB infection, but despite major advances these populations still require better characterization (Dartois and Rubin, 2022). The sputum culture is limiting not only as a representative sample of the numbers of viable bacilli in the body, but may be biased as a relative representation of the spectrum of phenotypes that exist deeper in tissues disconnected from larger airways. Sputum cultures may also be biased by the relative access to and susceptibility of those bacilli to treatment. It should be noted here that efforts to more fully characterize AFB found in sputa have found greater diversity than classically appreciated, as will be described below.

The situation is further complicated when considering the myriad environments in which TB disease can manifest outside the lung—the pleural space, in lymph nodes, the liver, the brain, etc—and the variable ease in obtaining useful culture. For example, in pleural TB, the yield of pleural fluid culture, which requires a thoracentesis, is variable but typically less than 30%, with the yield increasing in patients with HIV (Gil et al., 1995; Gopi et al., 2007; von Groote-Bidlingmaier et al., 2013). As with sputum, the yield improves by using liquid culture systems, but interestingly does not improve with increasing volume of pleural fluid (Luzze et al., 2001; Gopi et al., 2007; von Groote-Bidlingmaier et al., 2013). One interpretation of these findings, as hypothesized by the authors who tested Mtb recovery from differing pleural fluid volumes, is that the immune system produces a dichotomous result—either it will clear the pleural fluid completely or incompletely—and that patients with HIV are more commonly impaired in this pleural fluid clearance function, leading to increased culture yields (von Groote-Bidlingmaier et al., 2013). An alternative explanation is that the viable bacilli relatively sparsely found within the pleural space have specific growth requirements, and the need for such requirements is influenced by the immune system. The increased yield of liquid over solid culture already implies differential growth requirements for a subset of bacteria. Further support for this hypothesis comes from two studies which demonstrated that bedside inoculation of pleural fluid into liquid media as compared to later laboratory inoculation increased yield—suggesting yet another subpopulation of bacteria with specific cultivation needs (Maartens and Bateman, 1991; Augustine et al., 1999). Such variabilities in growth requirements are further explored in the next section.

How we are culturing

The practicalities of TB sample preparation ensure that quantification of viable *ex vivo* samples cannot be assumed equivalent to the number of culturable bacilli that existed *in situ*—even before they are placed in artificial media. Yield is affected by known factors, such as procedures to decontaminate sputa of non-mycobacterial organisms and room temperature storage (Damato et al., 1983; Paramasivan et al., 1983). The effect of refrigeration and freezing on culture yield of smear-positive sputa has been examined and generally show no significant loss of CFU; however, these studies did note increase in time to positivity of BacT/BACTEC liquid cultures in the same conditions (Tessema et al., 2011; Kolwijck et al., 2013). Sputum processing therefore alters phenotypic growth properties even when there is no apparent change in those bacilli robust enough to form CFU. Theoretically, this may render some bacillary subpopulations incapable of growing in culture and affect yield in paucibacillary cases.

Following such processing, the culture media and environment used in culture-based quantification also affects yield, as has already been touched upon. It has been well described that the sensitivity, specificity, and rapidity of growth in clinical culture systems are all affected by the type of culture media employed. For example, comparison of Middlebrook 7H12 broth, Lowenstein-Jensen, Middlebrook 7H10, and Middlebrook S7H11 media found variable recovery of mycobacteria from smear-negative patient sputa, ranging from 52.1% to 71.8% (Morgan et al., 1983). Efforts have been made to further optimize media composition, outgrowth environmental parameters, such as oxygen tension, and method of visualization, to speed growth and shorten time to detection (Ghodbane et al., 2014; Asmar et al., 2015; Ghodbane et al., 2015).

Recently, studies have found that some Mtb can only grow in liquid limiting dilution with or without supplementation with spent Mtb culture media or resuscitation promoting factors (Rpfs) (Biketov et al., 2000; Shleeva et al., 2002; Kana et al., 2008; Mukamolova et al., 2010; Nikitushkin et al., 2015; Chengalroyen et al., 2016; Dusthackeer et al., 2019). As summarized in the table, such differentially culturable (DC) or differentially detectable (DD) Mtb have been recovered in different *in vitro* models as well as in patient sputa before and

during treatment to varying degrees, and their quantity relative to CFU can be orders of magnitude higher. For those DD Mtb that are recovered by liquid limiting dilution, the population size can be estimated using the most probable number (MPN) method, which leverages technical replicates of dilution to extinction to calculate the original, undiluted concentration of viable organisms (Jarvis et al., 2010). While a review of *in vivo* animal models is beyond the scope of this review, it must be mentioned that the phenomena has been recognized for decades in mice who received treatment until bacilli could no longer be recovered in culture, but later relapsed off therapy (McCune et al., 1966). It should also be noted that in the literature, and in other organisms, another often used term is "viable but non-culturable" (VBNC) for similar phenotypes. The basic requirement of these terms is that they define a bacterial state in which the organism does not replicate on traditionally growth-supportive media but is found viable by another method, whether that method is based on resuscitation and return of culturability. A related but distinct phrase is "non-growing but metabolically active" (NGMA), which places focus on bacteria that are not replicating but shows evidence of metabolic activity at a single-cell level and does not require proof of viability (Manina and McKinney, 2013). For brevity and because we are focusing on growth phenotypes, in Mtb we will generally be using the term "DD Mtb," and in reference to literature from other organisms the term "VBNC," for the remainder of this review.

It is interesting that some DD Mtb phenotypes require Rpfs for resuscitation, but not others. Rpfs were first discovered in *Micrococcus luteus* as a bacterial equivalent of a cytokine, as it is released extracellularly (Mukamolova et al., 1998). Since then, Rpfs have been identified and characterized in bacteria with high guanine and cytosine content in their genomes, including *Corynebacterium glutamicum* and *Streptomyces* spp (Mukamolova et al., 1998; Mukamolova et al., 2002). Rpfs have been found to resuscitate cells from stress conditions such as prolonged stationary phase, residence in murine peritoneal macrophages, an *in vitro* hypoxia model and, as above, in sputum of patients with TB (Biketov et al., 2000; Shleeva et al., 2002; Chengalroyen et al., 2016; Dusthackeer et al., 2019). Mtb encodes five paralogues of Rpf (Kana et al., 2008). Structurally, Rpfs are similar to lysozyme and lytic transglycosylases (Nikitushkin et al., 2015) and are considered to be important determinants of TB pathogenesis (Rosser et al., 2017). The exact mechanisms of resuscitation with Rpfs and with spent culture filtrate, and why certain phenotypic states are stimulated to divide in their presence, are not fully elucidated but remain an important area of study (Gordhan et al., 2021).

Whether these methods of improving culture yield by finding DD Mtb can meaningfully improve rates of diagnosis and whether the more accurate quantification of mycobacterial load in some patients is clinically useful is being studied. It remains unclear and debated (Walter et al., 2018). McAulay et al. reported that the percentage of patients with DD Mtb in their sputum samples increased from 21% pretreatment to 69% after treatment with isoniazid, rifampin, pyrazinamide, and ethambutol (HRZE) (McAulay et al., 2018). Interestingly, one recent study evaluated for DD Mtb at the end of therapy for drug-susceptible TB *via* bronchoscopy and found that 5 of 41 patients had viable TB. Two of these patients relapsed within one year (Beltran et al., 2020). The relevance of these findings requires further evaluation but suggests that study of this subpopulation yet holds promise.

Mechanisms underlying heterogeneity in culturability

The above *ex vivo* studies have shown a broad range of growth phenotypes that MTBC organisms enter as defined by their rate of recovery and growth, need for Rpfs, and resuscitation in liquid but not solid culture media. Clinical outcomes have mirrored this broad range and rate of culturability within the human host. Here, we review some of the extensive efforts to probe this heterogeneity *in vitro*, with a focus on the relationship between reactive oxygen species (ROS), adaptations to cope with oxidative stress, genetic/epigenetic factors and cell division. Figure 2 places in a framework the interplay between stress, bacterial replication, and its clinical implications. Note that the rich, important literature regarding animal models will not be reviewed here but has been reviewed elsewhere (Fonseca et al., 2017; Zhan et al., 2017).

Extent of stress and cellular damage alters cultivability requirements

Damage caused by stress can be both oxidative and non-oxidative and can occur either during exposure to stress or during the recovery phase. The box inset provides an overview of reduction-oxidation balance in mycobacteria, the way mycobacteria attempt to maintain it, and the damage perturbations to it can cause. Studies in diverse bacterial species have shown that the extent of oxidative stress and damage can affect the cultivation requirements of cells to different levels. High oxygen tension during aerobic growth conditions in microaerophilic *Campylobacter jejuni* causes increases in ROS and in lipid and protein oxidation, and induces formation of coccoidal VBNC cells (Oh et al., 2015). Application of a density-gradient centrifugation technique in *E. coli* found that culturable and non-culturable cells can be separated into two distinct populations, and that proteins in non-culturable cells had increased carbonylation—a hallmark of oxidative damage—in stationary-phase (Desnues et al., 2003) and in heat-stressed cells (Bruhn-Olszewska et al., 2018). Nonthermal plasma (NTP) technology in *Staphylococcus*

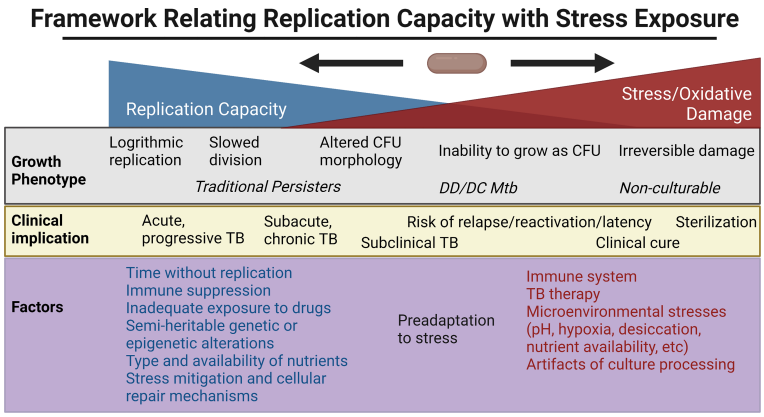


FIGURE 2
Framework for the relationship between replication capacity and stress/oxidative damage, with the related *in vitro* growth phenotype and *in vivo* clinical implication placed along the spectrum. Factors favoring increased replication capacity and increased stress are listed below. Created with BioRender.com.

aureus as an alternative to thermal pasteurization has been shown to cause oxidative stress and induce growth and metabolism quiescence leading to entry into the VBNC state (Liao et al., 2020). A study in *V. parahaemolyticus* further characterized 2 subtypes of VBNC populations, P1 and P2, which are induced by nutrient restriction (PBS starvation) at low temperature 6–8˚C over a period of 50 days (Wagley et al., 2021). 90% of the cells formed a P1 population, defined as small coccoid shaped bacteria that could be resuscitated for up to 14 days. 10% were a P2 population which consisted of bacteria that were large coccoids in shape and could be resuscitated for up to 50 days. While both types of VBNC populations could be resuscitated when subjected to an increase in temperature, the proportion of bacteria which recovered was higher in P2 (100%) as compared to P1 (14%). Proteome analysis of these populations revealed upregulation of the gene encoding lactate dehydrogenase (*lldD*), and an *lldD* deletion mutant entered the VBNC state earlier than the wild-type strain. Addition of sodium lactate resuscitated the VBNC population under conditions when thermal shift methods alone were not adequate. Hong et al. showed that diverse stresses like nalidixic acid, trimethoprim, ampicillin, and heat can induce ROS formation in *E. coli*, that ROS can increase even after the removal of stress, and mitigating ROS improves the recovery of cells (Hong et al., 2019). This indicated that cells could accumulate sub-lethal damage which can be repaired depending on how they are allowed to recover; moreover, it points to a threshold of ROS exposure beyond which the *E. coli* cannot recover replicative capacity. According to one hypothesis, persisters that can replicate normally with the withdrawal of stress differ from the VBNC population that require special cultivation conditions for resuscitation because of the relative amount of protein damage they accumulate (Dewachter et al., 2021). In that study, growth to stationary phase caused depletion of ATP and induced protein aggregation, resulting in the formation of persisters and a VBNC population in *E. coli*. However, while persisters in general exhibited early developmental stage aggregates, the VBNC population had more mature aggregates. This phenomenon of "dormancy depth" has been explored in detail in another study demonstrating that the cell's ability to disintegrate protein aggregates and restore proteostasis by recruiting DnaK-ClpB protein complexes to aggresomes is critical for its survival (Pu et al., 2019).

A similar observation was made in *Staphylococcus aureus* where host induced oxidative stress in the presence of antibiotics caused ATP depletion and induced formation of dormant states of persisters (Peyrusson et al., 2022). The degree of dormancy depth reached differed depending on the host cells, which induced in the bacteria different levels of oxidative stress. In high-oxidative-stress cells (for e.g., human macrophages, stimulated J774 macrophages, PMA-treated monocytes) ROS-induced ATP depletion caused higher protein aggregation, along with the recruitment of the DnaK-ClpB chaperone system. On the other hand, low-oxidative-stress cells (for example, untreated monocytes, unstimulated J774 macrophages epithelial cells, and osteoblasts) hosted a limited fraction of dormant persisters. The key difference between persisters isolated from low vs high-oxidative-stress cells was in the lag time before they resumed growth in liquid medium. Persisters from high-oxidative-stress cells took more time to regrow compared to those isolated from low-oxidative-stress cells, hypothesized to be either due to different levels of metabolism or the need for repair before regrowth. As in the *ex vivo* studies, time without replication appears to be a critical requirement for eventual growth in some phenotypes.

In mycobacteria, a relationship between exposure to stress and growth delay has been noted for decades. Studies in the

BOX Overview of redox balance within mycobacterial cells

Redox stress and balance

The redox environment of the cell is the sum total of the states of different redox couples as well as of the mechanisms which regulate the levels of these species, and disruptions to this environment can affect the culturability of Mtb (Sikri et al., 2018; Saito et al., 2021). More specifically, Sikri et al. reported the induction of stated Mtb upon treatment with vitamin C. This was accompanied by generation of non-cidal concentrations of H_2O_2, induction of ROS scavenging mechanisms, up-regulation of anabolic pathways of triacylglycerol (TAG) and sulfolipid (SL-1) synthesis. They also observed a reductive shift in intra-mycobacterial mycothiol redox potential upon vitamin C treatment in infected THP-1 cells. Saito et al., on the other hand, reported the formation of DD Mtb upon heat treatment, desiccation and starvation followed by rifampicin treatment. They further explored the mechanisms leading to the formation of DD Mtb and found production of ROS (superoxide), an oxidative shift in intra-mycobacterial mycothiol redox potential, and oxidative damage to major macromolecules and upregulation of oxidative stress response genes. Numerous studies have shed light on how the redox environment inside the cell affects Mtb's response to various stress conditions like starvation, antibiotic treatment, acidic pH, hypoxia, nitrosative stress and during infection (Pacl et al., 2018; Mehta and Singh, 2019; Mishra et al., 2019; Mishra et al., 2021). Any imbalance in this environment can cause oxidative or reductive stress (Kumar et al., 2011). Oxidative stress is defined as an increase in the levels reactive oxygen species (ROS) over and above the mechanisms which detoxify them. Oxidative stress can be caused by diverse species of reactive oxygen molecules like superoxide radical ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2), hydroxide ions (HO^-), organic hydroperoxides (ROOH), peroxyl radical ($RO_2^{\bullet}$) and alkoxyl radical ($RO^{\bullet}$). Reductive stress results from the accumulation of reducing equivalents of NADH, NADPH, $FADH_2$, MSH and EGH. These phenomena lie at the two extremes of the redox milieu. Increases in oxidative stress can lead to oxidative damage, affecting a cell's ability to repair and replicate. On the other hand, increases in reductive stress can cause growth arrest, and an increase in drug tolerance and virulence phenotypes (Singh et al., 2009; Bhaskar et al., 2014; Trivedi et al., 2016; Mishra et al., 2019; Shee et al., 2022). In a recent study, Shee et al. showed that moxifloxacin treatment causes reductive stress in Mtb as displayed by the rise in NADH/NAD^+ ratio, which in turn increases the labile, reduced form of Fe. This then fuels a Fenton reaction leading to ROS production. Addition of *N*-acetyl cysteine augmented ROS accumulation and apparent lethality when combined with moxifloxacin. High concentrations of cysteine had previously been shown to lead to increased levels of H_2O_2 (1.5- and 12-fold respectively for intracellular and extracellular H_2O_2) (Vilcheze et al., 2017). The combination of cysteine and isoniazid in that study shifted cells to a more reduced state with a higher menaquinol/menaquinone ratio and greater H_2O_2 levels (6- and 28-fold respectively for intracellular and extracellular H_2O_2) The role of reductive stress has also been studied in different aspects of Mtb biology (Trivedi et al., 2016; Coulson et al., 2017) and reviewed extensively elsewhere (Farhana et al., 2010; Mavi et al., 2020; Mishra et al., 2021; Singh et al., 2022). Thus, Mtb needs to sustain a delicate balance among different oxidizing and reducing species to maintain redox homeostasis.

Protective mechanisms

Mtb possess different mechanisms to deal with redox stress. These can be in the form of redox buffering systems like mycothiol, ergothioneine, thioredoxins and Dsb disulfide oxidoreductases. Mycothiol and ergothioneine are two low-molecular-weight thiols present in mycobacteria and they exist as oxidized–reduced redox couples (Cumming et al., 2018; Reyes et al., 2018). A mutant of mycothiol showed significantly more protein carbonylation and lipid peroxidation (Singh et al., 2016). Mtb also has thiol reductant proteins known as thioredoxins and Dsb disulfide oxidoreductases (Lu and Holmgren, 2014; Lin et al., 2016). Along with small molecules they are responsible for maintaining a reducing intracellular environment. Mtb also has antioxidant enzymes which can directly detoxify ROS, like catalase peroxidase (against H_2O_2), alkyl hydroperoxide reductases (against alkyl hydroperoxides) and superoxide dismutases (against $O_2^{\bullet -}$). Increasing evidence has shown the non-conventional role of metabolic enzymes in countering oxidative stress, as seen with the alpha-ketoglutarate (alpha-KG) dehydrogenase complex (KDHC) and isocitrate lyase (Nandakumar et al., 2014; Maksymiuk et al., 2015). Redox mediated transcriptional regulators like DosT/DosS, whiBs (whiB3, whiB4, whiB7), PknG also play a key role in regulating pathways to counter the deleterious effect of damage caused by oxidative stress (Morris et al., 2005; Singh et al., 2007; Singh et al., 2009; Chawla et al., 2012; Mishra et al., 2017; Khan et al., 2017).

Damage to macromolecules

Oxidative stress can damage all classes of cellular macromolecules. Damage to DNA can be *via* oxidation of guanine leading to formation of 8-oxo-dG (OG) or oxidation of dCTP leading to DNA breakage (Foti et al., 2012; Vilcheze et al., 2013; Fan et al., 2018). Proteins can undergo oxidative modification which can affect their activity (Hillion and Antelmann, 2015; Hillion et al., 2017). Cysteine oxidation leads to the formation of sulfenic acids, sulfinic acid or sulfonic acid (Ezraty et al., 2017). Methionine can be oxidized to methionine sulfoxide, methionine sulfone or methionine sulfone. Proline, lysine, threonine, and arginine are prone to carbonylation (Nystrom, 2005). Cysteine, lysine, and histidine can also undergo carbonylation by reacting with carbonyl compounds on carbohydrates and lipids (Frank et al., 2002; Grimsrud et al., 2008). Irreversibly damaged proteins forms aggregates which cannot be degraded by proteasome and inhibits its activity. Oxidative damage to lipids can lead to the production of lipid peroxides affecting the function of cell membrane and cell wall where they are most abundantly found.

1960s found that Mtb exposed to TB drugs such as streptomycin and INH could induce days of delay in replication and, in the case of rifampin, with as little as 2 hours of exposure (Dickinson and Mitchison, 1970). In sputa from patients with TB, non-replicating Mtb containing lipid bodies have been found in patient sputa, and this phenotype was reproduced *in vitro* in response to hypoxic stress. In that study, the percentage of AFB with lipid bodies positively correlated with time to positivity in BACTEC liquid cultures (Garton et al., 2008). Automated image analysis of individual colonies from sputum samples of patients undergoing treatment for pulmonary TB found that persisters demonstrated longer lag times in colony formation compared with bacilli which are rapidly eliminated by TB therapy (Barr et al., 2016). Furthermore, counts of longer lag-time colonies (>20 days) declined more slowly than shorter lag-time colonies. *In vitro* work in *Mycobacterium smegmatis* (Msm) found that incubation in mild nutrient starvation or suboptimal growth media to stationary phase created small cells that displayed growth lag or non-culturability (Shleeva et al., 2004; Wu et al., 2016b). Similar findings were reported in Mtb that underwent gradual acidification in stationary phase, leading first to persistence, and then to an eventual DD Mtb state requiring Rpfs for resuscitation (Shleeva et al., 2011). A recent study from our group has shown Mtb can enter the DD state under diverse stresses, including with nutrient starvation followed by rifampin treatment, heat stress at 45°C or desiccation (Saito et al., 2021). We found that the formation of this population was linked to the presence of an intermediate amount of oxidative stress—rifampin exposure produced levels of ROS and an oxidative shift in cell state that were above other antibiotics that did not produce DD Mtb, such as levofloxacin, but below levels of a direct RNA polymerase inhibitor (a Nα-aroyl-N-aryl-

phenylalaninamide compound) that apparently killed extensively and had no recoverable DD Mtb. *M. bovis* in the same conditions also suffered higher levels of oxidative stress than Mtb, and had no recoverable DD organisms. The results further suggested that a cell's ability to prevent or repair that damage altered its culturability. Cultivating DD Mtb after serial dilution in liquid nutrient rich media created a longer lag time, and we speculate this time allowed the DD Mtb to repair damage and resume cell division (Figure 2). Prolonged incubation in nutrient-deficient PBS similarly prevented regrowth and allowed cells to reverse the levels of oxidative DNA damage to the pre-antibiotic exposure state. These cells eventually regained culturability even on agar plates. See Table 1 for a summary of *in vitro* DD Mtb work; Figure 3 highlights the different mechanisms influencing growth heterogeneity in Mtb. While promising, much work remains to determine how the *in vitro* studies relate to *in vivo* findings; discrepancies in, for example, antibiotic response kinetics between one *in vitro* model of persistence and clinical sputum have been noted (Faraj et al., 2020).

Genetic and epigenetic factors

We have thus far noted the significant variabilities in mycobacterial growth between liquid and solid media, but bacilli can also demonstrate heterogeneity even as it grows as CFU. Generally, mycobacteria are known to grow as rough and dry, nonpigmented colonies. However, small colony variants (SCVs) and those with different colony morphologies can arise in response to host environments or drugs, as seen and studied in other bacteria such as *Staphylococcus aureus* (Proctor et al., 2006; Vulin et al., 2018). These morphologies can signal variability relevant to the behavior of the bacteria *in vivo*. Rough and smooth colony phenotypes have been identified in *Mycobacterium abscessus*, where the former stimulates the human macrophage innate immune response through TLR2, while the latter does not. This response is stimulated in part through phosphatidyl-myo-inositol mannosides which are present in both the variants but masked in the smooth variant by glycopeptidolipids present in their outermost portion of cell wall (Rhoades et al., 2009). This allows the smooth variant to restrict intraphagosomal acidification and induces less apoptosis and autophagy as compared to rough variant which induces the formation of autophagic vacuoles, rapidly acidification and apoptosis (Roux et al., 2016). The rough morphotype of *M. abscessus* forms clumps, unlike the smooth morphotype. Upon phagocytosis, the rough morphotype overwhelms the bactericidal capacities of J774 macrophages, killing them 72 hours after infection —while the macrophages infected with smooth morphotypes do not die even after 96 hours (Brambilla et al., 2016). The rough morphotype also produces proinflammatory cytokines and granuloma-like structures, while smooth morphotype does not. The rough colony morphotype of *Mycobacterium avium* also seems to be highly virulent in human macrophages and mice (Nishimura et al., 2020).

In Mtb, Safi et al. recently reported the presence of a sub-population of SCVs with a smooth morphology mixed with the large colony variants (LCVs) in clinical isolates of both drug sensitive and resistant Mtb strains. Whole genome sequencing of the SCVs identified frameshift mutations in *glpK* that disrupt its function. These mutations are reversible, and the mutants are tolerant to drugs and oxidative stress. They seem to represent a stress response which is activated when Mtb is starved for glycerol by either frameshifting *glpK* or by depleting glycerol from the medium (Safi et al., 2019). A similar mechanism of drug tolerance was found in reversible frameshift mutations in the Mtb *orn* gene which also produced SCVs (Safi et al., 2020). SCVs can also arise because of epigenetic regulation whereby posttranslational modification (lysine acetylation and methylation) of nucleoid-associated protein HupB results in a sub-population that exhibit heritable but semi-stable drug resistance (Sakatos et al., 2018).

While this review focuses on heterogeneity in growth in mycobacteria, and not phenotypic drug resistance per se, the interplay cannot be ignored. As previously mentioned, the mechanisms underlying the ability for persisters to survive drugs and their relationship to growth rate has been debated. Our discussion regarding the roles for stress and stress response in drug tolerance and growth rate point toward further questions that have been raised about these phenotypes. How much of the phenomenon is driven by direct damage to cellular processes, and how much is due to an adaptive response to that damage? (Nystrom, 2001) (Manina and McKinney, 2013) How much of any particular growth or drug resistance phenotype exists prior to a new stress in a stochastic manner, and how much is that subpopulation formed in response to the stress? How do the cells switch phenotypes (Dhar and McKinney, 2007)? Recent advances in microfluidics have shed light on some events which cause heterogeneity at the level of single cell (Santi et al., 2013). For example, using microfluidics, it was found that stochastic changes in the expression of catalase-peroxidase, *katG*, are negatively correlated with cell survival after INH treatment—but that growth rate prior to INH treatment was not correlated with the persistence phenotype (Wakamoto et al., 2013). Changes that cause drug tolerance among replicating mycobacterial subpopulations have also been described in bacilli subject to host induced stresses (Adams et al., 2011; Mishra et al., 2019). Adams et al. found that bacterial efflux pumps are responsible for the induction of drug tolerance in *Mycobacterium marinum* and Mtb during their replication inside zebrafish and macrophages, respectively. Mishra et al. extensively explored the role of redox heterogeneity in mediating drug tolerance in Mtb during infection. They found multiple mechanisms responsible for the induction of drug tolerance in a subpopulation exhibiting reduced mycothiol redox potential caused by phagosomal acidification. These included

TABLE 1 *In vitro* and *ex vivo* studies of differentially detectable Mtb and DD Msm.

	In vitro or *Ex vivo*	Description of the study	Reference
1	*In vitro*	Mtb isolated from murine peritoneal macrophages	(Biketov et al., 2000)
2	*In vitro/In vivo*	Stationary phase Mtb incubated for 100 days in microaerophilic conditions then treated with rifampin; mice infected with Mtb, then treated with pyrazinamide and isoniazid	(Hu et al., 2000)
3	*In vitro*	In Mtb after growth in Sauton's medium and prolonged incubation in stationary phase	(Shleeva et al., 2002)
4	*In vitro*	In Msm from stationary phase	(Shleeva et al., 2004)
5	*In vitro*	Wild type and the *rpf* deletion mutant Mtb strains aged in Sauton's medium without oxygen	(Downing et al., 2005)
6	*In vitro*	In Msm after prolonged storage at room temperature in a nitrogen-limited minimal medium	(Anuchin et al., 2009)
7	*In vitro*	In Mtb after prolonged incubation in Sauton's medium	(Salina et al., 2009)
8	*Ex vivo*	In sputum cultures of patients with TB before treatment, with and without Rpf supplementation	(Mukamolova et al., 2010)
9	*In vitro*	In Mtb after prolonged incubation in acidified Sauton's medium	(Shleeva et al., 2011)
10	*Ex vivo*	Viable but non-cultivable Mtb in pulmonary and extra-pulmonary samples	(Cubero et al., 2013)
11	*Ex vivo*	In sputum cultures of patients with TB before and during early treatment, without Rpf supplementation	(Dhillon et al., 2014)
12	*In vitro*	In Mtb grown in potassium-deficient media	(Salina et al., 2014)
13	*In vitro*	In lungs of mice chronically infected with *Mtb*	(Manina et al., 2015)
14	*Ex vivo*	Mtb in sputum from patient samples	(Chengalroyen et al., 2016)
15	*Ex vivo*	In bioaerosols from untreated TB patients	(Patterson et al., 2017)
16	*In vitro*	In PBS starved, rifampicin treated Mtb	(Saito et al., 2017)
17	*Ex vivo*	In patient sputum before and during treatment in Haitian cohort	(McAulay et al., 2018)
18	*Ex vivo*	In extrapulmonary tuberculosis samples from patients recruited before the onset of chemotherapy	(Rosser et al., 2018)
19	*In vitro*	In Mtb exposed to vitamin C	(Sikri et al., 2018)
20	*In vitro*	In Mtb under lipid diet model	(Khan et al., 2019)
21	*In vitro*	Nitrite induces non-cultivability in Mtb	(Gample et al., 2019)
22	*In vitro*	Diphenyleneiodonium, an inhibitor of NADH oxidase, induced a viable, but non-culturable state in mycobacteria	(Yeware et al., 2019)
23	*In vitro*	In clinical isolates under Wayne's model of hypoxia	(Dusthackeer et al., 2019)
24	*Ex vivo*	In pre- and post-treatment sputum samples from TB patients	(Almeida Junior et al., 2020)
25	*Ex vivo*	In induced sputum and bronchoalveolar lavage fluid from patients after therapy	(Beltran et al., 2020)
26	*Ex vivo*	Sputum from treatment naïve HIV-TB co-infected individuals	(McIvor et al., 2021)
27	*Ex vivo*	In the sputum of patients with drug-sensitive or drug-resistant TB	(Zainabadi et al., 2021)
28	*Ex vivo*	Sputum from individuals with drug susceptible TB	(Gordhan et al., 2021)
29	*Ex vivo*	Drug-Resistant Mtb in sputum from patients which only grew in glycerol-poor/lipid-rich medium	(Mesman et al., 2021)
30	*In vitro*	Mtb exposed to mild heat stress; Mtb desiccated on filters	(Saito et al., 2021)
31	*In vitro*	In Mtb from lung tissues derived from infected mice under specific drug treatments	(Evangelopoulos et al., 2022).

upregulation of genes encoding efflux pumps, oxidative stress response, DNA repair, protein quality control, envelope stress, sulfur metabolism, and SAM-dependent methyl transferases.

Single cell studies do, however, show growth rate diversity under *in vitro* growth conditions that is further amplified by stress conditions associated with infection and drug pressure (Manina et al., 2015). For example, a subpopulation of non-growing but metabolically active bacteria are found in chronically infected mice (Manina and McKinney, 2013; Manina et al., 2015). In another study, survival of

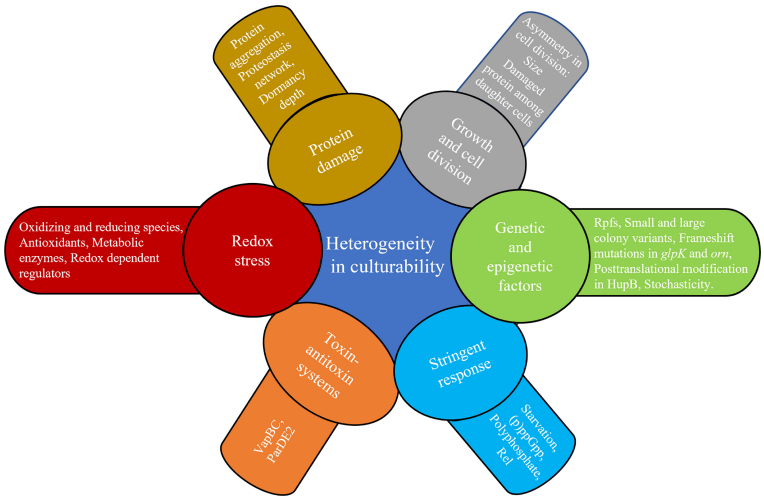


FIGURE 3
Factors that can introduce heterogeneity in culturability.

mycobacterial cells upon exposure to ciprofloxacin was found to be correlated with the highly heterogenous rate of intermittent pulsing of *recA* expression—with increased pulsing prior to antibiotic exposure suggesting a response to greater spontaneous DNA damage. The work underscores the complexity of survival mechanisms and the influence of baseline phenotypic heterogeneity: highly pulsing cells grew at slower rates but lead to a higher likelihood of death upon exposure to drug, and yet overall the survivors exhibited significantly lower growth rates and 20% stalled replication entirely before drug exposure (Manina et al., 2019). The phenotypes of persistence cannot be binned based solely on growth parameters or stress response markers, and a nuanced approach is needed. Combining micro co-culture systems with single cell microfluidics may reveal more answers regarding bacterial behavior during unexplored aspects of host-pathogen interaction (Delince et al., 2016; Toniolo et al., 2018).

What seems clear now is that the mechanisms underlying stochastic, deterministic, and semi-heritable growth phenotypes —and their relationship to the ability to survive drug exposure— can be incredibly complex and specific. For example, Msm exposed to usually lethal doses of rifampin and observed by microscopy found multiple potential fates for any particular bacillus—not only could a cell have arrested growth, it could also continue to divide and yield daughter cells that were then either capable of division or not (Zhu et al., 2018). These growth phenotypes in the face of rifampin were dependent on individual accumulation of RpoB, the target of rifampin, after rifampin exposure. This, in turn, was determined by initial survival of rifampin exposure—perhaps by stochastic mechanisms—followed by upregulation of *rpoB* by the differential impact of rifampin on the two *rpoB* promoters. This particular mechanism of bacillary diversity in growth is thus specific to rifampin, has profoundly different effects on apparently genetically identical bacilli, and can affect subsequent generations.

Asymmetric growth and division

Another source of heterogeneity on the single cell level is that mycobacteria grow and divide asymmetrically (Aldridge et al., 2012) (Joyce et al., 2012; Vijay et al., 2012; Vijay et al., 2014a; Vijay et al., 2014b; Rego et al., 2017; Priestman et al., 2017; Logsdon and Aldridge, 2018; Ufimtseva et al., 2019). The resultant cell size variability has been observed in *in vitro* models as well as in clinical samples (Vijay et al., 2017b; Ufimtseva et al., 2019; Pradhan et al., 2021). Differences in cell size yield differential susceptibilities to host and antibiotic stresses, which can in turn affect their survival and culturability (Richardson et al., 2016; Vijay et al., 2017a; Vijay et al., 2017b). For example, even log phase cultures of Msm and Mtb have been found to contain 2 sub-populations of cells which differ in size and density. Percoll density gradient centrifugation separated them into 2 distinct fractions—short-sized cells (SCs) and normal/long-sized cells (NCs). SCs were found to be more susceptible than NCs to antibiotics (rifampin and isoniazid), H_2O_2, and acidified $NaNO_2$. Additionally, drug resistant bacteria display a distinct mode of cell division and cell length heterogeneity when compared with drug sensitive bacteria (Vijay et al., 2017b; Jakkala et al., 2020). Notably, mycobacteria exhibit a unique kind of heterogeneity under stress where it asymmetrically distributes irreversibly oxidized proteins within bacteria and between their progeny (Vaubourgeix et al., 2015). Another study done primarily in

Msm was able to decrease the heterogeneity in cell size and growth rate by knocking out *lamA*, which was found to inhibit growth asymmetrically during the cell cycle (Rego et al., 2017). This in turn led to increased uniformity of killing by drugs that target the cell wall as well as less variability in growth rates in response to different concentrations of rifampin. These studies suggest that targeting mechanisms of heterogeneity may be vital to reducing treatment durations and improving relapse-free cure rates.

Stringent response

The stringent response refers to a signaling system initiated by bacteria after encountering stress conditions (Irving et al., 2021). ppGpp (guanosine tetraphosphate) is an alarmone produced by bacteria which regulates the stringent response and is synthesized by the protein Rel in Mtb (Avarbock et al., 1999). Multiple studies have shed light on the role played by ppGpp in diverse mycobacterial species (Chakrabarty, 1998; Ojha et al., 2000; Primm et al., 2000; Manganelli, 2007; Wu et al., 2016a; Prusa et al., 2018; Bhaskar et al., 2018; Danchik et al., 2021; Hunt-Serracin et al., 2022). In other organisms, the stringent response is known to play an important role in the formation of the VBNC population. For e.g. *E. coli* mutants lacking ppGpp are less efficient in entering VBNC state compared to overproducers of ppGpp (Boaretti et al., 2003). Mutant of polyphosphate kinase 1 in *Campylobacter jejuni* deficient in polyphosphate was compromised in its ability to form VBNC (Ayrapetyan et al., 2015). Mutants lacking the *rel* gene were also unable to form a DD Mtb population in nutrient-starved Mtb exposed to rifampin, highlighting the role played by the stringent response in affecting culturability in mycobacteria (Saito et al., 2021).

Toxin–antitoxin systems

Toxin-antitoxin (TA) systems have been identified in both bacteria and archaea (Gerdes et al., 2005; Yamaguchi et al., 2011), and play important roles in both physiology as well as pathogenesis. Mtb has more than 80 TA systems (Ramage et al., 2009; Sala et al., 2014) and many of them remain uncharacterized. TA systems have been implicated in the formation of a VBNC population in bacterial species like *E. coli* and *V. cholerae* (Ayrapetyan et al., 2015). In mycobacteria, transcriptome analysis of Mtb persisters showed upregulation of 10 TA modules (Keren et al., 2011). TA systems have also been implicated in the formation of a DD population in mycobacteria (Demidenok et al., 2014; Gupta et al., 2016). For example, overexpression of VapC toxin resulted in the production of ovoid cells which became non-culturable under potassium limiting conditions, while overexpression of VapB antitoxin prevented transition to this state. (Demidenok et al., 2014). Msm expressing the *parDE2* operon during oxidative stress also led to entry into the VBNC phenotype (Gupta et al., 2016). The same was found upon overexpression of parE2 toxin. Over-expression of MazF6 toxin resulted in a transcriptional profile which had significant overlap with the transcriptome of non-culturable cells (Ramirez et al., 2013). As we gain a better understanding of the role played by different TA systems in mycobacterial biology, future studies will reveal the mechanisms behind how they exert influence on culturability both *in vitro* and *in vivo*.

Conclusion

The act of replication, while seemingly consistent and reproducible in a bulk, logarithmically-dividing mycobacterial culture, is heterogenous when examined more closely—and even more dynamic and variable in physiologic and artificially stressed conditions. This has profound clinical and research implications. In this review, we have scratched the surface of our increasingly nuanced understanding of growth phenotypes, as well as the strengths and limitations of our current culture-based tools to assess mycobacterial replication. Complexity further increases when deeply considering host-pathogen interactions in both clinical manifestations (e.g., in children, in people living with HIV, in patients who have received BCG vaccination, etc) and laboratory settings (e.g., animal and other infection models).

Heterogeneity in growth as has been described here is not specific to TB disease or even to infectious diseases, and many parallels have been drawn to cutting edge research in cancer (Glickman and Sawyers, 2012; Lupoli et al., 2018). For example, a recently published study of lung cancer cells found wide heterogeneity among the population that survived drug treatment, including a rare population of persisters that divided several times despite drug pressure. These “cycling” persisters demonstrated a strong link with antioxidant gene signatures and less ROS levels as compared to non-cycling brethren, and offers tantalizing insight into relapse and time to recurrence in cancer (Oren et al., 2021). Similar studies of mycobacterial population structure at the single cell level have revealed comparable complexity, and at a higher resolution than bulk culture methods allow—further refinement and application of these techniques may be critical to further clinical breakthroughs (Bussi and Gutierrez, 2019). Applying more granular assessment of mycobacteria as they move across stresses and transition between phenotypes may reveal significant clues as to how bacilli maintain or restore replicative capacity in the varied and harsh environments of its life cycle. This line of inquiry has a high likelihood of discovering mycobacterial vulnerabilities that may then be exploited clinically. That the infective dose$_{50}$ is fewer than 10

bacilli mandates that a thorough understanding of TB infection and relapse cannot ignore the replication dynamics of even the smallest of subpopulations.

Author contributions

KS and SM researched, wrote and edited the manuscript and figures. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by NIH grant K08 AI139360 to KS.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Adams, K. N., Takaki, K., Connolly, L. E., Wiedenhoft, H., Winglee, K., Humbert, O., et al. (2011). Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. *Cell* 145 (1), 39–53. doi: 10.1016/j.cell.2011.02.022

Agency, E. M. (2017) *Addendum to the note for guidance on evaluation of medicinal products indicated for treatment of bacterial infections to specifically address the clinical development of new agents to treat disease due to mycobacterium tuberculosis*. Available at: https://www.ema.europa.eu/en/addendum-note-guidance-evaluation-medicinal-products-indicated-treatment-bacterial-infections.

Alcántara, R., Fuentes, P., Marin, L., Kirwan, D. E., Gilman, R. H., Zimic, M., et al. (2020). Direct determination of pyrazinamide (PZA) susceptibility by sputum microscopic observation drug susceptibility (MODS) culture at neutral pH: the MODS-PZA assay. *J. Clin. Microbiol.* 58 (5):e01165–19. doi: 10.1128/jcm.01165-19

Aldridge, B. B., Fernandez-Suarez, M., Heller, D., Ambravaneswaran, V., Irimia, D., Toner, M., et al. (2012). Asymmetry and aging of mycobacterial cells lead to variable growth and antibiotic susceptibility. *Science* 335 (6064), 100–104. doi: 10.1126/science.1216166

Almeida Junior, P. S., Schmidt Castellani, L. G., Peres, R. L., Combadao, J., Tristao, T. C., Dietze, R., et al. (2020). Differentially culturable tubercle bacteria dynamics during standard anti-tuberculosis treatment: A prospective cohort study. *Tuberc. (Edinb)* 124, 101945. doi: 10.1016/j.tube.2020.101945

Anuchin, A. M., Mulyukin, A. L., Suzina, N. E., Duda, V. I., El-Registan, G. I., and Kaprelyants, A. S. (2009). Dormant forms of *Mycobacterium smegmatis* with distinct morphology. *Microbiol. (Reading)* 155 (Pt 4), 1071–1079. doi: 10.1099/mic.0.023028-0

Asadi, L., Croxen, M., Heffernan, C., Dhillon, M., Paulsen, C., Egedahl, M. L., et al. (2022). How much do smear-negative patients really contribute to tuberculosis transmissions? re-examining an old question with new tools. *EClinicalMedicine* 43, 101250. doi: 10.1016/j.eclinm.2021.101250

Asmar, S., Chatellier, S., Mirande, C., Belkum, A. V., Canard, I., Raoult, D., et al. (2015). A novel solid medium for culturing *Mycobacterium tuberculosis* isolates from clinical specimens. *J. Clin. Microbiol.* 53 (8), 2566–2569. doi: 10.1128/JCM.01149-15

Augustine, F. B., Cheng, A. F., Tai, V. H., Li, M. S., Chan, C. H., Wong, C. F., et al. (1999). Improved recovery of mycobacterium tuberculosis from pleural aspirates: Bedside inoculation, heparinized containers and liquid culture media. *Scand. J. Infect. Dis.* 31 (5), 485–487. doi: 10.1080/00365549950164012

Avarbock, D., Salem, J., Li, L. S., Wang, Z. M., and Rubin, H. (1999). Cloning and characterization of a bifunctional RelA/SpoT homologue from *Mycobacterium tuberculosis*. *Gene* 233 (1-2), 261–269. doi: 10.1016/s0378-1119(99)00114-6

Ayrapetyan, M., Williams, T. C., and Oliver, J. D. (2015). Bridging the gap between viable but non-culturable and antibiotic persistent bacteria. *Trends Microbiol.* 23 (1), 7–13. doi: 10.1016/j.tim.2014.09.004

Baker, C. J. (2020). *Red book atlas of pediatric infectious diseases* (Itasca, IL: American Academy of Pediatrics).

Balaban, N. Q., Gerdes, K., Lewis, K., and McKinney, J. D. (2013). A problem of persistence: still more questions than answers? *Nat. Rev. Microbiol.* 11 (8), 587–591. doi: 10.1038/nrmicro3076

Balaban, N. Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D. I., et al. (2019). Definitions and guidelines for research on antibiotic persistence. *Nat. Rev. Microbiol.* 17 (7), 441–448. doi: 10.1038/s41579-019-0196-3

Bark, C. M., Gitta, P., and Johnson, J. L. (2014). Differences in EBA in the first two days of standard anti-tuberculosis treatment in different geographic regions. *Int. J. Tuberc. Lung Dis.* 18 (8), 1006–1007. doi: 10.5588/ijtld.13.0826

Bark, C. M., Thiel, B. A., and Johnson, J. L. (2012). Pretreatment time to detection of *Mycobacterium tuberculosis* in liquid culture is associated with relapse after therapy. *J. Clin. Microbiol.* 50 (2), 538–538. doi: 10.1128/JCM.06193-11

Barr, D. A., Kamdolozi, M., Nishihara, Y., Ndhlovu, V., Khonga, M., Davies, G. R., et al. (2016). Serial image analysis of *Mycobacterium tuberculosis* colony growth reveals a persistent subpopulation in sputum during treatment of pulmonary TB. *Tuberc. (Edinb)* 98, 110–115. doi: 10.1016/j.tube.2016.03.001

Behr, M. A., Warren, S. A., Salamon, H., Hopewell, P. C., Ponce de Leon, A., Daley, C. L., et al. (1999). Transmission of *Mycobacterium tuberculosis* from patients smear-negative for acid-fast bacilli. *Lancet* 353 (9151), 444–449. doi: 10.1016/s0140-6736(98)03406-0

Beltran, C. G. G., Heunis, T., Gallant, J., Venter, R., du Plessis, N., Loxton, A. G., et al. (2020). Investigating non-sterilizing cure in TB patients at the end of successful anti-TB therapy. *Front. Cell Infect. Microbiol.* 10. doi: 10.3389/fcimb.2020.00443

Bhaskar, A., Chawla, M., Mehta, M., Parikh, P., Chandra, P., Bhave, D., et al. (2014). Reengineering redox sensitive GFP to measure mycothiol redox potential of *Mycobacterium tuberculosis* during infection. *PloS Pathog.* 10 (1), e1003902. doi: 10.1371/journal.ppat.1003902

Bhaskar, A., De Piano, C., Gelman, E., McKinney, J. D., and Dhar, N. (2018). Elucidating the role of (p)ppGpp in mycobacterial persistence against antibiotics. *IUBMB Life* 70 (9), 836–844. doi: 10.1002/iub.1888

Biketov, S., Mukamolova, G. V., Potapov, V., Gilenkov, E., Vostroknutova, G., Kell, D. B., et al. (2000). Culturability of *Mycobacterium tuberculosis* cells isolated from murine macrophages: a bacterial growth factor promotes recovery. *FEMS Immunol. Med. Microbiol.* 29 (4), 233–240. doi: 10.1111/j.1574-695X.2000.tb01528.x

Blair, E. B., Brown, G. L., and Tull, A. H. (1976). Computer files and analyses of laboratory data from tuberculosis patients. II. analyses of six years' data on sputum specimens. *Am. Rev. Respir. Dis.* 113 (4), 427–432. doi: 10.1164/arrd.1976.113.4.427

Bloom, B. R., and McKinney, J. D. (1999). The death and resurrection of tuberculosis. *Nat. Med.* 5 (8), 872–874. doi: 10.1038/11309

Boaretti, M., Lleo, M. M., Bonato, B., Signoretto, C., and Canepari, P. (2003). Involvement of rpoS in the survival of *Escherichia coli* in the viable but non-culturable state. *Environ. Microbiol.* 5 (10), 986–996. doi: 10.1046/j.1462-2920.2003.00497.x

Bowness, R., Boeree, M. J., Aarnoutse, R., Dawson, R., Diacon, A., Mangu, C., et al. (2015). The relationship between *Mycobacterium tuberculosis* MGIT time to positivity and cfu in sputum samples demonstrates changing bacterial phenotypes potentially reflecting the impact of chemotherapy on critical sub-populations. *J. Antimicrob. Chemother.* 70 (2), 448–455. doi: 10.1093/jac/dku415

Brambilla, C., Llorens-Fons, M., Julian, E., Noguera-Ortega, E., Tomas-Martinez, C., Perez-Trujillo, M., et al. (2016). Mycobacteria clumping increase their capacity to damage macrophages. *Front. Microbiol.* 7. doi: 10.3389/fmicb.2016.01562

Bruhn-Olszewska, B., Szczepaniak, P., Matuszewska, E., Kuczynska-Wisnik, D., Stojowska-Swedrzynska, K., Moruno Algara, M., et al. (2018). Physiologically distinct subpopulations formed in *Escherichia coli* cultures in response to heat shock. *Microbiol. Res.* 209, 33–42. doi: 10.1016/j.micres.2018.02.002

Bussi, C., and Gutierrez, M. G. (2019). *Mycobacterium tuberculosis* infection of host cells in space and time. *FEMS Microbiol. Rev.* 43 (4), 341–361. doi: 10.1093/femsre/fuz006

Canetti, G. (1955). *The tubercle bacillus in the pulmonary lesion of man; histobacteriology and its bearing on the therapy of pulmonary tuberculosis* (New York: Springer Pub. Co).

Cascina, A., Fietta, A., and Casali, L. (2000). Is a Large number of sputum specimens necessary for the bacteriological diagnosis of tuberculosis? *J. Clin. Microbiol.* 38 (1), 466–466. doi: 10.1128/JCM.38.1.466-466.2000

Caviedes, L., Lee, T.-S., Gilman, R. H., Sheen, P., Spellman, E., Lee, E. H., et al. (2000). Rapid, efficient detection and drug susceptibility testing of *Mycobacterium tuberculosis* in sputum by microscopic observation of broth cultures. *J. Clin. Microbiol.* 38 (3), 1203–1208. doi: 10.1128/JCM.38.3.1203-1208.2000

Chakrabarty, A. M. (1998). Nucleoside diphosphate kinase: role in bacterial growth, virulence, cell signalling and polysaccharide synthesis. *Mol. Microbiol.* 28 (5), 875–882. doi: 10.1046/j.1365-2958.1998.00846.x

Chawla, M., Parikh, P., Saxena, A., Munshi, M., Mehta, M., Mai, D., et al. (2012). *Mycobacterium tuberculosis* WhiB4 regulates oxidative stress response to modulate survival and dissemination *in vivo*. *Mol. Microbiol.* 85 (6), 1148–1165. doi: 10.1111/j.1365-2958.2012.08165.x

Chengalroyen, M. D., Beukes, G. M., Gordhan, B. G., Streicher, E. M., Churchyard, G., Hafner, R., et al. (2016). Detection and quantification of differentially culturable tubercle bacteria in sputum from patients with tuberculosis. *Am. J. Respir. Crit. Care Med.* 194 (12), 1532–1540. doi: 10.1164/rccm.201604-0769OC

Colangeli, R., Jedrey, H., Kim, S., Connell, R., Ma, S., Chippada Venkata, U. D., et al. (2018). Bacterial factors that predict relapse after tuberculosis therapy. *N Engl. J. Med.* 379 (9), 823–833. doi: 10.1056/NEJMoa1715849

Cornfield, D. B., Beavis, K. G., Greene, J. A., Bojak, M., and Bondi, J. (1997). Mycobacterial growth and bacterial contamination in the mycobacteria growth indicator tube and BACTEC 460 culture systems. *J. Clin. Microbiol.* 35 (8), 2068–2071. doi: 10.1128/jcm.35.8.2068-2071.1997

Coulson, G. B., Johnson, B. K., Zheng, H., Colvin, C. J., Fillinger, R. J., Haiderer, E. R., et al. (2017). Targeting mycobacterium tuberculosis sensitivity to thiol stress at acidic pH kills the bacterium and potentiates antibiotics. *Cell Chem. Biol.* 24 (8), 993–1004.e1004. doi: 10.1016/j.chembiol.2017.06.018

Cruciani, M., Scarparo, C., Malena, M., Bosco, O., Serpelloni, G., and Mengoli, C. (2004). Meta-analysis of BACTEC MGIT 960 and BACTEC 460 TB, with or without solid media, for detection of mycobacteria. *J. Clin. Microbiol.* 42 (5), 2321–2325. doi: 10.1128/JCM.42.5.2321-2325.2004

Cubero, N., Esteban, J., Palenque, E., Rosell, A., and Garcia, M. J. (2013). Evaluation of the detection of *Mycobacterium tuberculosis* with metabolic activity in culture-negative human clinical samples. *Clin. Microbiol. Infect.* 19 (3), 273–278. doi: 10.1111/j.1469-0691.2012.03779.x

Cumming, B. M., Chinta, K. C., Reddy, V. P., and Steyn, A. J. C. (2018). Role of ergothioneine in microbial physiology and pathogenesis. *Antioxid. Redox Signal* 28 (6), 431–444. doi: 10.1089/ars.2017.7300

Daley, C. L., Iaccarino, J. M., Lange, C., Cambau, E., Wallace, R. J., Andrejak, C., et al. (2020). Treatment of nontuberculous mycobacterial pulmonary disease: An official ATS/ERS/ESCMID/IDSA clinical practice guideline. *Clin. Infect. Dis.* 71 (4), e1–e36. doi: 10.1093/cid/ciaa241

Damato, J. J., Collins, M. T., Rothlauf, M. V., and McClatchy, J. K. (1983). Detection of mycobacteria by radiometric and standard plate procedures. *J. Clin. Microbiol.* 17 (6), 1066–1073. doi: 10.1128/jcm.17.6.1066-1073.1983

Danchik, C., Wang, S., and Karakousis, P. C. (2021). Targeting the *Mycobacterium tuberculosis* stringent response as a strategy for shortening tuberculosis treatment. *Front. Microbiol.* 12. doi: 10.3389/fmicb.2021.744167

Dartois, V. A., and Rubin, E. J. (2022). Anti-tuberculosis treatment strategies and drug development: challenges and priorities. *Nat. Rev. Microbiol.* 20, 685–701. doi: 10.1038/s41579-022-00731-y

Delince, M. J., Bureau, J. B., Lopez-Jimenez, A. T., Cosson, P., Soldati, T., and McKinney, J. D. (2016). A microfluidic cell-trapping device for single-cell tracking of host-microbe interactions. *Lab. Chip* 16 (17), 3276–3285. doi: 10.1039/c6lc00649c

Demidenok, O. I., Kaprelyants, A. S., and Goncharenko, A. V. (2014). Toxin-antitoxin vapBC locus participates in formation of the dormant state in *Mycobacterium smegmatis*. *FEMS Microbiol. Lett.* 352 (1), 69–77. doi: 10.1111/1574-6968.12380

Desnues, B., Cuny, C., Gregori, G., Dukan, S., Aguilaniu, H., and Nystrom, T. (2003). Differential oxidative damage and expression of stress defence regulons in culturable and non-culturable *Escherichia coli* cells. *EMBO Rep.* 4 (4), 400–404. doi: 10.1038/sj.embor.embor799

Dewachter, L., Bollen, C., Wilmaerts, D., Louwagie, E., Herpels, P., Matthay, P., et al. (2021). The dynamic transition of persistence toward the viable but nonculturable state during stationary phase is driven by protein aggregation. *mBio* 12 (4), e0070321. doi: 10.1128/mBio.00703-21

Dhar, N., and McKinney, J. D. (2007). Microbial phenotypic heterogeneity and antibiotic tolerance. *Curr. Opin. Microbiol.* 10 (1), 30–38. doi: 10.1016/j.mib.2006.12.007

Dhillon, J., Fourie, P. B., and Mitchison, D. A. (2014). Persister populations of *Mycobacterium tuberculosis* in sputum that grow in liquid but not on solid culture media. *J. Antimicrob. Chemother.* 69 (2), 437–440. doi: 10.1093/jac/dkt357

Diacon, A. H., and Donald, P. R. (2014). The early bactericidal activity of antituberculosis drugs. *Expert Rev. Anti Infective Ther.* 12 (2), 223–237. doi: 10.1586/14787210.2014.870884

Diacon, A. H., Maritz, J. S., Venter, A., van Helden, P. D., Andries, K., McNeeley, D. F., et al. (2010). Time to detection of the growth of *Mycobacterium tuberculosis* in MGIT 960 for determining the early bactericidal activity of antituberculosis agents. *Eur. J. Clin. Microbiol. Infect. Dis.* 29 (12), 1561–1565. doi: 10.1007/s10096-010-1043-7

Dickinson, J. M., and Mitchison, D. A. (1970). Suitability of rifampicin for intermittent administration in the treatment of tuberculosis. *Tubercle* 51 (1), 82–94. doi: 10.1016/0041-3879(70)90131-5

DiNardo, A. R., Detjen, A., Ustero, P., Ngo, K., Bacha, J., and Mandalakas, A. M. (2016). Culture is an imperfect and heterogeneous reference standard in pediatric tuberculosis. *Tuberculosis* 101, S105–S108. doi: 10.1016/j.tube.2016.09.021

Dorman, S. E., Nahid, P., Kurbatova, E. V., Phillips, P. P. J., Bryant, K., Dooley, K. E., et al. (2021). Four-month rifapentine regimens with or without moxifloxacin for tuberculosis. *New Engl. J. Med.* 384 (18), 1705–1718. doi: 10.1056/NEJMoa2033400

Downing, K. J., Mischenko, V. V., Shleeva, M. O., Young, D. I., Young, M., Kaprelyants, A. S., et al. (2005). Mutants of *Mycobacterium tuberculosis* lacking three of the five rpf-like genes are defective for growth *in vivo* and for resuscitation *in vitro*. *Infect. Immun.* 73 (5), 3038–3043. doi: 10.1128/IAI.73.5.3038-3043.2005

Dusthackeer, A., Balasubramanian, M., Shanmugam, G., Priya, S., Nirmal, C. R., Sam Ebenezer, R., et al. (2019). Differential culturability of *Mycobacterium tuberculosis* in culture-negative sputum of patients with pulmonary tuberculosis and in a simulated model of dormancy. *Front. Microbiol.* 10, 2381. doi: 10.3389/fmicb.2019.02381

Epstein, M. D., Schluger, N. W., Davidow, A. L., Bonk, S., Rom, W. N., and Hanna, B. (1998). Time to detection of *Mycobacterium tuberculosis* in sputum culture correlates with outcome in patients receiving treatment for pulmonary tuberculosis. *Chest* 113 (2), 379–386. doi: 10.1378/chest.113.2.379

Evangelopoulos, D., Shoen, C. M., Honeyborne, I., Clark, S., Williams, A., Mukamolova, G. V., et al. (2022). Culture-free enumeration of *Mycobacterium tuberculosis* in mouse tissues using the molecular bacterial load assay for preclinical drug development. *Microorganisms* 10 (2). doi: 10.3390/microorganisms10020460

Ezraty, B., Gennaris, A., Barras, F., and Collet, J. F. (2017). Oxidative stress, protein damage and repair in bacteria. *Nat. Rev. Microbiol.* 15 (7), 385–396. doi: 10.1038/nrmicro.2017.26

Fan, X. Y., Tang, B. K., Xu, Y. Y., Han, A. X., Shi, K. X., Wu, Y. K., et al. (2018). Oxidation of dCTP contributes to antibiotic lethality in stationary-phase mycobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 115 (9), 2210–2215. doi: 10.1073/pnas.1719627115

Faraj, A., Clewe, O., Svensson, R. J., Mukamolova, G. V., Barer, M. R., and Simonsson, U. S. H. (2020). Difference in persistent tuberculosis bacteria between *in vitro* and sputum from patients: implications for translational predictions. *Sci. Rep.* 10 (1), 15537. doi: 10.1038/s41598-020-72472-y

Farhana, A., Guidry, L., Srivastava, A., Singh, A., Hondalus, M. K., and Steyn, A. J. (2010). Reductive stress in microbes: implications for understanding *Mycobacterium tuberculosis* disease and persistence. *Adv. Microb. Physiol.* 57, 43–117. doi: 10.1016/B978-0-12-381045-8.00002-3

FDA (2013). *Pulmonary tuberculosis: Developing drugs for treatment*. Silver Spring: Center for Drug Evaluation and Research Food and Drug Administration.

Fenner, F., Martin, S. P., and Pierce, C. H. (1949). The enumeration of viable tubercle bacilli in cultures and infected tissues. *Ann. New York Acad. Sci.* 52 (5), 751–764. doi: 10.1111/j.17496632.1949.tb53966.x

Fitzwater, S. P., Caviedes, L., Gilman, R. H., Coronel, J., LaChira, D., Salazar, C., et al. (2010). Prolonged infectiousness of tuberculosis patients in a directly observed therapy short-course program with standardized therapy. *Clin. Infect. Dis.* 51 (4), 371–378. doi: 10.1086/655127

Fonseca, K. L., Rodrigues, P. N. S., Olsson, I. A. S., and Saraiva, M. (2017). Experimental study of tuberculosis: From animal models to complex cell systems and organoids. *PloS Pathog.* 13 (8), e1006421. doi: 10.1371/journal.ppat.1006421

Foti, J. J., Devadoss, B., Winkler, J. A., Collins, J. J., and Walker, G. C. (2012). Oxidation of the guanine nucleotide pool underlies cell death by bactericidal antibiotics. *Science* 336 (6079), 315–319. doi: 10.1126/science.1219192

Frank, J., Pompella, A., and Biesalski, H. K. (2002). Immunohistochemical detection of protein oxidation. *Methods Mol. Biol.* 196, 35–40. doi: 10.1385/1-59259-274-0:35

Frascella, B., Richards, A. S., Sossen, B., Emery, J. C., Odone, A., Law, I., et al. (2021). Subclinical tuberculosis disease-a review and analysis of prevalence surveys to inform definitions, burden, associations, and screening methodology. *Clin. Infect. Dis.* 73 (3), e830–e841. doi: 10.1093/cid/ciaa1402

Gample, S. P., Agrawal, S., and Sarkar, D. (2019). Evidence of nitrite acting as a stable and robust inducer of non-cultivability in *Mycobacterium tuberculosis* with physiological relevance. *Sci. Rep.* 9 (1), 9261. doi: 10.1038/s41598-019-45652-8

Garton, N. J., Waddell, S. J., Sherratt, A. L., Lee, S. M., Smith, R. J., Senner, C., et al. (2008). Cytological and transcript analyses reveal fat and lazy persister-like bacilli in tuberculous sputum. *PloS Med.* 5 (4), e75. doi: 10.1371/journal.pmed.0050075

Gerdes, K., Christensen, S. K., and Lobner-Olesen, A. (2005). Prokaryotic toxin-antitoxin stress response loci. *Nat. Rev. Microbiol.* 3 (5), 371–382. doi: 10.1038/nrmicro1147

Ghodbane, R., Asmar, S., Betzner, M., Linet, M., Pierquin, J., Raoult, D., et al. (2015). Rapid diagnosis of tuberculosis by real-time high-resolution imaging of *Mycobacterium tuberculosis* colonies. *J. Clin. Microbiol.* 53 (8), 2693–2696. doi: 10.1128/JCM.00684-15

Ghodbane, R., Raoult, D., and Drancourt, M. (2014). Dramatic reduction of culture time of *Mycobacterium tuberculosis*. *Sci. Rep.* 4, 4236. doi: 10.1038/srep04236

Gil, V., Cordero, P. J., Greses, J. V., and Soler, J. J. (1995). Pleural tuberculosis in HIV-infected patients. *Chest* 107 (6), 1775–1776. doi: 10.1378/chest.107.6.1775-b

Gillespie, S. H., Crook, A. M., McHugh, T. D., Mendel, C. M., Meredith, S. K., Murray, S. R., et al. (2014). Four-month moxifloxacin-based regimens for drug-sensitive tuberculosis. *N Engl. J. Med.* 371 (17), 1577–1587. doi: 10.1056/NEJMoa1407426

Glickman, M. S., and Sawyers, C. L. (2012). Converting cancer therapies into cures: lessons from infectious diseases. *Cell* 148, 1089–1098. doi: 10.1016/j.cell.2012.02.015

Gold, B., and Nathan, C. (2017). Targeting phenotypically tolerant *Mycobacterium tuberculosis*. *Microbiol. Spectr.* 5 (1). doi: 10.1128/microbiolspec.TBTB2-0031-2016

Gopi, A., Madhavan, S. M., Sharma, S. K., and Sahn, S. A. (2007). Diagnosis and treatment of tuberculous pleural effusion in 2006. *Chest* 131 (3), 880–889. doi: 10.1378/chest.06-2063

Gordhan, B. G., Peters, J. S., McIvor, A., Machowski, E. E., Ealand, C., Waja, Z., et al. (2021). Detection of differentially culturable tubercle bacteria in sputum using mycobacterial culture filtrates. *Sci. Rep.* 11 (1), 6493. doi: 10.1038/s41598-021-86054-z

Graham, S. M., Cuevas, L. E., Jean-Philippe, P., Browning, R., Casenghi, M., Detjen, A. K., et al. (2015). Clinical case definitions for classification of intrathoracic tuberculosis in children: An update. *Clin. Infect. Dis.* 61 (suppl_3), S179–S187. doi: 10.1093/cid/civ581

Grimsrud, P. A., Xie, H., Griffin, T. J., and Bernlohr, D. A. (2008). Oxidative stress and covalent modification of protein with bioactive aldehydes. *J. Biol. Chem.* 283 (32), 21837–21841. doi: 10.1074/jbc.R700019200

Gupta, M., Nayyar, N., Chawla, M., Sitaraman, R., Bhatnagar, R., and Banerjee, N. (2016). The chromosomal parDE2 toxin-antitoxin system of *Mycobacterium tuberculosis* H37Rv: Genetic and functional characterization. *Front. Microbiol.* 7. doi: 10.3389/fmicb.2016.00886

Hafner, R., Cohn, J. A., Wright, D. J., Dunlap, N. E., Egorin, M. J., Enama, M. E., et al. (1997). Early bactericidal activity of isoniazid in pulmonary tuberculosis. optimization of methodology. the DATRI 008 study group. *Am. J. Respir. Crit. Care Med.* 156 (3 Pt 1), 918–923. doi: 10.1164/ajrccm.156.3.9612016

Harvell, J. D., Hadley, W. K., and Ng, V. L. (2000). Increased sensitivity of the BACTEC 460 mycobacterial radiometric broth culture system does not decrease the number of respiratory specimens required for a definitive diagnosis of pulmonary tuberculosis. *J. Clin. Microbiol.* 38 (10), 3608–3611. doi: 10.1128/JCM.38.10.3608-3611.2000

Heifets, L., Linder, T., Sanchez, T., Spencer, D., and Brennan, J. (2000). Two liquid medium systems, mycobacteria growth indicator tube and MB redox tube, for *Mycobacterium tuberculosis* isolation from sputum specimens. *J. Clin. Microbiol.* 38 (3), 1227–1230. doi: 10.1128/JCM.38.3.1227-1230.2000

Hernandez-Garduno, E., Cook, V., Kunimoto, D., Elwood, R. K., Black, W. A., and FitzGerald, J. M. (2004). Transmission of tuberculosis from smear negative patients: a molecular epidemiology study. *Thorax* 59 (4), 286–290. doi: 10.1136/thx.2003.011759

Hesseling, A. C., Walzl, G., Enarson, D. A., Carroll, N. M., Duncan, K., Lukey, P. T., et al. (2010). Baseline sputum time to detection predicts month two culture conversion and relapse in non-HIV-infected patients. *Int. J. Tuberc. Lung Dis.* 14 (5), 560–570.

Heyckendorf, J., Georghiou, S. B., Frahm, N., Heinrich, N., Kontsevaya, I., Reimann, M., et al. (2022). Tuberculosis treatment monitoring and outcome measures: New interest and new strategies. *Clin. Microbiol. Rev.* 35, e0022721. doi: 10.1128/cmr.00227-21

Hillion, M., and Antelmann, H. (2015). Thiol-based redox switches in prokaryotes. *Biol. Chem.* 396 (5), 415–444. doi: 10.1515/hsz-2015-0102

Hillion, M., Bernhardt, J., Busche, T., Rossius, M., Maass, S., Becher, D., et al. (2017). Monitoring global protein thiol-oxidation and protein s-mycothiolation in *Mycobacterium smegmatis* under hypochlorite stress. *Sci. Rep.* 7 (1), 1195. doi: 10.1038/s41598-017-01179-4

Hong, Y., Zeng, J., Wang, X., Drlica, K., and Zhao, X. (2019). Post-stress bacterial cell death mediated by reactive oxygen species. *Proc. Natl. Acad. Sci. U.S.A.* 116 (20), 10064–10071. doi: 10.1073/pnas.1901730116

Horne, D. J., Royce, S. E., Gooze, L., Narita, M., Hopewell, P. C., Nahid, P., et al. (2010). Sputum monitoring during tuberculosis treatment for predicting outcome: systematic review and meta-analysis. *Lancet Infect. Dis.* 10 (6), 387–394. doi: 10.1016/S1473-3099(10)70071-2

Hu, Y., Mangan, J. A., Dhillon, J., Sole, K. M., Mitchison, D. A., Butcher, P. D., et al. (2000). Detection of mRNA transcripts and active transcription in persistent *Mycobacterium tuberculosis* induced by exposure to rifampin or pyrazinamide. *J. Bacteriol.* 182 (22), 6358–6365. doi: 10.1128/JB.182.22.6358-6365.2000

Hunt-Serracin, A. C., Kazi, M. I., Boll, J. M., and Boutte, C. C. (2022). In *Mycobacterium abscessus*, the stringent factor rel regulates metabolism but is not the only (p)ppGpp synthase. *J. Bacteriol.* 204 (2), e0043421. doi: 10.1128/JB.00434-21

Ichiyama, S., Shimokata, K., and Takeuchi, J. (1993). Comparative study of a biphasic culture system (Roche MB check system) with a conventional egg medium for recovery of mycobacteria. aichi mycobacteriosis research group. *Tuber. Lung Dis.* 74 (5), 338–341. doi: 10.1016/0962-8479(93)90109-b

Irving, S. E., Choudhury, N. R., and Corrigan, R. M. (2021). The stringent response and physiological roles of (pp)pGpp in bacteria. *Nat. Rev. Microbiol.* 19 (4), 256–271. doi: 10.1038/s41579-020-00470-y

Jakkala, K., Paul, A., Pradhan, A., Nair, R. R., Sharan, D., Swaminath, S., et al. (2020). Unique mode of cell division by the mycobacterial genetic resister clones emerging *De novo* from the antibiotic-surviving population. *mSphere* 5 (6):e00994–20. doi: 10.1128/mSphere.00994-20

Jarvis, B., Wilrich, C., and Wilrich, P. T. (2010). Reconsideration of the derivation of most probable numbers, their standard deviations, confidence bounds and rarity values. *J. Appl. Microbiol.* 109 (5), 1660–1667. doi: 10.1111/j.1365-2672.2010.04792.x

Jindani, A., Aber, V. R., Edwards, E. A., and Mitchison, D. A. (1980). The early bactericidal activity of drugs in patients with pulmonary tuberculosis. *Am. Rev. Respir. Dis.* 121 (6), 939–949. doi: 10.1164/arrd.1980.121.6.939

Jindani, A., Dore, C. J., and Mitchison, D. A. (2003). Bactericidal and sterilizing activities of antituberculosis drugs during the first 14 days. *Am. J. Respir. Crit. Care Med.* 167 (10), 1348–1354. doi: 10.1164/rccm.200210-1125OC

Jindani, A., Harrison, T. S., Nunn, A. J., Phillips, P. P., Churchyard, G. J., Charalambous, S., et al. (2014). High-dose rifapentine with moxifloxacin for pulmonary tuberculosis. *N Engl. J. Med.* 371 (17), 1599–1608. doi: 10.1056/NEJMoa1314210

Johnson, J. L., Hadad, D. J., Dietze, R., Maciel, E. L., Sewali, B., Gitta, P., et al. (2009). Shortening treatment in adults with noncavitary tuberculosis and 2-month culture conversion. *Am. J. Respir. Crit. Care Med.* 180 (6), 558–563. doi: 10.1164/rccm.200904-0536OC

Joyce, G., Williams, K. J., Robb, M., Noens, E., Tizzano, B., Shahrezaei, V., et al. (2012). Cell division site placement and asymmetric growth in mycobacteria. *PloS One* 7 (9), e44582. doi: 10.1371/journal.pone.0044582

Kana, B. D., Gordhan, B. G., Downing, K. J., Sung, N., Vostroktunova, G., Machowski, E. E., et al. (2008). The resuscitation-promoting factors of *Mycobacterium tuberculosis* are required for virulence and resuscitation from dormancy but are collectively dispensable for growth *in vitro*. *Mol. Microbiol.* 67 (3), 672–684. doi: 10.1111/j.1365-2958.2007.06078.x

Kendall, E. A., Kitonsa, P. J., Nalutaaya, A., Erisa, K. C., Mukiibi, J., Nakasolya, O., et al. (2021). The spectrum of tuberculosis disease in an urban Ugandan community and its health facilities. *Clin. Infect. Dis.* 72 (12), e1035–e1043. doi: 10.1093/cid/ciaa1824

Keren, I., Minami, S., Rubin, E., and Lewis, K. (2011). Characterization and transcriptome analysis of *Mycobacterium tuberculosis* persisters. *Mbio* 2 (3): e00100–11. doi: 10.1128/mBio.00100-11

Khan, M. Z., Bhaskar, A., Upadhyay, S., Kumari, P., Rajmani, R. S., Jain, P., et al. (2017). Protein kinase G confers survival advantage to *Mycobacterium tuberculosis* during latency-like conditions. *J. Biol. Chem.* 292 (39), 16093–16108. doi: 10.1074/jbc.M117.797563

Khan, S. R., Venugopal, U., Chandra, G., Bharti, S., Maurya, R. K., and Krishnan, M. Y. (2019). Effect of various drugs on differentially detectable persisters of *Mycobacterium tuberculosis* generated by long-term lipid diet. *Tuberc. (Edinb)* 115, 89–95. doi: 10.1016/j.tube.2019.02.007

Kolwijck, E., Mitchell, M., Venter, A., Friedrich, S. O., Dawson, R., and Diacon, A. H. (2013). Short-term storage does not affect the quantitative yield of *Mycobacterium tuberculosis* in sputum in early-bactericidal-activity studies. *J. Clin. Microbiol.* 51 (4), 1094–1098. doi: 10.1128/jcm.02751-12

Kumar, A., Farhana, A., Guidry, L., Saini, V., Hondalus, M., and Steyn, A. J. (2011). Redox homeostasis in mycobacteria: the key to tuberculosis control? *Expert Rev. Mol. Med.* 13, e39. doi: 10.1017/S1462399411002079

Lanoix, J. P., Chaisson, R. E., and Nuermberger, E. L. (2015). Shortening tuberculosis treatment with fluoroquinolones: Lost in translation? *Clin. Infect. Dis.* 62 (4), 484–490. doi: 10.1093/cid/civ911

Lenaerts, A., Barry, C. E.3rd, and Dartois, V. (2015). Heterogeneity in tuberculosis pathology, microenvironments and therapeutic responses. *Immunol. Rev.* 264 (1), 288–307. doi: 10.1111/imr.12252

Levy, H., Feldman, C., Sacho, H., van der Meulen, H., Kallenbach, J., and Koornhof, H. (1989). A reevaluation of sputum microscopy and culture in the diagnosis of pulmonary tuberculosis. *Chest* 95 (6), 1193–1197. doi: 10.1378/chest.95.6.1193

Lewinsohn, D. M., Leonard, M. K., LoBue, P. A., Cohn, D. L., Daley, C. L., Desmond, E., et al. (2017). Official American thoracic Society/Infectious diseases society of America/Centers for disease control and prevention clinical practice guidelines: Diagnosis of tuberculosis in adults and children. *Clin. Infect. Dis.* 64 (2), e1–e33. doi: 10.1093/cid/ciw694

Liao, X., Liu, D., and Ding, T. (2020). Nonthermal plasma induces the viable-but-Nonculturable state in *Staphylococcus aureus via* metabolic suppression and the oxidative stress response. *Appl. Environ. Microbiol.* 86 (5):e02216–19. doi: 10.1128/AEM.02216-19

Lin, K., O'Brien, K. M., Trujillo, C., Wang, R., Wallach, J. B., Schnappinger, D., et al. (2016). *Mycobacterium tuberculosis* thioredoxin reductase is essential for thiol redox homeostasis but plays a minor role in antioxidant defense. *PloS Pathog.* 12 (6), e1005675. doi: 10.1371/journal.ppat.1005675

Logsdon, M. M., and Aldridge, B. B. (2018). Stable regulation of cell cycle events in mycobacteria: Insights from inherently heterogeneous bacterial populations. *Front. Microbiol.* 9. doi: 10.3389/fmicb.2018.00514

Loring, W. W., Melvin, I., Vandiviere, H. M., and Willis, H. S. (1955). The death and resurrection of the tubercle bacillus. *Trans. Am. Clin. Climatol. Assoc.* 67, 132–138.

Lu, J., and Holmgren, A. (2014). The thioredoxin antioxidant system. *Free Radic. Biol. Med.* 66, 75–87. doi: 10.1016/j.freeradbiomed.2013.07.036

Lupoli, T. J., Vaubourgeix, J., Burns-Huang, K., and Gold, B. (2018). Targeting the proteostasis network for mycobacterial drug discovery. *ACS Infect. Dis.* 4 (4), 478–498. doi: 10.1021/acsinfecdis.7b00231

Luzze, H., Elliott A, M., Joloba M, L., Odida, M., Oweka-Onyee, J., Nakiyingi, J., et al. (2001). Evaluation of suspected tuberculous pleurisy: clinical and diagnostic findings in HIV-1-positive and HIV-negative adults in Uganda. *Int. J. Tuberc. Lung Dis.* 5 (8), 746–753.

Maartens, G., and Bateman, E. D. (1991). Tuberculous pleural effusions: increased culture yield with bedside inoculation of pleural fluid and poor diagnostic value of adenosine deaminase. *Thorax* 46 (2), 96–99. doi: 10.1136/thx.46.2.96

Mac Kenzie, W. R., Heilig, C. M., Bozeman, L., Johnson, J. L., Muzanye, G., Dunbar, D., et al. (2011). Geographic differences in time to culture conversion in liquid media: Tuberculosis trials consortium study 28. culture conversion is delayed in Africa. *PloS One* 6 (4), e18358. doi: 10.1371/journal.pone.0018358

Maksymiuk, C., Balakrishnan, A., Bryk, R., Rhee, K. Y., and Nathan, C. F. (2015). E1 of alpha-ketoglutarate dehydrogenase defends *Mycobacterium tuberculosis* against glutamate anaplerosis and nitroxidative stress. *Proc. Natl. Acad. Sci. U.S.A.* 112 (43), E5834–E5843. doi: 10.1073/pnas.1510932112

Manganelli, R. (2007). Polyphosphate and stress response in mycobacteria. *Mol. Microbiol.* 65 (2), 258–260. doi: 10.1111/j.1365-2958.2007.05819.x

Manina, G., Dhar, N., and McKinney, J. D. (2015). Stress and host immunity amplify *Mycobacterium tuberculosis* phenotypic heterogeneity and induce nongrowing metabolically active forms. *Cell Host Microbe* 17 (1), 32–46. doi: 10.1016/j.chom.2014.11.016

Manina, G., Griego, A., Singh, L. K., McKinney, J. D., and Dhar, N. (2019). Preexisting variation in DNA damage response predicts the fate of single mycobacteria under stress. *EMBO J.* 38 (22), e101876. doi: 10.15252/embj.2019101876

Manina, G., and McKinney, J. D. (2013). A single-cell perspective on non-growing but metabolically active (NGMA) bacteria. *Curr. Top. Microbiol. Immunol.* 374, 135–161. doi: 10.1007/82_2013_333

Mavi, P. S., Singh, S., and Kumar, A. (2020). Reductive stress: New insights in physiology and drug tolerance of mycobacterium. *Antioxid. Redox Signal* 32 (18), 1348–1366. doi: 10.1089/ars.2019.7867

McAulay, K., Saito, K., Warrier, T., Walsh, K. F., Mathurin, L. D., Royal-Mardi, G., et al. (2018). Differentially detectable *Mycobacterium tuberculosis* cells in sputum from treatment-naive subjects in Haiti and their proportionate increase after initiation of treatment. *MBio* 9 (6):e02192–18. doi: 10.1128/mBio.02192-18

McCune, R. M., Feldmann, F. M., Lambert, H. P., and McDermott, W. (1966). Microbial persistence : I. the capacity of tubercle bacilli to survive sterilization in mouse tissues. *J. Exp. Med.* 123 (3), 445–468. doi: 10.1084/jem.123.3.445

McIvor, A., Gordhan, B. G., Waja, Z., Otwombe, K., Martinson, N. A., and Kana, B. D. (2021). Supplementation of sputum cultures with culture filtrate to detect tuberculosis in a cross-sectional study of HIV-infected individuals. *Tuberc. (Edinb)* 129, 102103. doi: 10.1016/j.tube.2021.102103

Mehta, M., and Singh, A. (2019). *Mycobacterium tuberculosis* WhiB3 maintains redox homeostasis and survival in response to reactive oxygen and nitrogen species. *Free Radic. Biol. Med.* 131, 50–58. doi: 10.1016/j.freeradbiomed.2018.11.032

Merle, C. S., Fielding, K., Sow, O. B., Gninafon, M., Lo, M. B., Mthiyane, T., et al. (2014). A four-month gatifloxacin-containing regimen for treating tuberculosis. *New Engl. J. Med.* 371 (17), 1588–1598. doi: 10.1056/NEJMoa1315817

Mesman, A. W., Baek, S. H., Huang, C. C., Kim, Y. M., Cho, S. N., Ioerger, T. R., et al. (2021). Characterization of drug-resistant lipid-dependent differentially detectable *Mycobacterium tuberculosis*. *J. Clin. Med.* 10 (15). doi: 10.3390/jcm10153249

Minion, J., Leung, E., Menzies, D., and Pai, M. (2010). Microscopic-observation drug susceptibility and thin layer agar assays for the detection of drug resistant tuberculosis: a systematic review and meta-analysis. *Lancet Infect. Dis.* 10 (10), 688–698. doi: 10.1016/s1473-3099(10)70165-1

Mishra, R., Kohli, S., Malhotra, N., Bandyopadhyay, P., Mehta, M., Munshi, M., et al. (2019). Targeting redox heterogeneity to counteract drug tolerance in replicating *Mycobacterium tuberculosis*. *Sci. Transl. Med.* 11 (518). doi: 10.1126/scitranslmed.aaw6635

Mishra, S., Shukla, P., Bhaskar, A., Anand, K., Baloni, P., Jha, R. K., et al. (2017). Efficacy of beta-lactam/beta-lactamase inhibitor combination is linked to WhiB4-mediated changes in redox physiology of *Mycobacterium tuberculosis*. *Elife* 6. doi: 10.7554/eLife.25624

Mishra, R., Yadav, V., Guha, M., and Singh, A. (2021). Heterogeneous host-pathogen encounters coordinate antibiotic resilience in *Mycobacterium tuberculosis*. *Trends Microbiol.* 29 (7), 606–620. doi: 10.1016/j.tim.2020.10.013

Monkongdee, P., McCarthy, K. D., Cain, K. P., Tasaneeyapan, T., Dung, N. H., Lan, N. T. N., et al. (2009). Yield of acid-fast smear and mycobacterial culture for tuberculosis diagnosis in people with human immunodeficiency virus. *Am. J. Respir. Crit. Care Med.* 180 (9), 903–908. doi: 10.1164/rccm.200905-0692OC

Moore, D. A., Evans, C. A., Gilman, R. H., Caviedes, L., Coronel, J., Vivar, A., et al. (2006). Microscopic-observation drug-susceptibility assay for the diagnosis of TB. *N Engl. J. Med.* 355 (15), 1539–1550. doi: 10.1056/NEJMoa055524

Moore, D. A., Mendoza, D., Gilman, R. H., Evans, C. A., Hollm Delgado, M. G., Guerra, J., et al. (2004). Microscopic observation drug susceptibility assay, a rapid, reliable diagnostic test for multidrug-resistant tuberculosis suitable for use in resource-poor settings. *J. Clin. Microbiol.* 42 (10), 4432–4437. doi: 10.1128/jcm.42.10.4432-4437.2004

Morgan, M. A., Horstmeier, C. D., DeYoung, D. R., and Roberts, G. D. (1983). Comparison of a radiometric method (BACTEC) and conventional culture media for recovery of mycobacteria from smear-negative specimens. *J. Clin. Microbiol.* 18 (2), 384–388. doi: 10.1128/jcm.18.2.384-388.1983

Morris, R. P., Nguyen, L., Gatfield, J., Visconti, K., Nguyen, K., Schnappinger, D., et al. (2005). Ancestral antibiotic resistance in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U.S.A.* 102 (34), 12200–12205. doi: 10.1073/pnas.0505446102

Mukamolova, G. V., Kaprelyants, A. S., Young, D. I., Young, M., and Kell, D. B. (1998). A bacterial cytokine. *Proc. Natl. Acad. Sci. U.S.A.* 95 (15), 8916–8921. doi: 10.1073/pnas.95.15.8916

Mukamolova, G. V., Turapov, O., Malkin, J., Woltmann, G., and Barer, M. R. (2010). Resuscitation-promoting factors reveal an occult population of tubercle

bacilli in sputum. *Am. J. Respir. Crit. Care Med.* 181 (2), 174–180. doi: 10.1164/rccm.200905-0661OC

Mukamolova, G. V., Turapov, O. A., Young, D. I., Kaprelyants, A. S., Kell, D. B., and Young, M. (2002). A family of autocrine growth factors in *Mycobacterium tuberculosis*. *Mol. Microbiol.* 46 (3), 623–635. doi: 10.1046/j.1365-2958.2002.03184.x

Nahid, P., Mase, S. R., Migliori, G. B., Sotgiu, G., Bothamley, G. H., Brozek, J. L., et al. (2019). Treatment of drug-resistant tuberculosis. an official ATS/CDC/ERS/IDSA clinical practice guideline. *Am. J. Respir. Crit. Care Med.* 200 (10), e93–e142. doi: 10.1164/rccm.201909-1874ST

Nandakumar, M., Nathan, C., and Rhee, K. Y. (2014). Isocitrate lyase mediates broad antibiotic tolerance in *Mycobacterium tuberculosis*. *Nat. Commun.* 5, 4306. doi: 10.1038/ncomms5306

Nikitushkin, V. D., Demina, G. R., Shleeva, M. O., Guryanova, S. V., Ruggiero, A., Berisio, R., et al. (2015). A product of RpfB and RipA joint enzymatic action promotes the resuscitation of dormant mycobacteria. *FEBS J.* 282 (13), 2500–2511. doi: 10.1111/febs.13292

Nishimura, T., Shimoda, M., Tamizu, E., Uno, S., Uwamino, Y., Kashimura, S., et al. (2020). The rough colony morphotype of *Mycobacterium avium* exhibits high virulence in human macrophages and mice. *J. Med. Microbiol.* 69 (7), 1020–1033. doi: 10.1099/jmm.0.001224

Nystrom, T. (2001). Not quite dead enough: on bacterial life, culturability, senescence, and death. *Arch. Microbiol.* 176 (3), 159–164. doi: 10.1007/s002030100314

Nystrom, T. (2005). Role of oxidative carbonylation in protein quality control and senescence. *EMBO J.* 24 (7), 1311–1317. doi: 10.1038/sj.emboj.7600599

O'Shea, M. K., Koh, G. C. K. W., Munang, M., Smith, G., Banerjee, A., and Dedicoat, M. (2014). Time-to-Detection in culture predicts risk of *Mycobacterium tuberculosis* transmission: A cohort study. *Clin. Infect. Dis.* 59 (2), 177–185. doi: 10.1093/cid/ciu244

Oh, E., McMullen, L., and Jeon, B. (2015). Impact of oxidative stress defense on bacterial survival and morphological change in *Campylobacter jejuni* under aerobic conditions. *Front. Microbiol.* 6. doi: 10.3389/fmicb.2015.00295

Ojha, A. K., Mukherjee, T. K., and Chatterji, D. (2000). High intracellular level of guanosine tetraphosphate in *Mycobacterium smegmatis* changes the morphology of the bacterium. *Infect. Immun.* 68 (7), 4084–4091. doi: 10.1128/IAI.68.7.4084-4091.2000

Olaru, I. D., Heyckendorf, J., Grossmann, S., and Lange, C. (2014). Time to culture positivity and sputum smear microscopy during tuberculosis therapy. *PloS One* 9 (8), e106075. doi: 10.1371/journal.pone.0106075

Oren, Y., Tsabar, M., Cuoco, M. S., Amir-Zilberstein, L., Cabanos, H. F., Hutter, J. C., et al. (2021). Cycling cancer persister cells arise from lineages with distinct programs. *Nature* 596 (7873), 576–582. doi: 10.1038/s41586-021-03796-6

Pacl, H. T., Reddy, V. P., Saini, V., Chinta, K. C., and Steyn, A. J. C. (2018). Host-pathogen redox dynamics modulate *Mycobacterium tuberculosis* pathogenesis. *Pathog. Dis.* 76 (5). doi: 10.1093/femspd/fty036

Palaci, M., Ueki, S. Y., Sato, D. N., Telles, M. A. D. S., Curcio, M., and Silva, E. A. (1996). Evaluation of mycobacteria growth indicator tube for recovery and drug susceptibility testing of *Mycobacterium tuberculosis* isolates from respiratory specimens. *J. Clin. Microbiol.* 34 (3), 762–764. doi: 10.1128/jcm.34.3.762-764.1996

Panel on Guidelines for the Prevention and Treatment of Opportunistic Infections in Adults and Adolescents with HIV *Guidelines for the prevention and treatment of opportunistic infections in adults and adolescents with HIV. national institutes of health, centers for disease control and prevention, HIV medicine association, and infectious diseases society of America*. Available at: https://clinicalinfo.hiv.gov/en/guidelines/adult-and-adolescent-opportunistic-infection (Accessed October 2022).

Paramasivan, C. N., Narayana, A. S. L., Prabhakar, R., Rajagopal, M. S., Somasundaram, P. R., and Tripathy, S. P. (1983). Effect of storage of sputum specimens at room temperature on smear and culture results. *Tubercle* 64 (2), 119–124. doi: 10.1016/0041-3879(83)90036-3

Patterson, B., Morrow, C., Singh, V., Moosa, A., Gqada, M., Woodward, J., et al. (2017). Detection of *Mycobacterium tuberculosis* bacilli in bio-aerosols from untreated TB patients. *Gates Open Res.* 1, 11. doi: 10.12688/gatesopenres.12758.2

Peyrusson, F., Nguyen, T. K., Najdovski, T., and Van Bambeke, F. (2022). Host cell oxidative stress induces dormant *Staphylococcus aureus* persisters. *Microbiol. Spectr.* 10 (1), e0231321. doi: 10.1128/spectrum.02313-21

Pfyffer, G. E., Welscher, H. M., Kissling, P., Cieslak, C., Casal, M. J., Gutierrez, J., et al. (1997). Comparison of the mycobacteria growth indicator tube (MGIT) with radiometric and solid culture for recovery of acid-fast bacilli. *J. Clin. Microbiol.* 35 (2), 364–368. doi: 10.1128/jcm.35.2.364-368.1997

Pheiffer, C., Carroll, N. M., Beyers, N., Donald, P., Duncan, K., Uys, P., et al. (2008). Time to detection of *Mycobacterium tuberculosis* in BACTEC systems as a viable alternative to colony counting. *Int. J. Tuberc. Lung Dis.* 12 (7), 792–798.

Phillips, P. P. J., Mendel, C. M., Burger, D. A., Crook, A. M., Nunn, A. J., Dawson, R., et al. (2016). Limited role of culture conversion for decision-making in individual patient care and for advancing novel regimens to confirmatory clinical trials. *BMC Med.* 14 (1), 19. doi: 10.1186/s12916-016-0565-y

Pradhan, A., Mukkayyan, N., Jakkala, K., and Ajitkumar, P. (2021). Mycobacterial populations partly change the proportions of the cells undergoing Asymmetric/Symmetric divisions in response to glycerol levels in growth medium. *Cells* 10 (5). doi: 10.3390/cells10051160

Priestman, M., Thomas, P., Robertson, B. D., and Shahrezaei, V. (2017). Mycobacteria modify their cell size control under Sub-optimal carbon sources. *Front. Cell Dev. Biol.* 5. doi: 10.3389/fcell.2017.00064

Primm, T. P., Andersen, S. J., Mizrahi, V., Avarbock, D., Rubin, H., and Barry, C. (2000). The stringent response of mycobacterium tuberculosis is required for long-term survival. *J. Bacteriol.* 182 (17), 4889–4898. doi: 10.1128/JB.182.17.4889-4898.2000

Proctor, R. A., von Eiff, C., Kahl, B. C., Becker, K., McNamara, P., Herrmann, M., et al. (2006). Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat. Rev. Microbiol.* 4 (4), 295–305. doi: 10.1038/nrmicro1384

Prusa, J., Zhu, D. X., and Stallings, C. L. (2018). The stringent response and *Mycobacterium tuberculosis* pathogenesis. *Pathog. Dis.* 76 (5). doi: 10.1093/femspd/fty054

Pu, Y., Li, Y., Jin, X., Tian, T., Ma, Q., Zhao, Z., et al. (2019). ATP-dependent dynamic protein aggregation regulates bacterial dormancy depth critical for antibiotic tolerance. *Mol. Cell* 73 (1), 143–156.e144. doi: 10.1016/j.molcel.2018.10.022

Ramage, H. R., Connolly, L. E., and Cox, J. S. (2009). Comprehensive functional analysis of *Mycobacterium tuberculosis* toxin-antitoxin systems: implications for pathogenesis, stress responses, and evolution. *PloS Genet.* 5 (12), e1000767. doi: 10.1371/journal.pgen.1000767

Ramirez, M. V., Dawson, C. C., Crew, R., England, K., and Slayden, R. A. (2013). MazF6 toxin of *Mycobacterium tuberculosis* demonstrates antitoxin specificity and is coupled to regulation of cell growth by a soj-like protein. *BMC Microbiol.* 13. doi: 10.1186/1471-2180-13-240

Rego, E. H., Audette, R. E., and Rubin, E. J. (2017). Deletion of a mycobacterial divisome factor collapses single-cell phenotypic heterogeneity. *Nature* 546 (7656), 153–157. doi: 10.1038/nature22361

Reyes, A. M., Pedre, B., De Armas, M. I., Tossounian, M. A., Radi, R., Messens, J., et al. (2018). Chemistry and redox biology of mycothiol. *Antioxid. Redox Signal* 28 (6), 487–504. doi: 10.1089/ars.2017.7074

Rhoades, E. R., Archambault, A. S., Greendyke, R., Hsu, F. F., Streeter, C., and Byrd, T. F. (2009). *Mycobacterium abscessus* glycopeptidolipids mask underlying cell wall phosphatidyl-myo-inositol mannosides blocking induction of human macrophage TNF-alpha by preventing interaction with TLR2. *J. Immunol.* 183 (3), 1997–2007. doi: 10.4049/jimmunol.0802181

Richardson, K., Bennion, O. T., Tan, S., Hoang, A. N., Cokol, M., and Aldridge, B. B. (2016). Temporal and intrinsic factors of rifampicin tolerance in mycobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 113 (29), 8302–8307. doi: 10.1073/pnas.1600372113

Ritchie, S. R., Harrison, A. C., Vaughan, R. H., Calder, L., and Morris, A. J. (2007). New recommendations for duration of respiratory isolation based on time to detect *Mycobacterium tuberculosis* in liquid culture. *Eur. Respir. J.* 30 (3), 501–507. doi: 10.1183/09031936.00131406

Romanowski, K., Balshaw, R. F., Benedetti, A., Campbell, J. R., Menzies, D., Ahmad Khan, F., et al. (2019). Predicting tuberculosis relapse in patients treated with the standard 6-month regimen: an individual patient data meta-analysis. *Thorax* 74 (3), 291–297. doi: 10.1136/thoraxjnl-2017-211120

Rosser, A., Pareek, M., Turapov, O., Wiselka, M. J., and Mukamolova, G. V. (2018). Differentially culturable tubercule bacilli are generated during nonpulmonary tuberculosis infection. *Am. J. Respir. Crit. Care Med.* 197 (6), 818–821. doi: 10.1164/rccm.201705-1048LE

Rosser, A., Stover, C., Pareek, M., and Mukamolova, G. V. (2017). Resuscitation-promoting factors are important determinants of the pathophysiology in *Mycobacterium tuberculosis* infection. *Crit. Rev. Microbiol.* 43 (5), 621–630. doi: 10.1080/1040841X.2017.1283485

Roux, A. L., Viljoen, A., Bah, A., Simeone, R., Bernut, A., Laencina, L., et al. (2016). The distinct fate of smooth and rough *Mycobacterium abscessus* variants inside macrophages. *Open Biol.* 6 (11). doi: 10.1098/rsob.160185

Rubin, E. J. (2018). Of MICs and men. *N Engl. J. Med.* 379 (9), 882–883. doi: 10.1056/NEJMe1808564

Rubin, E. J., and Mizrahi, V. (2021). Shortening the short course of tuberculosis treatment. *N Engl. J. Med.* 384 (18), 1764–1765. doi: 10.1056/NEJMe2104499

Rustomjee, R., Diacon, A. H., Allen, J., Venter, A., Reddy, C., Patientia, R. F., et al. (2008). Early bactericidal activity and pharmacokinetics of the diarylquinoline

TMC207 in treatment of pulmonary tuberculosis. *Antimicrob. Agents Chemother.* 52 (8), 2831–2835. doi: 10.1128/AAC.01204-07

Safi, H., Gopal, P., Lingaraju, S., Ma, S., Levine, C., Dartois, V., et al. (2019). Phase variation in *Mycobacterium tuberculosis* glpK produces transiently heritable drug tolerance. *Proc. Natl. Acad. Sci. U.S.A.* 116 (39), 19665–19674. doi: 10.1073/pnas.1907631116

Safi, H., Lingaraju, S., Ma, S., Husain, S., Hoque, M., Soteropoulos, P., et al. (2020). Rapidly correcting frameshift mutations in the *Mycobacterium tuberculosis* orn gene produce reversible ethambutol resistance and small-Colony-Variant morphology. *Antimicrob. Agents Chemother.* 64 (9), e00213–20. doi: 10.1128/AAC.00213-20

Saito, K., Mishra, S., Warrier, T., Cicchetti, N., Mi, J., Weber, E., et al. (2021). Oxidative damage and delayed replication allow viable *Mycobacterium tuberculosis* to go undetected. *Sci. Transl. Med.* 13 (621), eabg2612. doi: 10.1126/scitranslmed.abg2612

Saito, K., Warrier, T., Somersan-Karakaya, S., Kaminski, L., Mi, J., Jiang, X., et al. (2017). Rifamycin action on RNA polymerase in antibiotic-tolerant *Mycobacterium tuberculosis* results in differentially detectable populations. *Proc. Natl. Acad. Sci. U.S.A.* 114 (24), E4832–E4840. doi: 10.1073/pnas.1705385114

Sakatos, A., Babunovic, G. H., Chase, M. R., Dills, A., Leszyk, J., Rosebrock, T., et al. (2018). Posttranslational modification of a histone-like protein regulates phenotypic resistance to isoniazid in mycobacteria. *Sci. Adv.* 4 (5), eaao1478. doi: 10.1126/sciadv.aao1478

Sala, A., Bordes, P., and Genevaux, P. (2014). Multiple toxin-antitoxin systems in *Mycobacterium tuberculosis*. *Toxins (Basel)* 6 (3), 1002–1020. doi: 10.3390/toxins6031002

Salina, E. G., Mollenkopf, H. J., Kaufmann, S. H., and Kaprelyants, A. S. (2009). *M. tuberculosis* gene expression during transition to the "Non-culturable" state. *Acta Naturae* 1 (2), 73–77.

Salina, E. G., Waddell, S. J., Hoffmann, N., Rosenkrands, I., Butcher, P. D., and Kaprelyants, A. S. (2014). Potassium availability triggers *Mycobacterium tuberculosis* transition to, and resuscitation from, non-culturable (dormant) states. *Open Biol.* 4 (10). doi: 10.1098/rsob.140106

Santi, I., Dhar, N., Bousbaine, D., Wakamoto, Y., and McKinney, J. D. (2013). Single-cell dynamics of the chromosome replication and cell division cycles in mycobacteria. *Nat. Commun.* 4, 2470. doi: 10.1038/ncomms3470

Sarathy, J. P., and Dartois, V. (2020). Caseum: a niche for *Mycobacterium tuberculosis* drug-tolerant persisters. *Clin. Microbiol. Rev.* 33 (3):e00159–19. doi: 10.1128/CMR.00159-19

Schrader, S. M., Vaubourgeix, J., and Nathan, C. (2020). Biology of antimicrobial resistance and approaches to combat it. *Sci. Transl. Med.* 12 (549). doi: 10.1126/scitranslmed.aaz6992

Shah, N. S., Moodley, P., Babaria, P., Moodley, S., Ramtahal, M., Richardson, J., et al. (2011). Rapid diagnosis of tuberculosis and multidrug resistance by the microscopic-observation drug-susceptibility assay. *Am. J. Respir. Crit. Care Med.* 183 (10), 1427–1433. doi: 10.1164/rccm.201009-1449OC

Shee, S., Singh, S., Tripathi, A., Thakur, C., Kumar, T. A., Das, M., et al. (2022). Moxifloxacin-mediated killing of *Mycobacterium tuberculosis* involves respiratory downshift, reductive stress, and accumulation of reactive oxygen species. *Antimicrob. Agents Chemother.* 66 (9), e0059222. doi: 10.1128/aac.00592-22

Shleeva, M. O., Bagramyan, K., Telkov, M. V., Mukamolova, G. V., Young, M., Kell, D. B., et al. (2002). Formation and resuscitation of "non-culturable" cells of rhodococcus rhodochrous and *Mycobacterium tuberculosis* in prolonged stationary phase. *Microbiol. (Reading)* 148 (Pt 5), 1581–1591. doi: 10.1099/00221287-148-5-1581

Shleeva, M. O., Kudykina, Y. K., Vostroknutova, G. N., Suzina, N. E., Mulyukin, A. L., and Kaprelyants, A. S. (2011). Dormant ovoid cells of *Mycobacterium tuberculosis* are formed in response to gradual external acidification. *Tuberc. (Edinb)* 91 (2), 146–154. doi: 10.1016/j.tube.2010.12.006

Shleeva, M., Mukamolova, G. V., Young, M., Williams, H. D., and Kaprelyants, A. S. (2004). Formation of 'non-culturable' cells of *Mycobacterium smegmatis* in stationary phase in response to growth under suboptimal conditions and their rpf-mediated resuscitation. *Microbiol. (Reading)* 150 (Pt 6), 1687–1697. doi: 10.1099/mic.0.26893-0

Sikri, K., Duggal, P., Kumar, C., Batra, S. D., Vashist, A., Bhaskar, A., et al. (2018). Multifaceted remodeling by vitamin c boosts sensitivity of *Mycobacterium tuberculosis* subpopulations to combination treatment by anti-tubercular drugs. *Redox Biol.* 15, 452–466. doi: 10.1016/j.redox.2017.12.020

Singh, A., Crossman, D. K., Mai, D., Guidry, L., Voskuil, M. I., Renfrow, M. B., et al. (2009). *Mycobacterium tuberculosis* WhiB3 maintains redox homeostasis by regulating virulence lipid anabolism to modulate macrophage response. *PloS Pathog.* 5 (8), e1000545. doi: 10.1371/journal.ppat.1000545

Singh, A., Guidry, L., Narasimhulu, K. V., Mai, D., Trombley, J., Redding, K. E., et al. (2007). *Mycobacterium tuberculosis* WhiB3 responds to O2 and nitric oxide *via* its [4Fe-4S] cluster and is essential for nutrient starvation survival. *Proc. Natl. Acad. Sci. U.S.A.* 104 (28), 11562–11567. doi: 10.1073/pnas.0700490104

Singh, A. R., Strankman, A., Orkusyan, R., Purwantini, E., and Rawat, M. (2016). Lack of mycothiol and ergothioneine induces different protective mechanisms in *Mycobacterium smegmatis*. *Biochem. Biophys. Rep.* 8, 100–106. doi: 10.1016/j.bbrep.2016.08.006

Singh, A., Zhao, X., and Drlica, K. (2022). Fluoroquinolone heteroresistance, antimicrobial tolerance, and lethality enhancement. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.938032

Sirgel, F. A., Fourie, P. B., Donald, P. R., Padayatchi, N., Rustomjee, R., Levin, J., et al. (2005). The early bactericidal activities of rifampin and rifapentine in pulmonary tuberculosis. *Am. J. Respir. Crit. Care Med.* 172 (1), 128–135. doi: 10.1164/rccm.200411-1557OC

Sirgel, F., Venter, A., and Mitchison, D. (2001). Sources of variation in studies of the early bactericidal activity of antituberculosis drugs. *J. Antimicrob. Chemother.* 47 (2), 177–182. doi: 10.1093/jac/47.2.177

Song, R., Click, E. S., McCarthy, K. D., Heilig, C. M., McHembere, W., Smith, J. P., et al. (2021). Sensitive and feasible specimen collection and testing strategies for diagnosing tuberculosis in young children. *JAMA Pediatr.* 175 (5), e206069. doi: 10.1001/jamapediatrics.2020.6069

Sotgiu, G., Nahid, P., Loddenkemper, R., Abubakar, I., Miravitlles, M., and Migliori, G. B. (2016). The ERS-endorsed official ATS/CDC/IDSA clinical practice guidelines on treatment of drug-susceptible tuberculosis. *Eur. Respir. J.* 48 (4), 963–971. doi: 10.1183/13993003.01356-2016

Starke, J. R., and Cruz, A. T. (2021). Diagnosing childhood tuberculosis: A small step forward. *JAMA Pediatr.* 175 (5), e206078. doi: 10.1001/jamapediatrics.2020.6078

Stewart, S. M., Murdoch, J. M., Crofton, J. W., and Hay, D. (1957). Pyrazinamide together with oxytetracycline in patients with tubercle bacilli resistant to streptomycin, pas and isoniazid. *Br. J. Tuberc. Dis. Chest* 51 (2), 158–167. doi: 10.1016/S0366-0869(57)80031-8

Tessema, B., Beer, J., Emmrich, F., Sack, U., and Rodloff, A. C. (2011). Rate of recovery of *Mycobacterium tuberculosis* from frozen acid-Fast-Bacillus smear-positive sputum samples subjected to long-term storage in Northwest Ethiopia. *J. Clin. Microbiol.* 49 (7), 2557–2561. doi: 10.1128/JCM.00059-11

Thomas, T. A. (2017). Tuberculosis in children. *Pediatr. Clin. North Am.* 64 (4), 893–909. doi: 10.1016/j.pcl.2017.03.010

Toniolo, C., Delince, M., and McKinney, J. D. (2018). A microfluidic cell-trapping device to study dynamic host-microbe interactions at the single-cell level. *Methods Cell Biol.* 147, 199–213. doi: 10.1016/bs.mcb.2018.06.008

Tostmann, A., Kik, S. V., Kalisvaart, N. A., Sebek, M. M., Verver, S., Boeree, M. J., et al. (2008). Tuberculosis transmission by patients with smear-negative pulmonary tuberculosis in a large cohort in the Netherlands. *Clin. Infect. Dis.* 47 (9), 1135–1142. doi: 10.1086/591974

Trivedi, A., Mavi, P. S., Bhatt, D., and Kumar, A. (2016). Thiol reductive stress induces cellulose-anchored biofilm formation in *Mycobacterium tuberculosis*. *Nat. Commun.* 7, 11392. doi: 10.1038/ncomms11392

Turkova, A., Wills, G. H., Wobudeya, E., Chabala, C., Palmer, M., Kinikar, A., et al. (2022). Shorter treatment for nonsevere tuberculosis in African and Indian children. *N Engl. J. Med.* 386 (10), 911–922. doi: 10.1056/NEJMoa2104535

Ufimtseva, E., Eremeeva, N., Vakhrusheva, D., and Skornyakov, S. (2019). *Mycobacterium tuberculosis* shape and size variations in alveolar macrophages of tuberculosis patients. *Eur. Respir. J.* 54. doi: 10.1183/13993003.congress-2019.PA4605

Vaubourgeix, J., Lin, G., Dhar, N., Chenouard, N., Jiang, X., Botella, H., et al. (2015). Stressed mycobacteria use the chaperone ClpB to sequester irreversibly oxidized proteins asymmetrically within and between cells. *Cell Host Microbe* 17 (2), 178–190. doi: 10.1016/j.chom.2014.12.008

Vijay, S., Anand, D., and Ajitkumar, P. (2012). Unveiling unusual features of formation of septal partition and constriction in mycobacteria–an ultrastructural study. *J. Bacteriol.* 194 (3), 702–707. doi: 10.1128/JB.06184-11

Vijay, S., Mukkayyan, N., and Ajitkumar, P. (2014a). Highly deviated asymmetric division in very low proportion of mycobacterial mid-log phase cells. *Open Microbiol. J.* 8, 40–50. doi: 10.2174/1874285801408010040

Vijay, S., Nagaraja, M., Sebastian, J., and Ajitkumar, P. (2014b). Asymmetric cell division in *Mycobacterium tuberculosis* and its unique features. *Arch. Microbiol.* 196 (3), 157–168. doi: 10.1007/s00203-014-0953-7

Vijay, S., Nair, R. R., Sharan, D., Jakkala, K., Mukkayyan, N., Swaminath, S., et al. (2017a). Mycobacterial cultures contain cell size and density specific Sub-populations of cells with significant differential susceptibility to antibiotics, oxidative and nitrite stress. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.00463

Vijay, S., Vinh, D. N., Hai, H. T., Ha, V. T. N., Dung, V. T. M., Dinh, T. D., et al. (2017b). Influence of stress and antibiotic resistance on cell-length distribution in *Mycobacterium tuberculosis* clinical isolates. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.02296

Vilcheze, C., Hartman, T., Weinrick, B., and Jacobs, W. R.Jr. (2013). Mycobacterium tuberculosis is extraordinarily sensitive to killing by a vitamin c-induced fenton reaction. *Nat. Commun.* 4, 1881. doi: 10.1038/ncomms2898

Vilcheze, C., Hartman, T., Weinrick, B., Jain, P., Weisbrod, T. R., Leung, L. W., et al. (2017). Enhanced respiration prevents drug tolerance and drug resistance in mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. U.S.A.* 114 (17), 4495–4500. doi: 10.1073/pnas.1704376114

von Groote-Bidlingmaier, F., Koegelenberg, C. F., Bolliger, C. T., Chung, P. K., Rautenbach, C., Wasserman, E., et al. (2013). The yield of different pleural fluid volumes for *Mycobacterium tuberculosis* culture. *Thorax* 68 (3), 290–291. doi: 10.1136/thoraxjnl-2012-202338

Vulin, C., Leimer, N., Huemer, M., Ackermann, M., and Zinkernagel, A. S. (2018). Prolonged bacterial lag time results in small colony variants that represent a sub-population of persisters. *Nat. Commun.* 9 (1), 4074. doi: 10.1038/s41467-018-06527-0

Wagley, S., Morcrette, H., Kovacs-Simon, A., Yang, Z. R., Power, A., Tennant, R. K., et al. (2021). Bacterial dormancy: A subpopulation of viable but non-culturable cells demonstrates better fitness for revival. *PloS Pathog.* 17 (1), e1009194. doi: 10.1371/journal.ppat.1009194

Wakamoto, Y., Dhar, N., Chait, R., Schneider, K., Signorino-Gelo, F., Leibler, S., et al. (2013). Dynamic persistence of antibiotic-stressed mycobacteria. *Science* 339 (6115), 91–95. doi: 10.1126/science.1229858

Wallis, R. S., Doherty, T. M., Onyebujoh, P., Vahedi, M., Laang, H., Olesen, O., et al. (2009). Biomarkers for tuberculosis disease activity, cure, and relapse. *Lancet Infect. Dis.* 9 (3), 162–172. doi: 10.1016/S1473-3099(09)70042-8

Wallis, R. S., Wang, C., Doherty, T. M., Onyebujoh, P., Vahedi, M., Laang, H., et al. (2010). Biomarkers for tuberculosis disease activity, cure, and relapse. *Lancet Infect. Dis.* 10 (2), 68–69. doi: 10.1016/S1473-3099(10)70003-7

Wallis, R. S., Wang, C., Meyer, D., and Thomas, N. (2013). Month 2 culture status and treatment duration as predictors of tuberculosis relapse risk in a meta-regression model. *PloS One* 8 (8), e71116. doi: 10.1371/journal.pone.0071116

Walter, N. D., Moore, C. M., Kayigire, X. A., Dide-Agossou, C., Worodria, W., Huang, L., et al. (2018). Does discovery of differentially culturable *M tuberculosis* really demand a new treatment paradigm? longitudinal analysis of DNA clearance from sputum. *BMC Infect. Dis.* 18 (1), 293. doi: 10.1186/s12879-018-3213-7

Weiner, M., Prihoda, T. J., Burman, W., Johnson, J. L., Goldberg, S., Padayatchi, N., et al. (2010). Evaluation of time to detection of *Mycobacterium tuberculosis* in broth culture as a determinant for end points in treatment trials. *J. Clin. Microbiol.* 48 (12), 4370–4376. doi: 10.1128/JCM.00757-10

WHO (2019). "WHO guidelines approved by the guidelines review committee," in *WHO consolidated guidelines on drug-resistant tuberculosis treatment*(Geneva: World Health Organization).

WHO (2021). *WHO consolidated guidelines on tuberculosis: Module 3: diagnosis - rapid diagnostics for tuberculosis detection* (Geneva: World Health Organization).

WHO (2022). *WHO consolidated guidelines on tuberculosis: Module 4: Treatment - drug-susceptible tuberculosis treatment* (Geneva: World Health Organization).

Wong, E. B. (2021). It is time to focus on asymptomatic tuberculosis. *Clin. Infect. Dis.* 72 (12), e1044–e1046. doi: 10.1093/cid/ciaa1827

Wu, M. L., Chan, C. L., and Dick, T. (2016a). Rel is required for morphogenesis of resting cells in *Mycobacterium smegmatis*. *Front. Microbiol.* 7. doi: 10.3389/fmicb.2016.01390

Wu, M. L., Gengenbacher, M., and Dick, T. (2016b). Mild nutrient starvation triggers the development of a small-cell survival morphotype in mycobacteria. *Front. Microbiol.* 7. doi: 10.3389/fmicb.2016.00947

Xie, Y. L., de Jager, V. R., Chen, R. Y., Dodd, L. E., Paripati, P., Via, L. E., et al. (2021). Fourteen-day PET/CT imaging to monitor drug combination activity in treated individuals with tuberculosis. *Sci. Transl. Med.* 13 (579). doi: 10.1126/scitranslmed.abd7618

Yamaguchi, Y., Park, J. H., and Inouye, M. (2011). Toxin-antitoxin systems in bacteria and archaea. *Annu. Rev. Genet.* 45, 61–79. doi: 10.1146/annurev-genet-110410-132412

Yeware, A., Gample, S., Agrawal, S., and Sarkar, D. (2019). Using diphenyleneiodonium to induce a viable but non-culturable phenotype in *Mycobacterium tuberculosis* and its metabolomics analysis. *PloS One* 14 (8), e0220628. doi: 10.1371/journal.pone.0220628

Zainabadi, K., Walsh, K. F., Vilbrun, S. C., Mathurin, L. D., Lee, M. H., Saito, K., et al. (2021). Characterization of differentially detectable *Mycobacterium tuberculosis* in the sputum of subjects with drug-sensitive or drug-resistant tuberculosis before and after two months of therapy. *Antimicrob. Agents Chemother.* 65 (8), e0060821. doi: 10.1128/AAC.00608-21

Zhan, L., Tang, J., Sun, M., and Qin, C. (2017). Animal models for tuberculosis in translational and precision medicine. *Front. Microbiol.* 8. doi: 10.3389/fmicb.2017.00717

Zhu, J.-H., Wang, B.-W., Pan, M., Zeng, Y.-N., Rego, H., and Javid, B. (2018). Rifampicin can induce antibiotic tolerance in mycobacteria *via* paradoxical changes in rpoB transcription. *Nat. Commun.* 9 (1), 4218–4218. doi: 10.1038/s41467-018-06667-3

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Review
PUBLISHED 06 January 2023
DOI 10.3389/fcimb.2022.1085946

OPEN ACCESS

EDITED BY
Ben Gold,
Weill Cornell Medicine, United States

REVIEWED BY
Junhao Zhu,
Harvard University, United States
Babak Javid,
University of California, San Francisco, United States
Karl Drlica,
Rutgers, The State University of New Jersey, United States

*CORRESPONDENCE
Bree B. Aldridge
bree.aldridge@tufts.edu

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 31 October 2022
ACCEPTED 20 December 2022
PUBLISHED 06 January 2023

CITATION
Greenstein T and Aldridge BB (2023) Tools to develop antibiotic combinations that target drug tolerance in *Mycobacterium tuberculosis*. *Front. Cell. Infect. Microbiol.* 12:1085946. doi: 10.3389/fcimb.2022.1085946

Tools to develop antibiotic combinations that target drug tolerance in *Mycobacterium tuberculosis*

Talia Greenstein[1,2] and Bree B. Aldridge[1,2,3,4]*

[1]Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA, United States, [2]Graduate School of Biomedical Sciences, Tufts University School of Medicine, Boston, MA, United States, [3]Stuart B. Levy Center for Integrated Management of Antimicrobial Resistance, Boston, MA, United States, [4]Department of Biomedical Engineering, Tufts University School of Engineering, Medford, MA, United States

Combination therapy is necessary to treat tuberculosis to decrease the rate of disease relapse and prevent the acquisition of drug resistance, and shorter regimens are urgently needed. The adaptation of *Mycobacterium tuberculosis* to various lesion microenvironments in infection induces various states of slow replication and non-replication and subsequent antibiotic tolerance. This non-heritable tolerance to treatment necessitates lengthy combination therapy. Therefore, it is critical to develop combination therapies that specifically target the different types of drug-tolerant cells in infection. As new tools to study drug combinations earlier in the drug development pipeline are being actively developed, we must consider how to best model the drug-tolerant cells to use these tools to design the best antibiotic combinations that target those cells and shorten tuberculosis therapy. In this review, we discuss the factors underlying types of drug tolerance, how combination therapy targets these populations of bacteria, and how drug tolerance is currently modeled for the development of tuberculosis multidrug therapy. We highlight areas for future studies to develop new tools that better model drug tolerance in tuberculosis infection specifically for combination therapy testing to bring the best drug regimens forward to the clinic.

KEYWORDS

tuberculosis, drug combinations, drug tolerance, dormancy, pharmacodynamics (PD), pharmacokinetics (PK), drug interaction

Introduction

Tuberculosis (TB) remains notoriously difficult to treat and, until the COVID-19 pandemic, was the leading cause of death by a single infectious agent, *Mycobacterium tuberculosis* (Mtb) (WHO, 2021). TB requires lengthy combination therapy to prevent the acquisition of heritable drug resistance [addressed by others in this collection, including

(Bhagwat et al., 2022; Jones et al., 2022; Liebenberg et al., 2022)] and to effectively target inherent heterogeneity in infection that results in drug tolerance to prevent treatment failure and subsequent disease relapse (Fox et al., 1999; Kerantzas and Jacobs, 2017). Genotypic resistance is defined by the heritable ability to grow in the presence of high concentrations of antibiotics beyond the minimum inhibitory concentration (MIC) (Balaban et al., 2019). Antibiotic tolerance is defined as the non-heritable ability of bacteria to survive transient exposure to drugs at concentrations that would otherwise be lethal (Brauner et al., 2016; Balaban et al., 2019). The current regimen for drug-sensitive Mtb requires a 2-month intensive phase of rifampicin, isoniazid, pyrazinamide, and ethambutol followed by a four or seven-month continuation phase of rifampicin and isoniazid. The phase III clinical trial ("Study 31") for a four-month regimen with rifapentine, moxifloxacin, isoniazid, and pyrazinamide recently demonstrated non-inferiority to the six-month standard of care and is now recommended for some patients with drug-sensitive TB (Dorman et al., 2021; Carr et al., 2022). This progress notwithstanding, shorter and more effective therapies are urgently needed for both drug-sensitive and particularly for drug-resistant TB.

A hallmark of TB pathogenesis is the formation of different lesion types that have varied structures and provide different microenvironmental conditions to resident Mtb (Figure 1). Mtb readily adapts to these different niches to withstand environmental stressors, often by slowing or halting replication and metabolic activity. These adaptations enable Mtb to tolerate drug treatment (Lenaerts et al., 2015; Sarathy and Dartois, 2020). Because the bacteria occupy separate niches with different environments, multiple states of drug tolerance exist together, influencing potential drug activity (pharmacodynamics) (Cadena et al., 2017). Furthermore, the physical structures of lesions vary, and drug penetration and accumulation in each lesion are dependent on the lesion structure and chemical properties of each drug (pharmacokinetics) (Prideaux et al., 2015; Sarathy et al., 2016) (Figure 1). Therefore, drug combinations with varied pharmacokinetic and pharmacodynamic properties are necessary to target the bacteria in all their locations.

More than twenty-five new TB drugs are at various stages across the developmental pipeline (newtbdrugs.org). There are 2,300 possible three-way combinations for twenty-five drugs, far too many to test *in vivo*. Single-drug responses may not be indicative of drug combination activity and potential to reduce relapse, and drugs may not behave in combination as they do alone due to drug interactions such as synergies and antagonisms. For example, pyrazinamide, a crucial member of the current standard of care regimen, is inactive against Mtb in many *in vitro* growth conditions and as a monotherapy *in vivo* (Steenken and Wolinsky, 1954; Cho et al., 2007; Lanoix et al., 2016). However, pyrazinamide contributes significant sterilizing activity when used for the first two months of treatment in combination with the other drugs in the standard of care (Fox et al., 1999). On the other hand, pyrazinamide unexpectedly exhibited antagonism when combined with rifampicin in a 14-day PET/CT clinical study (Xie et al., 2021). This finding was consistent with the prior evidence that the combination of rifampicin + pyrazinamide + isoniazid for the continuation phase of treatment displayed slightly higher relapse rates than rifampicin + isoniazid without pyrazinamide (East African- British medical research councils, 1973). Because combination therapy is necessary to treat TB and single-drug responses are not informative of combination activity or interactions, we need to be able to systematically screen and optimize combination therapies. However, it is logistically impossible to screen the entire drug combination space in animal infection models. Therefore, it is critical to be able to perform systematic combination screens using *in vitro* and computational tools that translate to animal and clinical outcomes. There is an urgent need for *in vitro* and computational tools that capture the complex pharmacodynamics (due to drug tolerance) and pharmacokinetics (due to lesion structure) in infection to design drug combinations that successfully target the bacteria that are hardest to access and kill.

Animal models have been a critical part of the preclinical development of drug combinations. Multiple models are used in the drug development pipeline to balance throughput, cost, and information gained. Heterogeneity across animal models and even within species complicates comparison across models. Mouse models have been used for decades; the most commonly used models until recently were the C57BL/6 and BALB/c models. These mice are genetically resistant to TB infection and make only cellular granulomas, thus presenting a major limitation in their ability to recapitulate human disease. The C3HeB/FeJ model has recently grown in popularity and use; this model is genetically susceptible to TB and makes both cellular and caseous granulomas, more similar to human disease (Driver et al., 2012). Larger animals (i.e., the New Zealand White rabbit, marmosets, cynomolgus and rhesus macaques) recapitulate human disease more closely than smaller animals but are logistically prohibitive for comprehensive early screens (Lenaerts et al., 2015). Given the massive combination landscape and logistical limitation of screening every combination in animal models, many *in vitro* models have been developed to mimic the different stressors encountered in lesions. The utility of these models in predicting animal and clinical outcomes is not always clear. Standard minimum inhibitory concentration assays using glucose-based media optimized for Mtb growth were the gold standard for a long time. However, it is well-established that glucose is not the primary carbon source for Mtb *in vivo*, and Pethe et al. demonstrated the importance of making measurements of drug response in more *in vivo*-like conditions when they identified a class of drugs with glycerol-specific activity against Mtb that was inactive *in vivo* (Pethe et al., 2010). This study also highlighted the importance of validating the predictive power of *in vitro* measurements of *in vivo* outcomes.

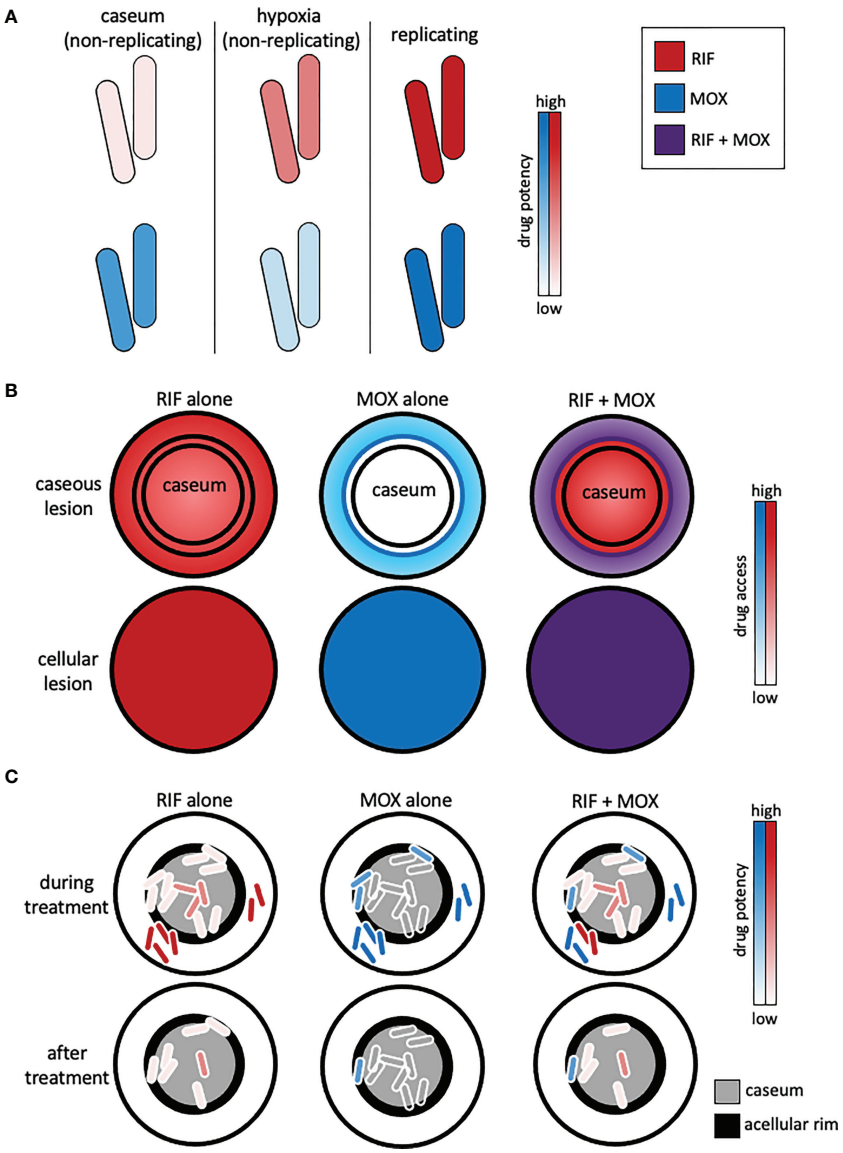


FIGURE 1
Representation of complex lesion pharmacokinetics and pharmacodynamics and how combination therapy must be optimized to reach different populations of drug-tolerant bacteria. **(A)** Drug pharmacodynamics are dependent on the bacterial state. Rifampicin (RIF) and moxifloxacin (MOX) exhibit different potencies against non-replicating intracaseum bacilli, non-replicating bacilli (e.g., induced by the Wayne hypoxia model), and replicating bacilli (e.g., in standard culture media). **(B)** Drug pharmacokinetics are lesion-dependent. Rifampicin (red) and moxifloxacin (blue) exhibit different levels of access to caseous lesions due to their drug-specific pharmacokinetic profiles. Inner concentric circles represent the acellular rim of caseous lesions. Drug combinations (e.g., RIF + MOX, purple) can overcome the pharmacokinetic limitations of single drugs. **(C)** Mtb that evade treatment and cause relapse are mainly found in the acellular rim (black) of caseum and in caseum. Drug combinations should be chosen to capitalize on pharmacokinetic and pharmacodynamic properties such that they access all the bacteria and kill the different types of drug-tolerant bacteria induced by the microenvironments in those locations.

In this review, we will discuss how heterogeneous lesion microenvironments give rise to non-replication and subsequent drug tolerance, why this tolerance requires combination therapy for effective TB treatment, and the implications for screening assay design. We review the current state of *in vitro* and computational tools for drug screens and speculate on areas of improvement as they relate to drug tolerance and non-replication. New tools have been developed to allow for systematic study of higher-order drug combinations, including the DiaMOND methodology to reduce the number of measurements required in the higher-order combination screening space, a mathematical "dose" model to predict higher-order combination activity from drug pairs, and chemo-genomic and regulatory network models to predict drug interactions from single-drug transcriptomics (Peterson et al.,

2016; Zimmer et al., 2016; Cokol et al., 2017; Ma et al., 2019). Beyond drug interactions, additional tools have been developed to consider single-drug and drug combination potency (Wooten et al., 2021; Li et al., 2022). Computational models have advanced to incorporate complex pharmacokinetics and pharmacodynamics to predict drug response outcomes informed by animal data and clinical data (Pienaar et al., 2017; Savic et al., 2017; Lyons, 2022). With all these developments, we have the opportunity to consider how to best utilize all these tools in the context of drug tolerance for the rational development of drug combinations that will be most effective in treating TB.

Lesion microenvironments give rise to non-replication and subsequent drug tolerance

To identify drugs and combinations that will best target the bacteria that are most tolerant to treatment, it is critical to understand the underlying causes of the drug tolerance that is most difficult to treat, how we currently model environments that induce drug tolerance, and to fill the knowledge and technical gaps that will improve our current models. Heterogeneous lesions are the hallmark of TB infection, and these lesions present a variety of microenvironments to which Mtb adapts. TB granulomas are classically categorized as fibrotic, cellular/non-necrotic, caseous/necrotizing, or cavitating, each with distinct characteristics (Lenaerts et al., 2015). However, the reality of infection is more of a spectrum than discrete classes, and the microenvironmental components are not homogenous even within one lesion type (Barry et al., 2009). Mtb adaptation to these environments results in varied drug tolerance that requires combination therapy to treat effectively. A critical effect of many of the environmental components that the bacteria experience is slowing or halting replication. Non-replication is associated with extreme drug tolerance (Sarathy et al., 2018). In this section, we detail how different environments slow and halt Mtb growth, their influence on drug tolerance, and how they are modeled.

Lipids

Lipids provide a crucial carbon source to Mtb in infection. Mtb has a remarkable capacity to metabolize different carbon sources (Wilburn et al., 2018). TB lesions are lipid-rich environments with varied lipid composition. Mtb induces the differentiation of infected macrophages into lipid-loaded foamy macrophages. These infected foam cells contain an abundance of triglycerides (TAG), cholesterol esters, and free cholesterol, and the composition is highly conserved across species (Guerrini et al., 2018). Fatty acid metabolism is required for Mtb to establish and maintain infection *in vivo* and cholesterol metabolism is required for Mtb persistence (Pandey and Sassetti, 2008; Marrero et al., 2010). Mtb co-metabolize both; they incorporate propionyl-CoA (an otherwise toxic byproduct of cholesterol metabolism) into their cell wall virulence lipids (e.g., PDIM) by utilizing host-derived long-chain fatty acids (LCFAs) to provide primers for their synthesis. Quinonez et al. showed that the accumulation of intermediates from the methylcitrate cycle, the pathway by which propionyl-CoA is formed, was associated with drug tolerance (Quinonez et al., 2022). It should be noted that though Mtb can co-metabolize cholesterol and many LCFAs, some LCFAs contribute to the slowing or arrest of Mtb growth (Lee et al., 2013; Rodriguez et al., 2014). Mtb resides in foamy macrophages in a non-replicating state (Peyron et al., 2008). As foamy macrophages become necrotic, they release the bacilli and their lipid-rich content, which accumulates to form lipid-rich caseum (Russell et al., 2009). Mtb is generally slowly replicating or non-replicating in caseum (Sarathy and Dartois, 2020). Mtb in lipid-rich conditions accumulate intracellular lipid inclusions (ILIs), which are associated with a dormant state and tolerance to rifampicin and isoniazid (Deb et al., 2009; Daniel et al., 2011). Intracaseum Mtb are remarkably tolerant to drugs; many antibiotics that are bactericidal in standard replicating conditions fail to sterilize caseum Mtb, and the concentrations of drugs required to kill Mtb are markedly higher (Sarathy et al., 2018).

Several *in vitro* models of Mtb lipid metabolism and drug response in lipid-rich conditions have been developed and utilized. As the importance of lipids in triggering Mtb "dormancy" (non-replication) has become more apparent, there has also been an increase in studies of Mtb transcriptome in lipid-rich conditions. These experiments have helped illuminate which lipids drive certain transcriptional and physiological responses (Rodriguez et al., 2014; Soto-Ramirez et al., 2017). For example, one study demonstrated that Mtb adaptation to a lipid-rich (LCFA-based) environment resulted in the overexpression of five genes in the DosR (dormancy) regulon. The transcriptional signature of Mtb adapted to LCFA was also compared against the signatures under hypoxia and starvation, and it was noted that the LCFA-adapted Mtb had more genes from the DosR regulon in common with Mtb under hypoxia than starvation (Rodriguez et al., 2014). These studies provide insight for choosing lipids to model Mtb adaptation to stressors encountered *in vivo*. A few high-throughput single-lipid models for drug screens have been developed, either short-chain fatty acid-based or cholesterol-based (Gold et al., 2015; Early et al., 2016; Larkins-Ford et al., 2021). One study identified anti-tubercular compounds with butyrate-specific activity that were not active against Mtb when cultured with glucose (Early et al., 2016). The caveat of single-lipid studies is that Mtb do not experience one lipid at a time, and it is plausible that outcomes from single-lipid studies might not translate across different lipids. Single-lipid models are typically growth models, but

intracaseum Mtb, which experience a combination of many lipids, are slow or non-replicating. A combination of lipids, with or without additional stressors, might be more reflective of the environment(s) that Mtb experience *in vivo* and therefore provide a better model of lipid-driven dormancy and drug tolerance for drug combination studies. A notable such model is *ex vivo* rabbit caseum, where intracaseum Mtb from *ex vivo* rabbit caseum are treated with drugs. These bacilli were demonstrated to be non-replicating, but unlike simpler non-replicating models, the drug tolerance observed in these bacilli was more similar to that observed *in vivo*, making this a highly valuable tool for drug screens (Sarathy et al., 2018). High-throughput multi-stress models that achieve similar results to *ex vivo* caseum could therefore be an excellent tool for combination drug screens.

pH

The pH in different lesions (and even within lesions) varies widely, from as low as 4.5 in the phagosomal compartment of the macrophages to 8 in caseum. The pH in caseum varies across animal models and from patient-to-patient, ranging from acidic to mildly basic (Lanoix et al., 2016; Kempker et al., 2017; Sarathy and Dartois, 2020). Recently, it was demonstrated that even within one lesion, the pH varies; a pH/Cl^- reporter strain was used to show that Mtb in infected C3HeB/FeJ mice experience more acidic pH at the lesion cuff than in the caseous core, and this correlated with reduced bacterial replication and increased antibiotic tolerance at the lesion cuff (Lavin and Tan, 2022). Mtb adaptation to acidic pH results in altered drug susceptibility (Baker et al., 2019). Mtb is viable at acidic pH but slows its growth with increasing acidity and halts growth entirely at pH 5.0 (Piddington et al., 2000; Baker et al., 2014). Notably, growth arrest at low pH is carbon source-specific; Mtb halts replication at pH 5.7 with glucose, glycerol, and TCA cycle intermediates but grows at pH 5.7 with host-associated carbon sources that function at the intersection of glycolysis and the TCA cycle (Baker et al., 2014). Mtb under acidic growth arrest are metabolically active (Baker and Abramovitch, 2018). Therefore, these cells are non-replicating but not necessarily "dormant" by the classical definition and may be metabolically or transcriptionally distinct from other populations of non-replicating Mtb induced by other microenvironments.

Though caseum is acidic in some lesions, the observation of neutral caseum in some lesions suggests that acidity is not the primary driving factor of non-replication and drug tolerance in caseum. A few *in vitro* models of Mtb adaptation to acidic pH have been developed for drug screens (Gold et al., 2015; de Miranda Silva et al., 2019; Early et al., 2019b; Larkins-Ford et al., 2021). One such model uses pH 4.5 to induce a non-replicating state and luminescent activity or a fluorescent reporter to measure viable bacteria (Early et al., 2019a; Early et al., 2019b). This assay was used to identify compounds with specific activity against non-replicating bacteria. Compounds with pH-dependent activity were also identified (Early et al., 2019a). A caveat of this model is that the acidic medium is low-nutrient. Therefore, it cannot be stated with certainty if the acidic pH was the primary driver of this particular non-replicating model, as starvation independently induces non-replication (Betts et al., 2002; Grant et al., 2013). A recent study demonstrated that LCFAs palmitic acid, oleic acid, and arachidonic acid enabled growth at pH 5.5, and supplementation of cholesterol to palmitic acid or oleic acid further enhanced this growth. Repeated supplementation of oleic acid permitted growth at pH as low as 4.5, albeit with decreasing growth rate (Gouzy et al., 2021). Therefore, in a low pH, low-nutrient model, the lack of nutrients potentially influences the bacterial ability to adapt to acidic pH and induces non-replication. A comparison of the transcriptome of different models of acid-adapted Mtb and other non-replication models may provide insight into the driving component of Mtb's response in those models. Understanding the driving components of non-replication may be valuable in predicting drug tolerance profiles (e.g., how tolerant the bacteria are to a set of drugs). For example, if each non-replicative state induced by different conditions is transcriptionally distinct, will they exhibit unique drug tolerance profiles? Furthermore, is there a primary driver in multi-stress-induced non-replication that results in a similar transcriptome and drug tolerance profile for the multi-stress to that of the primary driver, or are multi-stress models of non-replication transcriptionally distinct from the single-stress models? This information could be used to predict the drug tolerance profile of bacteria in specific locations of a lesion based on the type of non-replication induced by the local environmental components.

Ion and metal availability

Macrophage response to infection includes changes in ion flux, and Mtb has a variety of mechanisms to adapt to these responses (Neyrolles et al., 2015). Mtb responds to high chloride in a linked transcriptional response to acidic pH during phagosomal maturation, and Mtb potassium uptake is shown to play a role in host colonization (Tan et al., 2013; MacGilvary et al., 2019). Zinc-limited Mtb display decreased sensitivity to oxidative stress and some antibiotics (Dow et al., 2021). Zinc limitation signals Mtb to build alternative ribosomes (Prisic et al., 2015). These alternative ribosomes were shown to be essential for *M. smegmatis* growth in an iron-depleted environment (Chen et al., 2020). Mtb upregulate virulence factors in response to iron limitation (Rodriguez et al., 2022); in turn, iron starvation leads to the transition of Mtb to a non-replicative state and subsequent drug tolerance (Kurthkoti et al., 2017). Intracellular (macrophage) Mtb exhibit a similar

transcriptional response to Mtb cultured in low-iron media, indicating Mtb experience a low-iron environment in the phagosome (Schnappinger et al., 2003). These different states may therefore represent a critical component of the host response to infection that should be considered in *in vitro* models.

Ion perturbation models are largely overlooked for drug screens; ion studies tend to be mechanistic or study effects on host colonization rather than drug susceptibility. However, a recent macrophage infection drug screen demonstrated that one of the hit compounds limited Mtb's access to iron by acting as an iron chelator. Mtb also has a greater dependency on iron when cultured with cholesterol as the sole carbon source, linking iron uptake and cholesterol metabolism (Theriault et al., 2022). Another recent study demonstrated that iron levels play a role in modulating transcriptional responses to growth arrest when Mtb transition from exponential growth to stationary phase (Alebouyeh et al., 2022). Iron uptake's role in non-replication and the influence of iron availability on drug tolerance warrant further investigation and incorporation into drug response screens.

Oxygenation

Hypoxia has been recognized as an important feature within some granulomas. Caseous necrotic granulomas in guinea pigs, rabbits, and non-human primates were all demonstrated to be hypoxic (Via et al., 2008). C57BL/6 and BALB/c mice only develop cellular lesions, which are not hypoxic, but C3HeB/FeJ mice develop hypoxic caseous necrotic lesions (Harper et al., 2012). Mtb exposure to hypoxia induces expression of the Mtb dormancy survival regulator *dosR*. Hypoxia induces dormancy by the more classical definition of non-replication and decreased cellular functions, including DNA and protein synthesis (Sherman et al., 2001; Rao et al., 2008). Mtb is markedly more tolerant to drugs in an anaerobic environment relative to aerobic (Cho et al., 2007).

One of the first major models of Mtb survival in hypoxia is the Wayne model, in which Mtb descend into a hypoxic environment in a controlled, gradual manner. They describe two distinct states of non-replicating persistence, NRP1 and NRP2, each with distinct drug-dependent drug tolerance profiles (Wayne and Hayes, 1996). While both are non-replicating, they have distinct transcriptional profiles (Muttucumaru et al., 2004). Though this model is not amenable to high-throughput screens, it highlights how Mtb survives using transcriptionally distinct states of non-replication and how this translates to different types of drug tolerance. Abramovitch and colleagues utilize another model of gradual descent into hypoxia in which Mtb grows in multi-well plates for six days, resulting in oxygen consumption and promoting hypoxic conditions at the bottom of the wells (Zheng et al., 2017). The low-oxygen-recovery assay (LORA) is a drug screen assay where drug-treated autoluminescent bacteria are placed under anaerobic conditions for ten days and recovered with oxygen for 28 hours, after which luminescence is measured (Cho et al., 2007). The LORA can be used to identify drugs with activity against non-replicating bacteria. This assay does not specifically distinguish between NRP1 and NRP2; based on the timescale of the assay, it is a model of NRP1. A caveat of the model is that there could be drug carryover during the recovery phase. Given the evidence that non-replicating bacteria are more drug-tolerant than replicating, drug carryover into the recovery phase could potentially confound the results. This might explain the noted discrepancies between luminescence assay-based minimum inhibitory concentrations (MICs) and colony-forming unit (CFU)-based MICs (Cho et al., 2007).

It is unlikely that Mtb experience sudden anaerobiosis *in vivo*; rather, access to oxygen likely decreases gradually. Sudden anaerobiosis is lethal to Mtb cultures. The Wayne and LORA models both create anaerobic conditions gradually, allowing Mtb to adapt and halt replication. Mtb can respire nitrate in the absence of oxygen and nitrate enhances Mtb survival under anaerobiosis (Sohaskey, 2008). Therefore, some hypoxic models use sodium nitrate as an alternate electron acceptor for Mtb. Gold et al. developed a multi-stress model that includes a lipid carbon source (butyrate), hypoxia, sodium nitrate, and acidic pH (5.5). This combination of stressors induces non-replication. The multi-stress model is unique in that three of the media components are independent drivers of dormancy (hypoxia, reactive nitrogen intermediates, and acidic pH without starvation). The assay compares replicating and non-replicating bacteria and distinguishes bacteriostatic and bactericidal compounds. In lieu of plating and counting colony-forming units to quantify drug effect, which is too resource-intensive, this model was paired with the charcoal agar resazurin assay (CARA), wherein the drug-treated bacteria are transferred to agar with activated charcoal in multi-well format for outgrowth for seven days, and then fluorescence is measured. The activated charcoal serves to inactivate any carryover drug (Gold et al., 2015). The CARA is a useful method for measuring drug activity against non-replicating bacteria in a high throughput manner. The multi-stress model developed by Gold et al. was recently modified to use sodium nitrate and hypoxia in a lipid-rich environment to induce dormancy for drug combination screening (Larkins-Ford et al., 2022).

Many interesting questions remain unanswered: is there a distinction between combination-stressor-induced dormancy versus single-component-induced dormancy as they relate to drug tolerance? If one stressor (i.e., hypoxia) can induce multiple states of non-replicating drug tolerance (i.e., NRP1 and NRP2), then it is plausible that multiple stressors could, as well. Understanding the different transcriptomic and drug response profiles of different dormancy/non-replication states is key to

identifying which are most important to model for drug combination screens. Mtb can survive in a dormant state for a remarkably long time; one study demonstrated that after one year of dormancy, Mtb's proteome remained similar to the proteome of 4-month dormant bacteria (Trutneva et al., 2020). Though many different models of non-replication have been developed, the time of bacterial exposure to the conditions is typically short (<= seven days), and an in-depth analysis of the relationship between time spent in a non-replicating or dormant state and drug tolerance is lacking. Furthermore, does the method of induction of non-replication influence the rate at which Mtb can resume replication from a non-replicating state? Soto-Ramirez et al. have shown differential gene expression and different rates of change in gene expression upon re-oxygenation from NRP1 and NRP2 states dependent on lipid carbon source, indicating that the growth environment may play a role in when and how Mtb exits a non-replicative state (Soto-Ramirez et al., 2017). When caseous lesions cavitate, the bacteria rapidly switch from a hypoxic environment to high oxygenation, allowing them to exit dormancy and resume replication. Mtb's ability to rapidly recover from non-replication therefore influences its survival.

Combination therapy is key to preventing relapse: The connection to drug tolerance

Combination therapy's superiority over monotherapy in bactericidal activity and preventing relapse is well-established; however, drug screens and animal and clinical studies typically focus on outcomes and rarely investigate and understand the relationship between this superiority and targeting drug tolerance. Early studies in the development of the current standard of care (isoniazid + rifampicin + pyrazinamide + ethambutol, or HRZE) found that the addition of rifampicin or pyrazinamide to the treatment regimen with streptomycin and isoniazid reduced disease relapse and including both allowed for shorter treatment duration (Fox et al., 1999). Though it was initially thought that this success was due to drug synergy (East African- British medical research councils, 1974), it was ultimately attributed to activity against semi-dormant bacteria (Dickinson and Mitchison, 1981). This was demonstrated using *in vitro* studies of isoniazid- or rifampicin-treated Mtb whose growth rate was stalled by decreasing incubation temperature or culture in acidic conditions. Short exposure to optimal growth conditions resulted in short recovery bursts, during which rifampicin had greater bactericidal activity than isoniazid, suggesting that rifampicin is better at killing these bacilli that are semi-dormant, that is, dormant much of the time with occasional metabolic bursts. Pyrazinamide has also been demonstrated specifically to target dormant (non-replicating) bacilli and loses its sterilizing activity when metabolic activity resumes (Hu et al., 2006). Therefore, using multiple drugs in combination may reduce the incidence of relapse by targeting multiple bacterial states in infection. In support of this, Mitchison and colleagues used a guinea pig model and showed that the addition of rifampicin to isoniazid or isoniazid + ethambutol did not increase the bactericidal activity of the combination but reduced relapse, suggesting that the success of the combination was not in additional killing, but in killing particular cells that would have otherwise caused relapse (Dickinson and Mitchison, 1976). Walter et al. proposed a ribosomal RNA synthesis ratio as a metric to distinguish sterilizing and non-sterilizing drugs and drug combinations and demonstrated its utility both *in vitro* and in the relapsing mouse model (Walter et al., 2021). This method could be used to provide molecular insight into how sterilizing combinations modulate cellular processes in subpopulations of drug-tolerant Mtb. Furthermore, the combination of the ribosomal RNA synthesis ratio with CFU measurement in the mouse model was recently found to be more informative of treatment-shortening potential than either metric alone (Dide-Agossou et al., 2022). A combination of pharmacodynamic markers could enhance our understanding of how combination therapy targets drug-tolerant cells both *in vitro* and *in vivo*.

Successful treatment of TB requires killing the bacteria that would otherwise remain to cause relapse. These cells may reside in different lesions and therefore exhibit different types of drug tolerance due to adaptation to different microenvironments. Combination therapy with multiple modes of action offers greater potential over monotherapy to kill multiple types of drug tolerance, and the best combinations will be those that successfully target different types of drug tolerance. Though this has not been demonstrated systematically, empirical evidence supports this concept: bedaquiline and pretomanid, which have performed remarkably well in both animal and clinical studies and have been approved in combination with linezolid for MDR-TB (NiX-TB) (Conradie et al., 2020), target both replicating and non-replicating bacteria, and have been shown to be particularly potent against non-replicating bacteria induced by different microenvironmental conditions (Cho et al., 2007; Koul et al., 2008; Tasneen et al., 2011; Gold et al., 2015). Their superior sterilizing performance could be attributed to their ability to target multiple types of drug tolerance. Recently, a four-month regimen with rifapentine and moxifloxacin was found to be non-inferior to the standard of care ("Study 31") (Dorman et al., 2021). In preclinical studies, moxifloxacin exhibited potent early bactericidal activity in treatment (Nuermberger et al., 2004). Moxifloxacin's potent activity against both replicating and non-replicating Mtb may contribute to this more rapid clearance. Taken together, the evidence suggests that combination therapy is required to reduce relapse, and the most effective regimens will include drugs that target multiple types of drug tolerance.

Lesion structure influences drug access: Consideration in drug screens

Heterogeneous lesions also result in varied drug access, absorption, and metabolism (i.e., pharmacokinetics) dependent on lesion structure, which drives the necessity of combination therapy to ensure that one or more of the drugs can access all the different locations and be effective in those locations (Figure 1). MALDI mass spectrometry imaging (MSI) has been used to show how different drugs penetrate caseum *in vivo* (Sarathy et al., 2016). Pyrazinamide and isoniazid penetrate fairly evenly, but bedaquiline, which is highly lipophilic, binds intracellular lipids and caseum macromolecules and penetrates caseum very poorly (Sarathy et al., 2016; Greenwood et al., 2019). Clofazimine, which is highly effective against Mtb in BALB/c mice (which make exclusively cellular lesions), is relatively ineffective against Mtb in C3HeB/FeJ mice (which develop caseous necrotic lesions) (Irwin et al., 2014). Pharmacokinetic modeling demonstrated that clofazimine accumulates in cellular layers and does not diffuse into necrotic foci, explaining the lack of efficacy in the C3HeB/FeJ mice (Prideaux et al., 2015). Therefore, modeling lesion drug access to ensure that sufficient drug(s) reach the different types of drug tolerance in all their locations is important for the design of effective combinations.

Pharmacokinetics are also important when considering drug interactions; if an antagonistic combination of drugs do not act in the same location, the antagonism may not be realized. Lesion-specific and caseum-specific pharmacokinetics are studied using a combination of MSI *in vivo*, *ex vivo* rabbit caseum, and *in vitro* caseum surrogate derived from foamy THP-1 macrophages (Sarathy et al., 2016). Lesion pharmacokinetics are also modeled using computational models, typically informed by *in vivo* data (Kjellsson et al., 2012; Prideaux et al., 2015). Most pharmacokinetic/dynamic (PK/PD) models do not consider drug interactions. Recently INDIGO-MTB, a computational tool to predict drug interactions using transcriptomics, was integrated with GranSim, a multi-scale model of tuberculosis granuloma formation. GranSim incorporates host immunity, Mtb growth dynamics, and drug PK/PD into one computational framework that describes interactions between these entities through space and time in granuloma evolution (Cicchese et al., 2021). Expanding this and other models to consider drug combinations, drug interactions, varied drug tolerance, and lesion-specific pharmacokinetics could provide invaluable information for the development and improvement of effective drug combinations.

Computational modeling to link *in vitro* models with animal and clinical outcomes

Though many lesion microenvironmental components are thought to affect drug susceptibility in some capacity, it may be that we do not need to model every single one to capture the drug tolerance that causes disease relapse, i.e., the bacteria that are hardest to treat. Instead, we should identify which of these environmental niches and resultant bacterial states represent the bacteria that are most difficult to treat. Using a guinea pig model, Lenaerts and colleagues showed that the bacteria that remained after treatment were extracellular, primarily in the hypoxic acellular rim of caseous necrosis (Lenaerts et al., 2007). A marmoset study showed that the difference between sterilizing and non-sterilizing regimens was rapid clearance of cavitating lesions by the sterilizing regimen, indicating that drug combinations must target the cavitating lesion for a better outcome (Via et al., 2015). Given the complexity of the tuberculosis heterogeneity, computational models are a critical tool to link *in vitro* drug responses to such treatment outcomes in animals and the clinic. Recently Larkins-Ford et al. used a machine learning approach to predict relapse outcomes in the BALB/c mouse model from a suite of drug combination potency and interaction measures made in a variety of growth conditions to model the different environments in TB lesions (Larkins-Ford et al., 2021). This study demonstrated that *in vitro* measurements of response to drug combinations made in a subset of growth conditions (as a "sum of parts") could be predictive of *in vivo* outcomes. Some sets of conditions were more predictive of *in vivo* response than others, thereby identifying validated sets of *in vitro* models as suitable for drug combination screening. We have yet to understand whether or not measurement in more complex growth conditions that combine these "parts" into one condition (i.e., a multi-stress model) will be more predictive than the sum of parts approach. Improving *in vitro* models to capture the environments that result in drug tolerance that enables the bacteria to survive beyond treatment and correlating response in these models to *in vivo* outcomes is a clear next step. Computational and mathematical modeling offer the advantage that they are inherently optimal for iterative learning and can leverage *in vivo* outcome data.

To get the best prediction from computational models, the input from *in vitro* models must be optimized and streamlined. Several tools have been developed to improve the quality and utility of metrics from *in vitro* models of drug combination activity (summarized in Table 1). Diagonal measurement of n-way drug interactions (DiaMOND) is a method to measure drug interactions using only a fraction of the drug-dose combination

matrix ("checkerboard"), which is logistically prohibitive to measure for higher-order of systematic studies (Cokol et al., 2017). DiaMOND measures dose response curves, enabling the collection of additional metrics beyond drug interaction, including combination potency metrics. MuSyC is another framework that calculates drug interactions and distinguishes between different types of combination effects (whether we consider dose or efficacy to evaluate drug synergy) to overcome conflicting assumptions of the widely used Loewe Additivity and Bliss Independence principles and to account for drug interactions and combination potencies (Wooten et al., 2021). This widening of combination effects from only measuring traditional synergy may be important in our ability to develop predictive models of *in vivo* outcomes from *in vitro* data. Larkins-Ford et al. recently demonstrated that combination potency metrics were important to accurately predict treatment outcomes in mouse models (Larkins-Ford et al., 2021; Larkins-Ford et al., 2022). Additional modeling techniques utilize pairwise drug response measurements to predict high-order drug interactions (Zimmer et al., 2016; Katzir et al., 2019) and *in vivo* treatment outcomes (Larkins-Ford et al., 2022) as a path to reduce the number of measurements required to study the increasingly large drug combination space and still obtain informative metrics beyond traditional drug interactions. Other new computational tools incorporate pathway-specific effects of drug action to predict drug interactions (Peterson et al., 2016). INDIGO (inferring drug interactions using chemo-genomics and orthology), for example, uses transcriptomic data from single-drug responses to predict drug interactions (Chandrasekaran et al., 2016; Ma et al., 2019). These integrated molecular approaches may help us understand the pathways underlying drug tolerance and combination drug response.

More complex, mechanistic computational models integrate host-pathogen interactions, pharmacodynamics, and pharmacokinetics (summarized in Table 1) (Ernest et al., 2021). GranSim is a multi-scale, agent-based model that captures the temporal and spatial dynamics of immune cell activity, bacterial growth, and bacterial killing by drugs in different lesion types, informed by pharmacokinetic and pharmacodynamic data from non-human primates and rabbits. These dynamics are captured at the molecular, scale, and whole lesion scales. As new *in vivo* data are acquired, the model may be updated. GranSim has demonstrated how changes in cytokines influence early infection and lesion formation (Fallahi-Sichani et al., 2011; Wong et al., 2020). GranSim can also be integrated with other computational frameworks, including INDIGO-MTB and a constraint-based model (CBM) that predicts Mtb metabolism and growth. GranSim-CBM is used to predict how environmental influence on Mtb metabolism and growth influences granuloma development and outcomes (Pienaar et al., 2016). The hollow fiber system model of tuberculosis is an *in vitro* drug development tool to optimize drug regimens and dose selection to maximize drug or combination efficacy and minimize the emergence of genotypic resistance. The *in vitro* system has a pharmacodynamic compartment that houses the bacteria and a pharmacokinetic compartment with semipermeable hollow fibers that allow drugs to diffuse to the pharmacodynamic compartment in a manner that mimics the appropriate concentration-time profile for the drugs. The results of the *in vitro* experiments are used in Monte Carlo simulations to predict outcomes in clinical populations (Gumbo et al., 2004; Gumbo et al., 2015). Another PK/PD model incorporates human clinical data to simulate the outcome of various multi-drug regimen scenarios (available at http://www.saviclab.org/systems-tb/) (Fors et al., 2020). Savic and colleagues have developed population PK/PD models that were used to determine the optimal dose of rifapentine with the most potential to shorten the duration of treatment based on outcomes from phase II clinical trials (Savic et al., 2017). This information was used to inform the dosing strategy for the phase III clinical trial for Study 31, which was found noninferior to the 6-month standard of care (Dorman et al., 2021). Incorporating validated *in vitro* model data that capture the drug tolerance *in vivo* in PK/PD models offers the potential to predict treatment outcomes at the granuloma level and to fine-tune and optimize regimens to target the bacteria that would otherwise withstand treatment either due to drug tolerance or poor drug access and cause relapse.

Though the field of computational models and mathematical frameworks for measuring combination drug response has seen tremendous progress and innovation, gaps remain. Tools like INDIGO-MTB and the dose model enable the prediction of higher-order interactions from single-drug transcriptomics or pairwise drug interactions and raise the question of the necessity of higher-order combination screens. Recently Larkins-Ford et al. demonstrated that synergy and potency metrics derived from pairwise combination measurements could be used to build successful higher-order combinations (Larkins-Ford et al., 2022). Streamlining predictive metrics of treatment outcomes (both animal and clinical) and the definition of "systematic" (is pairwise sufficient or is higher-order necessary)? is important to improve the *in vitro* data that will be used in modeling. There is also a need for a system to report failed regimens to improve computational models. Understanding why regimens failed will help inform model parameters to identify other combinations that might also fail.

Looking forward: The future of TB combination therapy and models of drug tolerance

The development of improved, streamlined *in vitro* models that inform computational models is not the end of the line.

TABLE 1 Summary of mathematical frameworks and computational tools used to study drug combinations and PK/PD in TB.

Method	Application	Advantage to pipeline	Limitations
Dose model (Zimmer et al., 2016; Katzir et al., 2019)	Mathematical model to predict higher-order *in vitro* drug combination response from pairwise data	★ Reduces the number of combination measurements needed by predicting higher-order measurements above pairwise	• Predicts *in vitro* values (not *in vivo*) •Not compatible with drugs that cannot achieve complete inhibition or killing
INDIGO (Chandrasekaran et al., 2016; Ma et al., 2019; Cicchese et al., 2021)	Machine learning model to predict drug interactions using single-drug transcriptomic data	★ Reduces the number of combination measurements needed □ Provides molecular insights ❖ predicted interactions shown to correlate with clinical efficacy ❖ Used with GranSim to predict influence of drug interaction on bacterial killing rate in granulomas and correlate to clinical efficacy	• Does not account for dose response or pharmacokinetics • Predicts only drug interaction metrics • Merger of gene expression data from different sources may require batch normalization
DiaMOND (Cokol et al., 2017; Larkins-Ford et al., 2021)	Methodology to reduce the number of *in vitro* measurements necessary to capture the drug combination checkerboard space	★ Increased efficiency in *in vitro* measurements enables more combinations and conditions to be screened □ Combination dose response offers multiple usable and interpretable metrics for prediction of *in vivo*/clinical outcomes ❖ Used in machine learning models to predict treatment outcomes of large numbers of combinations in preclinical models	• Ideal approximation of synergy requires equally potent concentrations of drug in combination; deviation from equipotency compromises accuracy • Equally potent concentrations of drugs in combination do not reflect pharmacokinetics • This geometric approximation will not be accurate for very asymmetric drug interaction
Hollow fiber model (Gumbo et al., 2004; Gumbo et al., 2015)	*In vitro* tool to model and measure PK/PD combined with computational modeling	□ Experimental approach incorporates PK *in vitro*, allowing for *in vitro* regimen design ❖ Used in Monte Carlo simulations to predict optimal doses of drugs ❖ Used to predict outcome in clinical patients from *in vitro* data	• Low-medium throughput • Does not model components of the immune response
GranSim (Fallahi-Sichani et al., 2011; Pienaar et al., 2017; Cicchese et al., 2021)	Multi-scale spatial-temporal model of Mtb-immune cell dynamics in granuloma formation and resolution with drug treatment	★ Parameters easily changed for new simulations (e.g., to modulate specific cytokine production) ❖ Outcome combines granuloma immune contribution with drug response ❖ Used to predict granuloma outcomes in response to drug treatment (e.g., bacterial burden, time to sterilization) ❖ Tool to design and optimize regimens for preclinical models	• Running agent-based models is computationally intensive • Low-medium throughput
PK/PD modeling (Savic et al., 2017; Strydom et al., 2019; Fors et al., 2020)	Model to analyze drug exposure-response relationship in clinical population	❖ Provides clinical dosing strategy based on treatment-shortening potential (used for this purpose for Study 31) ❖ Outcome combines host immune contribution with drug response ❖ Predicts clinical population and individual outcomes ❖ Tool to design and optimize regimens for preclinical models and clinical trials	• PK/PD models use animal data inputs to set model parameters, therefore relying on assumptions that scaling between species and host immune response across species are equivalent • Low-medium throughput

Symbols under "Advantage to pipeline" represent advantage class: □ novel combination effects (i.e., metrics) ★ increases efficiency of combination measurement ❖ capable of or used for prediction of *in vivo* or clinical outcomes.

Additional considerations that may improve models include single-cell heterogeneity and strain-to-strain differences. For example, innate growth and metabolic differences among closely related bacilli create subpopulations that are differentially susceptible to drug treatment (Aldridge et al., 2012; Manina et al., 2015; Rego et al., 2017; Shee et al., 2022). Antibiotic-stressed, replicating mycobacteria display drug tolerance at the single-cell level; antibiotic-induced tolerance and tolerance in replicating Mtb warrant further study (Wakamoto et al., 2013; Zhu et al., 2018). It is unknown if and how much single-cell heterogeneity contributes to the subpopulations of bacteria in humans that survive beyond treatment to cause relapse. Once we have improved and validated *in vitro* models of non-replicating Mtb that survive beyond treatment, single-cell studies can be used to determine if certain cells exhibit identifiable transcriptomic or morphological markers that pre-dispose them to greater drug tolerance. We might also consider the effects of resistance mutations on drug combination activity. Schrader et al. showed that mutations that arise due to exposure to one antibiotic can cause multiform antibiotic resistance, i.e., different types of drug tolerance that may not be heritable (Schrader et al., 2021).

Effective targeting of drug-tolerant Mtb will also require that we understand how Mtb lineage contributes to different manifestations of tolerance. Different Mtb strains exhibit differences in virulence, adaptation to environmental stressors, and drug tolerance (De Groote et al., 2012; Tizzano et al., 2021). Using genome-wide association studies (GWAS), Hicks et al. identified SNPs in clinical strains of the Beijing lineage associated with propionate metabolism that conferred drug

tolerance to certain drugs (Hicks et al., 2018). Recently Li et al. utilized CRISPRi technology to quantify the expression of Mtb genes and bacterial fitness in the presence of different drugs and discovered genetic mechanisms of intrinsic drug tolerance. Upon comparing this data against genomics of Mtb clinical isolates, strain-dependent mutations were discovered that conferred specific drug susceptibility (Li et al., 2022). These studies offer the possibility to tailor drug combinations to Mtb lineage based on genetic differences across lineages. Another recent study showed that the *resR* transcriptional regulator is a frequent target of positive selection for mutations and that strains with these mutations exhibit antibiotic "resilience," which describes the potential for antibiotic tolerance and genetic resistance. The bacilli harboring these mutations exhibited more rapid post-antibiotic recovery than wild-type cells (Liu et al., 2022). This new finding demonstrates that we must better understand the various strategies Mtb uses to recover from drug treatment beyond standard definitions of non-replication and dormancy so that we can design combination therapies that target these subpopulations. To ensure that drug combinations adequately target tolerance across lineages and resilient subpopulations, multiple (clinical and laboratory) strains could be used in *in vitro* models for combination drug screens. We propose an iterative learning process (Figure 2) to develop improved drug combinations where *in vitro* models of non-replicating-induced drug tolerance are used to screen drug combinations. Streamlined drug interaction and potency metrics would be used as input for computational models of complex PK/PD to identify better drug combinations to target drug-tolerant Mtb. Computational models should then identify potential populations that could cause relapse and then loop back to the *in vitro* models to redesign the combinations to better target those populations. Multiple strains could then be tested and used as additional input in the computational model so that the best regimens that target drug tolerance across lesions and strains are carried forward to test in animals and the clinic.

FIGURE 2
Paradigm for iterative modeling of drug combinations using *in vitro* and computational tools to reduce the drug combination space for *in vivo* testing and clinical trials. Each iterative step of drug combination design should reduce the number of combinations or regimens to test in the next step (represented with funnels). The tools in the *in vitro* exploration space (blue) allow for exhaustive combination screens while requiring fewer measurements. Specialized tools like the hollow fiber system allow for *in vitro* PK/PD exploration. The output of *in vitro* models is input into computational models (purple) for empirical prediction of *in vivo* efficacy and used with *in vivo* PK study data in mechanistic predictions of *in vivo* PK/PD outcomes. The best combinations from model predictions can then go back to the *in vitro* space (blue) for expanded testing (e.g., with different Mtb strains, different culture conditions), and the prioritized combinations from expanded *in vitro* testing would be input again through computational models (gray arrows). The best regimens from model predictions are tested in preclinical models (green), and the best regimens from preclinical models can go back to *in vitro* exploration and computational models for further refinement of the models and experimental design (gray arrows). Iterative improvement of regimens allows for optimized combinations to go to clinical trials.

Concluding remarks

TB requires lengthy multidrug treatment due to populations of drug-tolerant bacteria that arise from the heterogeneity of infection. Complex lesion structure and microenvironments induce multiple types of non-replicating bacterial populations with differential drug susceptibility and variable drug access. Combination therapy is required to target these populations in all their locations, and better tools for *in vitro* screens are urgently needed. The ability to screen combinations systematically will enable us to consider the best combinations that will target drug-tolerant cells in a data-driven manner. Incorporating these data in lesion-scale PK/PD models that simulate which bacteria evade kill will also help inform which niches in the lesions induce the drug tolerance that results in relapse. Understanding which bacterial subpopulations remain will inform better choices of compounds to target those types of tolerance. Those drugs can then be included in systematic combination screens to design optimized combinations that target the types of drug-tolerant bacteria that evade kill. To achieve this goal, we must fill the knowledge gaps delineated in this review about the drug-tolerant non-replicating populations, streamline measurements from combinations screens, and optimize computational models of PK/PD to account for drug combinations and multiple types of drug tolerance.

Author contributions

Writing and editing: TG and BA. All authors contributed to the article and approved the submitted version.

Funding

This work was supported in part by the Bill & Melinda Gates Foundation (INV-027276) and by the NIH (R01AI150684).

Acknowledgments

We thank T. Smith, J. Larkins-Ford, M-A. Nolan, T. Washington, V. Dartois, and D. Kirschner for insightful discussion.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Aldridge, B. B., Fernandez-Suarez, M., Heller, D., Ambravaneswaran, V., Irimia, D., Toner, M., et al. (2012). Asymmetry and aging of mycobacterial cells lead to variable growth and antibiotic susceptibility. *Science* 335 (6064), 100–104. doi: 10.1126/science.1216166

Alebouyeh, S., Cardenas-Pestana, J. A., Vazquez, L., Prados-Rosales, R., Del Portillo, P., Sanz, J., et al. (2022). Iron deprivation enhances transcriptional responses to *in vitro* growth arrest of mycobacterium tuberculosis. *Front. Microbiol.* 13. doi: 10.3389/fmicb.2022.956602

Baker, J. J., and Abramovitch, R. B. (2018). Genetic and metabolic regulation of mycobacterium tuberculosis acid growth arrest. *Sci. Rep.* 8 (1), 4168. doi: 10.1038/s41598-018-22343-4

Baker, J. J., Dechow, S. J., and Abramovitch, R. B. (2019). Acid fasting: Modulation of mycobacterium tuberculosis metabolism at acidic pH. *Trends Microbiol.* 27 (11), 942–953. doi: 10.1016/j.tim.2019.06.005

Baker, J. J., Johnson, B. K., and Abramovitch, R. B. (2014). Slow growth of mycobacterium tuberculosis at acidic pH is regulated by phoPR and host-associated carbon sources. *Mol. Microbiol.* 94 (1), 56–69. doi: 10.1111/mmi.12688

Balaban, N. Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D. I., et al. (2019). Definitions and guidelines for research on antibiotic persistence. *Nat. Rev. Microbiol.* 17 (7), 441–448. doi: 10.1038/s41579-019-0196-3

Barry, C. E.3rd, Boshoff, H. I., Dartois, V., Dick, T., Ehrt, S., Flynn, J., et al. (2009). The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat. Rev. Microbiol.* 7 (12), 845–855. doi: 10.1038/nrmicro2236

Betts, J. C., Lukey, P. T., Robb, L. C., McAdam, R. A., and Duncan, K. (2002). Evaluation of a nutrient starvation model of mycobacterium tuberculosis persistence by gene and protein expression profiling. *Mol. Microbiol.* 43 (3), 717–731. doi: 10.1046/j.1365-2958.2002.02779.x

Bhagwat, A., Deshpande, A., and Parish, T. (2022). How mycobacterium tuberculosis drug resistance has shaped anti-tubercular drug discovery. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.974101

Brauner, A., Fridman, O., Gefen, O., and Balaban, N. Q. (2016). Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat. Rev. Microbiol.* 14 (5), 320–330. doi: 10.1038/nrmicro.2016.34

Cadena, A. M., Fortune, S. M., and Flynn, J. L. (2017). Heterogeneity in tuberculosis. *Nat. Rev. Immunol.* 17 (11), 691–702. doi: 10.1038/nri.2017.69

Carr, W., Kurbatova, E., Starks, A., Goswami, N., Allen, L., and Winston, C. (2022). Interim guidance: 4-month rifapentine-moxifloxacin regimen for the treatment of drug-susceptible pulmonary tuberculosis - united states 2022. *MMWR Morb Mortal Wkly Rep.* 71 (8), 285–289. doi: 10.15585/mmwr.mm7108a1

Chandrasekaran, S., Cokol-Cakmak, M., Sahin, N., Yilancioglu, K., Kazan, H., Collins, J. J., et al. (2016). Chemogenomics and orthology-based design of antibiotic combination therapies. *Mol. Syst. Biol.* 12 (5), 872. doi: 10.15252/msb.20156777

Chen, Y. X., Xu, Z. Y., Ge, X., Sanyal, S., Lu, Z. J., and Javid, B. (2020). Selective translation by alternative bacterial ribosomes. *Proc. Natl. Acad. Sci. U.S.A.* 117 (32), 19487–19496. doi: 10.1073/pnas.2009607117

Cho, S. H., Warit, S., Wan, B., Hwang, C. H., Pauli, G. F., and Franzblau, S. G. (2007). Low-oxygen-recovery assay for high-throughput screening of compounds

against nonreplicating mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 51 (4), 1380–1385. doi: 10.1128/AAC.00055-06

Cicchese, J. M., Sambarey, A., Kirschner, D., Linderman, J. J., and Chandrasekaran, S. (2021). A multi-scale pipeline linking drug transcriptomics with pharmacokinetics predicts *in vivo* interactions of tuberculosis drugs. *Sci. Rep.* 11 (1), 5643. doi: 10.1038/s41598-021-84827-0

Cokol, M., Kuru, N., Bicak, E., Larkins-Ford, J., and Aldridge, B. B. (2017). Efficient measurement and factorization of high-order drug interactions in mycobacterium tuberculosis. *Sci. Adv.* 3 (10), e1701881. doi: 10.1126/sciadv.1701881

Conradie, F., Diacon, A. H., Ngubane, N., Howell, P., Everitt, D., Crook, A. M., et al. (2020). Treatment of highly drug-resistant pulmonary tuberculosis. *N Engl. J. Med.* 382 (10), 893–902. doi: 10.1056/NEJMoa1901814

Daniel, J., Maamar, H., Deb, C., Sirakova, T. D., and Kolattukudy, P. E. (2011). Mycobacterium tuberculosis uses host triacylglycerol to accumulate lipid droplets and acquires a dormancy-like phenotype in lipid-loaded macrophages. *PLoS Pathog.* 7 (6), e1002093. doi: 10.1371/journal.ppat.1002093

Deb, C., Lee, C. M., Dubey, V. S., Daniel, J., Abomoelak, B., Sirakova, T. D., et al. (2009). A novel *in vitro* multiple-stress dormancy model for mycobacterium tuberculosis generates a lipid-loaded, drug-tolerant, dormant pathogen. *PLoS One* 4 (6), e6077. doi: 10.1371/journal.pone.0006077

De Groote, M. A., Gruppo, V., Woolhiser, L. K., Orme, I. M., Gilliland, J. C., and Lenaerts, A. J. (2012). Importance of confirming data on the *in vivo* efficacy of novel antibacterial drug regimens against various strains of mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 56 (2), 731–738. doi: 10.1128/AAC.05701-11

de Miranda Silva, C., Hajihosseini, A., Myrick, J., Nole, J., Louie, A., Schmidt, S., et al. (2019). Effect of moxifloxacin plus pretomanid against mycobacterium tuberculosis in log phase, acid phase, and nonreplicating-persister phase in an *In vitro* assay. *Antimicrob. Agents Chemother.* 63 (1). doi: 10.1128/AAC.01695-18

Dickinson, J. M., and Mitchison, D. A. (1976). Bactericidal activity *in vitro* and in the guinea-pig of isoniazid, rifampicin and ethambutol. *Tubercle* 57 (4), 251–258. doi: 10.1016/s0041-3879(76)80002-5

Dickinson, J. M., and Mitchison, D. A. (1981). Experimental models to explain the high sterilizing activity of rifampin in the chemotherapy of tuberculosis. *Am. Rev. Respir. Dis.* 123 (4 Pt 1), 367–371. doi: 10.1164/arrd.1981.123.4.367

Dide-Agossou, C., Bauman, A. A., Ramey, M. E., Rossmassler, K., Al Mubarak, R., Pauly, S., et al. (2022). Combination of mycobacterium tuberculosis RS ratio and CFU improves the ability of murine efficacy experiments to distinguish between drug treatments. *Antimicrob. Agents Chemother.* 66 (4), e0231021. doi: 10.1128/aac.02310-21

Dorman, S. E., Nahid, P., Kurbatova, E. V., Phillips, P. P. J., Bryant, K., Dooley, K. E., et al. (2021). Four-month rifapentine regimens with or without moxifloxacin for tuberculosis. *N Engl. J. Med.* 384 (18), 1705–1718. doi: 10.1056/NEJMoa2033400

Dow, A., Sule, P., O'Donnell, T. J., Burger, A., Mattila, J. T., Antonio, B., et al. (2021). Zinc limitation triggers anticipatory adaptations in mycobacterium tuberculosis. *PloS Pathog.* 17 (5), e1009570. doi: 10.1371/journal.ppat.1009570

Driver, E. R., Ryan, G. J., Hoff, D. R., Irwin, S. M., Basaraba, R. J., Kramnik, I., et al. (2012). Evaluation of a mouse model of necrotic granuloma formation using C3HeB/FeJ mice for testing of drugs against mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 56 (6), 3181–3195. doi: 10.1128/AAC.00217-12

Early, J. V., Casey, A., Martinez-Grau, M. A., Gonzalez Valcarcel, I. C., Vieth, M., Ollinger, J., et al. (2016). Oxadiazoles have butyrate-specific conditional activity against mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 60 (6), 3608–3616. doi: 10.1128/AAC.02896-15

Early, J. V., Mullen, S., and Parish, T. (2019b). A rapid, low pH, nutrient stress, assay to determine the bactericidal activity of compounds against non-replicating mycobacterium tuberculosis. *PloS One* 14 (10), e0222970. doi: 10.1371/journal.pone.0222970

Early, J., Ollinger, J., Darby, C., Alling, T., Mullen, S., Casey, A., et al. (2019a). Identification of compounds with pH-dependent bactericidal activity against mycobacterium tuberculosis. *ACS Infect. Dis.* 5 (2), 272–280. doi: 10.1021/acsinfecdis.8b00256

East African- British medical research councils (1973). Controlled clinical trial of four short-course (6-month) regimens of chemotherapy for treatment of pulmonary tuberculosis. second report. *Lancet* 1 (7816), 1331–1338.

East African- British medical research councils (1974). Controlled clinical trial of four short-course (6-month) regimens of chemotherapy for treatment of pulmonary tuberculosis. third report. East African-British medical research councils. *Lancet* 2 (7875), 237–240. doi: 10.1016/S0140-6736(74)91411-1

Ernest, J. P., Strydom, N., Wang, Q., Zhang, N., Nuermberger, E., Dartois, V., et al. (2021). Development of new tuberculosis drugs: Translation to regimen composition for drug-sensitive and multidrug-resistant tuberculosis. *Annu. Rev. Pharmacol. Toxicol.* 61, 495–516. doi: 10.1146/annurev-pharmtox-030920-011143

Fallahi-Sichani, M., El-Kebir, M., Marino, S., Kirschner, D. E., and Linderman, J. J. (2011). Multiscale computational modeling reveals a critical role for TNF-alpha receptor 1 dynamics in tuberculosis granuloma formation. *J. Immunol.* 186 (6), 3472–3483. doi: 10.4049/jimmunol.1003299

Fors, J., Strydom, N., Fox, W. S., Keizer, R. J., and Savic, R. M. (2020). Mathematical model and tool to explore shorter multi-drug therapy options for active pulmonary tuberculosis. *PLoS Comput. Biol.* 16 (8), e1008107. doi: 10.1371/journal.pcbi.1008107

Fox, W., Ellard, G. A., and Mitchison, D. A. (1999). Studies on the treatment of tuberculosis undertaken by the British medical research council tuberculosis units 1946-1986, with relevant subsequent publications. *Int. J. Tuberc Lung Dis.* 3 (10 Suppl 2), S231–S279.

Gold, B., Roberts, J., Ling, Y., Quezada, L. L., Glasheen, J., Ballinger, E., et al. (2015). Rapid, semiquantitative assay to discriminate among compounds with activity against replicating or nonreplicating mycobacterium tuberculosis. *Antimicrob. Agents Chemother.* 59 (10), 6521–6538. doi: 10.1128/AAC.00803-15

Gouzy, A., Healy, C., Black, K. A., Rhee, K. Y., and Ehrt, S. (2021). Growth of mycobacterium tuberculosis at acidic pH depends on lipid assimilation and is accompanied by reduced GAPDH activity. *Proc. Natl. Acad. Sci. U.S.A.* 118 (32). doi: 10.1073/pnas.2024571118

Grant, S. S., Kawate, T., Nag, P. P., Silvis, M. R., Gordon, K., Stanley, S. A., et al. (2013). Identification of novel inhibitors of nonreplicating mycobacterium tuberculosis using a carbon starvation model. *ACS Chem. Biol.* 8 (10), 2224–2234. doi: 10.1021/cb4004817

Greenwood, D. J., Dos Santos, M. S., Huang, S., Russell, M. R. G., Collinson, L. M., MacRae, J. I., et al. (2019). Subcellular antibiotic visualization reveals a dynamic drug reservoir in infected macrophages. *Science* 364 (6447), 1279–1282. doi: 10.1126/science.aat9689

Guerrini, V., Prideaux, B., Blanc, L., Bruiners, N., Arrigucci, R., Singh, S., et al. (2018). Storage lipid studies in tuberculosis reveal that foam cell biogenesis is disease-specific. *PloS Pathog.* 14 (8), e1007223. doi: 10.1371/journal.ppat.1007223

Gumbo, T., Louie, A., Deziel, M. R., Parsons, L. M., Salfinger, M., and Drusano, G. L. (2004). Selection of a moxifloxacin dose that suppresses drug resistance in mycobacterium tuberculosis, by use of an *in vitro* pharmacodynamic infection model and mathematical modeling. *J. Infect. Dis.* 190 (9), 1642–1651. doi: 10.1086/424849

Gumbo, T., Pasipanodya, J. G., Romero, K., Hanna, D., and Nuermberger, E. (2015). Forecasting accuracy of the hollow fiber model of tuberculosis for clinical therapeutic outcomes. *Clin. Infect. Dis.* 61 Suppl 1, S25–S31. doi: 10.1093/cid/civ427

Harper, J., Skerry, C., Davis, S. L., Tasneen, R., Weir, M., Kramnik, I., et al. (2012). Mouse model of necrotic tuberculosis granulomas develops hypoxic lesions. *J. Infect. Dis.* 205 (4), 595–602. doi: 10.1093/infdis/jir786

Hicks, N. D., Yang, J., Zhang, X., Zhao, B., Grad, Y. H., Liu, L., et al. (2018). Clinically prevalent mutations in mycobacterium tuberculosis alter propionate metabolism and mediate multidrug tolerance. *Nat. Microbiol.* 3 (9), 1032–1042. doi: 10.1038/s41564-018-0218-3

Hu, Y., Coates, A. R., and Mitchison, D. A. (2006). Sterilising action of pyrazinamide in models of dormant and rifampicin-tolerant mycobacterium tuberculosis. *Int. J. Tuberc Lung Dis.* 10 (3), 317–322.

Irwin, S. M., Gruppo, V., Brooks, E., Gilliland, J., Scherman, M., Reichlen, M. J., et al. (2014). Limited activity of clofazimine as a single drug in a mouse model of tuberculosis exhibiting caseous necrotic granulomas. *Antimicrob. Agents Chemother.* 58 (7), 4026–4034. doi: 10.1128/AAC.02565-14

Jones, R. M., Adams, K. N., Eldesouky, H. E., and Sherman, D. R. (2022). The evolving biology of mycobacterium tuberculosis drug resistance. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.1027394

Katzir, I., Cokol, M., Aldridge, B. B., and Alon, U. (2019). Prediction of ultra-high-order antibiotic combinations based on pairwise interactions. *PloS Comput. Biol.* 15 (1), e1006774. doi: 10.1371/journal.pcbi.1006774

Kempker, R. R., Heinrichs, M. T., Nikolaishvili, K., Sabulua, I., Bablishvili, N., Gogishvili, S., et al. (2017). Lung tissue concentrations of pyrazinamide among patients with drug-resistant pulmonary tuberculosis. *Antimicrob. Agents Chemother.* 61 (6). doi: 10.1128/AAC.00226-17

Kerantzas, C. A., and Jacobs, W. R.Jr (2017). Origins of combination therapy for tuberculosis: Lessons for future antimicrobial development and application. *mBio* 8 (2). doi: 10.1128/mBio.01586-16

Kjellsson, M. C., Via, L. E., Goh, A., Weiner, D., Low, K. M., Kern, S., et al. (2012). Pharmacokinetic evaluation of the penetration of antituberculosis agents in rabbit pulmonary lesions. *Antimicrob. Agents Chemother.* 56 (1), 446–457. doi: 10.1128/AAC.05208-11

Koul, A., Vranckx, L., Dendouga, N., Balemans, W., Van den Wyngaert, I., Vergauwen, K., et al. (2008). Diarylquinolines are bactericidal for dormant mycobacteria as a result of disturbed ATP homeostasis. *J. Biol. Chem.* 283 (37), 25273–25280. doi: 10.1074/jbc.M803899200

Kurthkoti, K., Amin, H., Marakalala, M. J., Ghanny, S., Subbian, S., Sakatos, A., et al. (2017). The capacity of mycobacterium tuberculosis to survive iron starvation might enable it to persist in iron-deprived microenvironments of human granulomas. *mBio* 8 (4). doi: 10.1128/mBio.01092-17

Lanoix, J. P., Ioerger, T., Ormond, A., Kaya, F., Sacchettini, J., Dartois, V., et al. (2016). Selective inactivity of pyrazinamide against tuberculosis in C3HeB/FeJ mice is best explained by neutral pH of caseum. *Antimicrob. Agents Chemother.* 60 (2), 735–743. doi: 10.1128/AAC.01370-15

Larkins-Ford, J., Degefu, Y. N., Van, N., Sokolov, A., and Aldridge, B. B. (2022). Design principles to assemble drug combinations for effective tuberculosis therapy using interpretable pairwise drug response measurements. *Cell Rep. Med.* 3 (9), 100737. doi: 10.1016/j.xcrm.2022.100737

Larkins-Ford, J., Greenstein, T., Van, N., Degefu, Y. N., Olson, M. C., Sokolov, A., et al. (2021). Systematic measurement of combination-drug landscapes to predict *in vivo* treatment outcomes for tuberculosis. *Cell Syst.* 12 (11), 1046–1063.e1047. doi: 10.1016/j.cels.2021.08.004

Lavin, R. C., and Tan, S. (2022). Spatial relationships of intra-lesion heterogeneity in mycobacterium tuberculosis microenvironment, replication status, and drug efficacy. *PloS Pathog.* 18 (3), e1010459. doi: 10.1371/journal.ppat.1010459

Lee, W., VanderVen, B. C., Fahey, R. J., and Russell, D. G. (2013). Intracellular mycobacterium tuberculosis exploits host-derived fatty acids to limit metabolic stress. *J. Biol. Chem.* 288 (10), 6788–6800. doi: 10.1074/jbc.M112.445056

Lenaerts, A., Barry, C. E.3rd, and Dartois, V. (2015). Heterogeneity in tuberculosis pathology, microenvironments and therapeutic responses. *Immunol. Rev.* 264 (1), 288–307. doi: 10.1111/imr.12252

Lenaerts, A. J., Hoff, D., Aly, S., Ehlers, S., Andries, K., Cantarero, L., et al. (2007). Location of persisting mycobacteria in a Guinea pig model of tuberculosis revealed by r207910. *Antimicrob. Agents Chemother.* 51 (9), 3338–3345. doi: 10.1128/AAC.00276-07

Liebenberg, D., Gordhan, B. G., and Kana, B. D. (2022). Drug resistant tuberculosis: Implications for transmission, diagnosis, and disease management. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.943545

Li, S., Poulton, N. C., Chang, J. S., Azadian, Z. A., DeJesus, M. A., Ruecker, N., et al. (2022). CRISPRi chemical genetics and comparative genomics identify genes mediating drug potency in mycobacterium tuberculosis. *Nat. Microbiol.* 7 (6), 766–779. doi: 10.1038/s41564-022-01130-y

Liu, Q., Zhu, J., Dulberger, C. L., Stanley, S., Wilson, S., Chung, E. S., et al. (2022). Tuberculosis treatment failure associated with evolution of antibiotic resilience. *Science* 378 (6624), 1111–1118. doi: 10.1126/science.abq2787

Lyons, M. A. (2022). Pharmacodynamics and bactericidal activity of bedaquiline in pulmonary tuberculosis. *Antimicrob. Agents Chemother.* 66 (2), e0163621. doi: 10.1128/AAC.01636-21

MacGilvary, N. J., Kevorkian, Y. L., and Tan, S. (2019). Potassium response and homeostasis in mycobacterium tuberculosis modulates environmental adaptation and is important for host colonization. *PLoS Pathog.* 15 (2), e1007591. doi: 10.1371/journal.ppat.1007591

Ma, S., Jaipalli, S., Larkins-Ford, J., Lohmiller, J., Aldridge, B. B., Sherman, D. R., et al. (2019). Transcriptomic signatures predict regulators of drug synergy and clinical regimen efficacy against tuberculosis. *mBio* 10 (6). doi: 10.1128/mBio.02627-19

Manina, G., Dhar, N., and McKinney, J. D. (2015). Stress and host immunity amplify mycobacterium tuberculosis phenotypic heterogeneity and induce nongrowing metabolically active forms. *Cell Host Microbe* 17 (1), 32–46. doi: 10.1016/j.chom.2014.11.016

Marrero, J., Rhee, K. Y., Schnappinger, D., Pethe, K., and Ehrt, S. (2010). Gluconeogenic carbon flow of tricarboxylic acid cycle intermediates is critical for mycobacterium tuberculosis to establish and maintain infection. *Proc. Natl. Acad. Sci. U.S.A.* 107 (21), 9819–9824. doi: 10.1073/pnas.1000715107

Muttucumaru, D. G., Roberts, G., Hinds, J., Stabler, R. A., and Parish, T. (2004). Gene expression profile of mycobacterium tuberculosis in a non-replicating state. *Tuberculosis (Edinb)* 84 (3-4), 239–246. doi: 10.1016/j.tube.2003.12.006

Neyrolles, O., Wolschendorf, F., Mitra, A., and Niederweis, M. (2015). Mycobacteria, metals, and the macrophage. *Immunol. Rev.* 264 (1), 249–263. doi: 10.1111/imr.12265

Nuermberger, E. L., Yoshimatsu, T., Tyagi, S., O'Brien, R. J., Vernon, A. N., Chaisson, R. E., et al. (2004). Moxifloxacin-containing regimen greatly reduces time to culture conversion in murine tuberculosis. *Am. J. Respir. Crit. Care Med.* 169 (3), 421–426. doi: 10.1164/rccm.200310-1380OC

Pandey, A. K., and Sassetti, C. M. (2008). Mycobacterial persistence requires the utilization of host cholesterol. *Proc. Natl. Acad. Sci. U.S.A.* 105 (11), 4376–4380. doi: 10.1073/pnas.0711159105

Peterson, E. J. R., Ma, S., Sherman, D. R., and Baliga, N. S. (2016). Network analysis identifies Rv0324 and Rv0880 as regulators of bedaquiline tolerance in mycobacterium tuberculosis. *Nat. Microbiol.* 1 (8), 16078. doi: 10.1038/nmicrobiol.2016.78

Pethe, K., Sequeira, P. C., Agarwalla, S., Rhee, K., Kuhen, K., Phong, W. Y., et al. (2010). A chemical genetic screen in mycobacterium tuberculosis identifies carbon-source-dependent growth inhibitors devoid of *in vivo* efficacy. *Nat. Commun.* 1, 57. doi: 10.1038/ncomms1060

Peyron, P., Vaubourgeix, J., Poquet, Y., Levillain, F., Botanch, C., Bardou, F., et al. (2008). Foamy macrophages from tuberculous patients' granulomas constitute a nutrient-rich reservoir for m. tuberculosis persistence. *PLoS Pathog.* 4 (11), e1000204. doi: 10.1371/journal.ppat.1000204

Piddington, D. L., Kashkouli, A., and Buchmeier, N. A. (2000). Growth of mycobacterium tuberculosis in a defined medium is very restricted by acid pH and Mg(2+) levels. *Infect. Immun.* 68 (8), 4518–4522. doi: 10.1128/IAI.68.8.4518-4522.2000

Pienaar, E., Matern, W. M., Linderman, J. J., Bader, J. S., and Kirschner, D. E. (2016). Multiscale model of mycobacterium tuberculosis infection maps metabolite and gene perturbations to granuloma sterilization predictions. *Infect. Immun.* 84 (5), 1650–1669. doi: 10.1128/IAI.01438-15

Pienaar, E., Sarathy, J., Prideaux, B., Dietzold, J., Dartois, V., Kirschner, D. E., et al. (2017). Comparing efficacies of moxifloxacin, levofloxacin and gatifloxacin in tuberculosis granulomas using a multi-scale systems pharmacology approach. *PloS Comput. Biol.* 13 (8), e1005650. doi: 10.1371/journal.pcbi.1005650

Prideaux, B., Via, L. E., Zimmerman, M. D., Eum, S., Sarathy, J., O'Brien, P., et al. (2015). The association between sterilizing activity and drug distribution into tuberculosis lesions. *Nat. Med.* 21 (10), 1223–1227. doi: 10.1038/nm.3937

Prisic, S., Hwang, H., Dow, A., Barnaby, O., Pan, T. S., Lonzanida, J. A., et al. (2015). Zinc regulates a switch between primary and alternative S18 ribosomal proteins in mycobacterium tuberculosis. *Mol. Microbiol.* 97 (2), 263–280. doi: 10.1111/mmi.13022

Quinonez, C. G., Lee, J. J., Lim, J., Odell, M., Lawson, C. P., Anyogu, A., et al. (2022). The role of fatty acid metabolism in drug tolerance of mycobacterium tuberculosis. *mBio* 13 (1), e0355921. doi: 10.1128/mbio.03559-21

Rao, S. P., Alonso, S., Rand, L., Dick, T., and Pethe, K. (2008). The protonmotive force is required for maintaining ATP homeostasis and viability of hypoxic, nonreplicating mycobacterium tuberculosis. *Proc. Natl. Acad. Sci. U.S.A.* 105 (33), 11945–11950. doi: 10.1073/pnas.0711697105

Rego, E. H., Audette, R. E., and Rubin, E. J. (2017). Deletion of a mycobacterial divisome factor collapses single-cell phenotypic heterogeneity. *Nature* 546 (7656), 153–157. doi: 10.1038/nature22361

Rodriguez, J. G., Hernandez, A. C., Helguera-Repetto, C., Aguilar Ayala, D., Guadarrama-Medina, R., Anzola, J. M., et al. (2014). Global adaptation to a lipid environment triggers the dormancy-related phenotype of mycobacterium tuberculosis. *mBio* 5 (3), e01125–e01114. doi: 10.1128/mBio.01125-14

Rodriguez, G. M., Sharma, N., Biswas, A., and Sharma, N. (2022). The iron response of mycobacterium tuberculosis and its implications for tuberculosis pathogenesis and novel therapeutics. *Front. Cell Infect. Microbiol.* 12. doi: 10.3389/fcimb.2022.876667

Russell, D. G., Cardona, P. J., Kim, M. J., Allain, S., and Altare, F. (2009). Foamy macrophages and the progression of the human tuberculosis granuloma. *Nat. Immunol.* 10 (9), 943–948. doi: 10.1038/ni.1781

Sarathy, J. P., and Dartois, V. (2020). Caseum: a niche for mycobacterium tuberculosis drug-tolerant persisters. *Clin. Microbiol. Rev.* 33 (3). doi: 10.1128/CMR.00159-19

Sarathy, J. P., Via, L. E., Weiner, D., Blanc, L., Boshoff, H., Eugenin, E. A., et al. (2018). Extreme drug tolerance of mycobacterium tuberculosis in caseum. *Antimicrob. Agents Chemother.* 62 (2). doi: 10.1128/AAC.02266-17

Sarathy, J. P., Zuccotto, F., Hsinpin, H., Sandberg, L., Via, L. E., Marriner, G. A., et al. (2016). Prediction of drug penetration in tuberculosis lesions. *ACS Infect. Dis.* 2 (8), 552–563. doi: 10.1021/acsinfecdis.6b00051

Savic, R. M., Weiner, M., MacKenzie, W. R., Engle, M., Whitworth, W. C., Johnson, J. L., et al. (2017). Defining the optimal dose of rifapentine for pulmonary tuberculosis: Exposure-response relations from two phase II clinical trials. *Clin. Pharmacol. Ther.* 102 (2), 321–331. doi: 10.1002/cpt.634

Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., et al. (2003). Transcriptional adaptation of mycobacterium tuberculosis within macrophages: Insights into the phagosomal environment. *J. Exp. Med.* 198 (5), 693–704. doi: 10.1084/jem.20030846

Schrader, S. M., Botella, H., Jansen, R., Ehrt, S., Rhee, K., Nathan, C., et al. (2021). Multiform antimicrobial resistance from a metabolic mutation. *Sci. Adv.* 7 (35). doi: 10.1126/sciadv.abh2037

Shee, S., Singh, S., Tripathi, A., Thakur, C., Kumar, T. A., Das, M., et al. (2022). Moxifloxacin-mediated killing of mycobacterium tuberculosis involves respiratory downshift, reductive stress, and accumulation of reactive oxygen species. *Antimicrob. Agents Chemother.* 66 (9), e0059222. doi: 10.1128/aac.00592-22

Sherman, D. R., Voskuil, M., Schnappinger, D., Liao, R., Harrell, M. I., and Schoolnik, G. K. (2001). Regulation of the mycobacterium tuberculosis hypoxic response gene encoding alpha -crystallin. *Proc. Natl. Acad. Sci. U.S.A.* 98 (13), 7534–7539. doi: 10.1073/pnas.121172498

Sohaskey, C. D. (2008). Nitrate enhances the survival of mycobacterium tuberculosis during inhibition of respiration. *J. Bacteriol.* 190 (8), 2981–2986. doi: 10.1128/JB.01857-07

Soto-Ramirez, M. D., Aguilar-Ayala, D. A., Garcia-Morales, L., Rodriguez-Peredo, S. M., Badillo-Lopez, C., Rios-Muniz, D. E., et al. (2017). Cholesterol plays a larger role during mycobacterium tuberculosis *in vitro* dormancy and reactivation than previously suspected. *Tuberculosis (Edinb)* 103, 1–9. doi: 10.1016/j.tube.2016.12.004

Steenken, W.Jr., and Wolinsky, E. (1954). The antituberculous activity of pyrazinamide *in vitro* and in the guinea pig. *Am. Rev. Tuberc* 70 (2), 367–369. doi: 10.1164/art.1954.70.2.367

Strydom, N., Gupta, S. V., Fox, W. S., Via, L. E., Bang, H., Lee, M., et al. (2019). Tuberculosis drugs' distribution and emergence of resistance in patient's lung lesions: A mechanistic model and tool for regimen and dose optimization. *PloS Med.* 16 (4), e1002773. doi: 10.1371/journal.pmed.1002773

Tan, S., Sukumar, N., Abramovitch, R. B., Parish, T., and Russell, D. G. (2013). Mycobacterium tuberculosis responds to chloride and pH as synergistic cues to the immune status of its host cell. *PloS Pathog.* 9 (4), e1003282. doi: 10.1371/journal.ppat.1003282

Tasneen, R., Li, S. Y., Peloquin, C. A., Taylor, D., Williams, K. N., Andries, K., et al. (2011). Sterilizing activity of novel TMC207- and PA-824-containing regimens in a murine model of tuberculosis. *Antimicrob. Agents Chemother.* 55 (12), 5485–5492. doi: 10.1128/AAC.05293-11

Theriault, M. E., Pisu, D., Wilburn, K. M., Le-Bury, G., MacNamara, C. W., Michael Petrassi, H., et al. (2022). Iron limitation in m. tuberculosis has broad impact on central carbon metabolism. *Commun. Biol.* 5 (1), 685. doi: 10.1038/s42003-022-03650-z

Tizzano, B., Dallenga, T. K., Utpatel, C., Behrends, J., Homolka, S., Kohl, T. A., et al. (2021). Survival of hypoxia-induced dormancy is not a common feature of all strains of the mycobacterium tuberculosis complex. *Sci. Rep.* 11 (1), 2628. doi: 10.1038/s41598-021-81223-6

Trutneva, K. A., Shleeva, M. O., Demina, G. R., Vostroknutova, G. N., and Kaprelyans, A. S. (2020). One-year old dormant, "Non-culturable" mycobacterium tuberculosis preserves significantly diverse protein profile. *Front. Cell Infect. Microbiol.* 10. doi: 10.3389/fcimb.2020.00026

Via, L. E., England, K., Weiner, D. M., Schimel, D., Zimmerman, M. D., Dayao, E., et al. (2015). A sterilizing tuberculosis treatment regimen is associated with faster clearance of bacteria in cavitary lesions in marmosets. *Antimicrob. Agents Chemother.* 59 (7), 4181–4189. doi: 10.1128/AAC.00115-15

Via, L. E., Lin, P. L., Ray, S. M., Carrillo, J., Allen, S. S., Eum, S. Y., et al. (2008). Tuberculous granulomas are hypoxic in guinea pigs, rabbits, and nonhuman primates. *Infect. Immun.* 76 (6), 2333–2340. doi: 10.1128/IAI.01515-07

Wakamoto, Y., Dhar, N., Chait, R., Schneider, K., Signorino-Gelo, F., Leibler, S., et al. (2013). Dynamic persistence of antibiotic-stressed mycobacteria. *Science* 339 (6115), 91–95. doi: 10.1126/science.1229858

Walter, N. D., Born, S. E. M., Robertson, G. T., Reichlen, M., Dide-Agossou, C., Ektnitphong, V. A., et al. (2021). Mycobacterium tuberculosis precursor rRNA as a measure of treatment-shortening activity of drugs and regimens. *Nat. Commun.* 12 (1), 2899. doi: 10.1038/s41467-021-22833-6

Wayne, L. G., and Hayes, L. G. (1996). An *in vitro* model for sequential study of shiftdown of mycobacterium tuberculosis through two stages of nonreplicating persistence. *Infect. Immun.* 64 (6), 2062–2069. doi: 10.1128/iai.64.6.2062-2069.1996

WHO (2021). *Global tuberculosis report 2021* (Geneva: World Health Organization).

Wilburn, K. M., Fieweger, R. A., and VanderVen, B. C. (2018). Cholesterol and fatty acids grease the wheels of mycobacterium tuberculosis pathogenesis. *Pathog. Dis.* 76 (2). doi: 10.1093/femspd/fty021

Wong, E. A., Evans, S., Kraus, C. R., Engelman, K. D., Maiello, P., Flores, W. J., et al. (2020). IL-10 impairs local immune response in lung granulomas and lymph nodes during early mycobacterium tuberculosis infection. *J. Immunol.* 204 (3), 644–659. doi: 10.4049/jimmunol.1901211

Wooten, D. J., Meyer, C. T., Lubbock, A. L. R., Quaranta, V., and Lopez, C. F. (2021). MuSyC is a consensus framework that unifies multi-drug synergy metrics for combinatorial drug discovery. *Nat. Commun.* 12 (1), 4607. doi: 10.1038/s41467-021-24789-z

Xie, Y. L., de Jager, V. R., Chen, R. Y., Dodd, L. E., Paripati, P., Via, L. E., et al. (2021). Fourteen-day PET/CT imaging to monitor drug combination activity in treated individuals with tuberculosis. *Sci. Transl. Med.* 13 (579). doi: 10.1126/scitranslmed.abd7618

Zheng, H., Colvin, C. J., Johnson, B. K., Kirchhoff, P. D., Wilson, M., Jorgensen-Muga, K., et al. (2017). Inhibitors of mycobacterium tuberculosis DosRST signaling and persistence. *Nat. Chem. Biol.* 13 (2), 218–225. doi: 10.1038/nchembio.2259

Zhu, J. H., Wang, B. W., Pan, M., Zeng, Y. N., Rego, H., and Javid, B. (2018). Rifampicin can induce antibiotic tolerance in mycobacteria *via* paradoxical changes in rpoB transcription. *Nat. Commun.* 9 (1), 4218. doi: 10.1038/s41467-018-06667-3

Zimmer, A., Katzir, I., Dekel, E., Mayo, A. E., and Alon, U. (2016). Prediction of multidimensional drug dose responses based on measurements of drug pairs. *Proc. Natl. Acad. Sci. U.S.A.* 113 (37), 10442–10447. doi: 10.1073/pnas.1606301113

frontiers | Frontiers in Cellular and Infection Microbiology

TYPE Original Research
PUBLISHED 12 January 2023
DOI 10.3389/fcimb.2022.1064148

OPEN ACCESS

EDITED BY
Helena Ingrid Boshoff,
National Institutes of Health (NIH),
United States

REVIEWED BY
Brian D. Robertson,
Imperial College London,
United Kingdom
Sarah Stanley,
University of California, Berkeley,
United States

*CORRESPONDENCE
Bavesh D. Kana
bavesh.kana@wits.ac.za

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Molecular Bacterial Pathogenesis, a section of the journal Frontiers in Cellular and Infection Microbiology

RECEIVED 07 October 2022
ACCEPTED 22 December 2022
PUBLISHED 12 January 2023

CITATION
Peters JS, McIvor A, Papadopoulos AO, Masangana T, Gordhan BG, Waja Z, Otwombe K, Letutu M, Kamariza M, Sterling TR, Bertozzi CR, Martinson NA and Kana BD (2023) Differentially culturable tubercle bacteria as a measure of tuberculosis treatment response.
Front. Cell. Infect. Microbiol.
12:1064148.
doi: 10.3389/fcimb.2022.1064148

Differentially culturable tubercle bacteria as a measure of tuberculosis treatment response

Julian S. Peters[1†], Amanda McIvor[1†], Andrea O. Papadopoulos[1], Tshepiso Masangana[1], Bhavna G. Gordhan[1], Ziyaad Waja[2], Kennedy Otwombe[2,3], Matebogo Letutu[2], Mireille Kamariza[4], Timothy R. Sterling[5], Carolyn R. Bertozzi[6,7], Neil A. Martinson[1,2,8] and Bavesh D. Kana[1*]

[1]Department of Science and Innovation/National Research Foundation Centre of Excellence for Biomedical Tuberculosis Research, The National Health Laboratory Service, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, [2]Perinatal HIV Research Unit, University of the Witwatersrand, Johannesburg, South Africa, [3]School of Public Health, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa, [4]Department of Biology, Stanford University, Stanford, CA, United States, [5]Vanderbilt University Medical Center, Nashville, TN, United States, [6]Department of Chemistry, Stanford University, Stanford, CA, United States, [7]Howard Hughes Medical Institute, Stanford University, Stanford, CA, United States, [8]Johns Hopkins University Center for TB Research, Baltimore, MD, United States

Introduction: Routine efficacy assessments of new tuberculosis (TB) treatments include quantitative solid culture or routine liquid culture, which likely miss quantification of drug tolerant bacteria. To improve these assessments, comparative analyses using additional measures such as quantification of differentially culturable tubercle bacteria (DCTB) are required. Essential for enabling this is a comparative measure of TB treatment responses using routine solid and liquid culture with liquid limiting dilutions (LLDs) that detect DCTB in sputum.

Methods: We recruited treatment-naïve TB patients, with and without HIV-infection, and serially quantified their sputum for DCTB over the course of treatment.

Results: Serial sputum sampling in 73 individuals during their first 14 days of treatment demonstrated that clearance of DCTB was slower compared to routine solid culture. Treatment response appeared to be characterized by four patterns: (1) Classic bi-phasic bacterial clearance; (2) early non-responders with slower clearance; (3) paradoxical worsening with an increase in bacterial count upon treatment initiation; and (4) non-responders with no change in bacterial load. During treatment, LLDs displayed greater bacterial yield when compared with quantitative solid culture. Upon treatment completion, 74% [46/62] of specimens displayed residual DCTB and within this group, two recurrences were diagnosed. Residual DCTB upon treatment completion was associated with a higher proportion of MGIT culture, GeneXpert, and smear positivity at two months post treatment. No recurrences occurred in the group without residual DCTB.

Discussion: These data indicate that DCTB assays detect distinct subpopulations of organisms in sputum that are missed by routine solid and liquid culture, and offer important alternatives for efficacy assessments of new TB treatments. The residual DCTB observed upon treatment completion suggests that TB treatment does not always eliminate all bacterial populations, a finding that should be investigated in larger cohorts.

KEYWORDS

tuberculosis, resuscitation, colony forming units, most probable number (MPN), differentially culturable tubercle bacteria (DCTB)

Introduction

Tuberculosis (TB) therapy requires a prolonged duration to clear drug-tolerant persister bacteria not easily eliminated during early treatment. The underlying assumption is that the microenvironments encountered during infection of the human host drives these bacterial populations into non-replicative, drug tolerant states (Turapov et al., 2014; Hu et al., 2015; Gold and Nathan, 2017). Given the growing body of evidence suggesting drug tolerance is associated with eventual emergence of resistance (Liu et al., 2020), further study of these tolerant organisms is essential for formulating new therapeutic approaches to combat drug resistant TB. Definitive evidence for the presence of drug-tolerant persister organisms during TB treatment has been difficult to obtain due to the complex nature of sputum specimens, the exact source of sampling *Mycobacterium tuberculosis* from the lung, and inherent difficulties in detecting and quantifying persister bacteria. Multiple studies have identified bacterial populations in sputum that are unable to form colonies on solid culture but able to grow in liquid media supplemented with growth factors (Mukamolova et al., 2010; Dhillon et al., 2013; Kolwijck et al., 2014; Chengalroyen et al., 2016; Turapov et al., 2016; Saito et al., 2017; Mcaulay et al., 2018; Dusthackeer et al., 2019; Gordhan et al., 2021; Mcivor et al., 2021; Mesman et al., 2021; Zainabadi et al., 2021). These organisms are termed differentially culturable tubercle bacteria (DCTB) (Chengalroyen et al., 2016) or differentially detectable tubercle bacteria (DDTB) (Saito et al., 2017). In addition to being detected in a variety of extra-pulmonary specimens, DCTB have been identified from sputum prior to initiation and during TB treatment, and recently in animal models of TB disease (Mcaulay et al., 2018; Rosser et al., 2018; Zainabadi et al., 2021; Evangelopoulos et al., 2022). Detection of DCTB is facilitated by supplementation of sputum with culture filtrate (CF) from axenically grown *M. tuberculosis*, which stimulates bacterial growth. DCTB display drug tolerance when compared to bacteria cultured on solid media and their recovery can be enhanced with lipid rich media (Turapov et al., 2016; Mesman et al., 2021). The generation of DCTB has been linked to intracellular oxidative stress, which damages macromolecules in the bacillus, leading to the inability to recover on solid media (Saito et al., 2021). These observations suggest that a rigorous analysis of DCTB populations over the full course of TB chemotherapy could provide valuable insight for measuring treatment response.

In previous work with South African TB patients, we demonstrated that treatment-naïve sputum harbors a significant proportion of DCTB and enhanced detection thereof improved *M. tuberculosis* identification in sputum smear negative patients (Chengalroyen et al., 2016). The growth stimulatory effect of CF has been ascribed to resuscitation promoting factors (Rpfs), a group of bacterial growth stimulatory enzymes found in CF (Mukamolova et al., 2010). However, the concurrent observation of sputum samples harboring DCTB that grow independently of stimulation with Rpfs suggests that other enzymes/molecules may be involved in the growth stimulatory effect of CF (Chengalroyen et al., 2016; Gordhan et al., 2021).

The ability to detect treatment response of all bacterial populations in sputum is critical to understanding the efficacy of current and novel TB therapeutics. New anti-TB drugs are currently tested using a standardized Colony Forming Unit (CFU) assay on solid media and automated liquid culture, both of which may not detect DCTB populations. Hence, an assay that detects DCTB, which could be used to determine how these bacteria respond to new treatments, would help in identifying the best drug combinations for treatment shortening. To address this, we determined the treatment response of DCTB in comparison with CFUs and routine measures of bacterial load (GeneXpert, Mycobacterial Growth Indicator Tube [MGIT, Becton Dickinson] and smear microscopy) in a well-characterized prospective clinical cohort of drug susceptible TB patients at two South African sites (Soweto and Matlosana).

Methods

Recruitment and sputum sampling

Ethics approval for this study was obtained from the Human Research Ethics Committee of the University of the Witwatersrand (clearance number M120256). Study participants were approached if they had a positive sputum GeneXpert result from a primary health care clinic which was routinely tested in the public sector, usually the National Health Laboratory Service laboratories. Additional inclusion criteria for this study were: adults at least 18 years of age, able to produce a sputum sample of ≥ 3 ml, either evidence of HIV infection or a recent hard copy HIV test result, and no prior history of treatment for TB. Patients with rifampin resistance at baseline and/or characteristics suggesting non-adherence to study protocol were excluded (Supplementary Table 1). Following informed consent, spot and overnight (collected early in the morning) sputum samples were collected prior to TB treatment initiation and participants were followed-up during the course of treatment. For the treatment responses analyses, results of overnight sputa are reported here, as we anticipated these would provide the best bacterial yield. Where overnight sputum was not available, or the resulting cultures contaminated, the spot sputum was used. Data from spot samples were also used when assessing standard measures of bacterial load (smear, GeneXpert and MGIT). Data from overnight and spot sputa are reported seperately serial sputum samples were collected whilst the patient was taking TB treatment (acceptable visit windows enclosed in parentheses): 3 (2 – 4), 7 (5 – 10), 14 (12 – 19), 35 (30 – 40), 56 (50 – 65) and 180 days (170-190) after treatment initiation (Supplementary Figure 1). Cure was defined by the presence of negative MGIT cultures and smears at day 180. Treatment failure was defined as a positive smear or MGIT culture at five- or six-months post treatment initiation. Disease recurrence was defined as the reoccurrence of TB after cure.

Liquid limiting dilution assays

LLDs entail determination of bacterial count using limiting dilutions of sputum and were carried out as previously described (Chengalroyen et al., 2016). These assays yield the Most probable number (MPN) of bacteria present in a sample. Briefly, CF was obtained from both wild-type and a quintuple *rpf* deletion mutant of *M. tuberculosis* H37Rv strains (Kana et al., 2008) and are referred to as CF and Rpf $^{-}$CF, respectively. Cultures of H37Rv were grown to an Optical Density$_{600nm}$ (OD_{600nm}) = 0.6-0.9, from which the cells were harvested by centrifugation at 4 200 x g for 8 minutes and the resulting CF was filtered using a 0.2 µm polyethersulfone (PES) filter (Amicon) to remove any residual cells. To replenish broth nutrients depleted by growing *M. tuberculosis*, CF preparations were diluted 1:1 with fresh Middlebrook 7H9 (Difco) media supplemented with OADC (BD diagnostics) containing 0.05% tween and 450 µl of this was dispensed across 8 wells of a 48 well micro-titre plate (Nunc Thermo) in triplicate. As a control, 450 µl of un-supplemented Middlebrook 7H9 was added across a second 48 well plate in triplicate. To each plate, 50 µl of decontaminated sputum sample was added to the first column of wells and a 10-fold serial dilution was carried out across the plate to the 8th dilution (Figure 1A). Growth was assessed at 6 weeks by visual scoring and bacterial counts were calculated using an algorithm based on the Poisson distribution (https://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/professoren/wilrich/index.html). CFU assays were performed by plating 100 µl of a ten-fold serial dilution series of decontaminated sputum onto Middlebrook 7H11 plates (Figure 1A). Further detail can be found in the supplementary material. None of the experimental assays were used to guide participant treatment or care.

LLD analysis and statistical methods

We assessed the treatment response of bacteria emerging from LLD assays using the MPN value and other readouts. Data were analyzed using Graphpad Prism 7 Medians, interquartile ranges (IQRs) and proportions were determined for continuous measures and categorical variables, respectively. Continuous measures were compared (between CF-supplemented LLDs, Rpf $^{-}$ CF-supplemented LLDs, un-supplemented LLDs and CFUs) using the Mann-Whitney or Wilcoxon signed-rank tests whereas categorical measures were compared by the Chi-square test. Correlations between pairs of continuous measures were conducted using the Spearman's rank correlation test. The Kruskal-Wallis test was used to compare the hypothesis of no difference in multiple non-parametric comparisons of LLD derived MPN values and CFU values. LOESS curves were plotted for MPN, CFU, DCTB, the inverse of the MGIT days to positivity (1/MGIT) and the inverse of the GeneXpert cycle threshold (1/GeneXpert). Additional plots were generated and stratified by the following groups: Classic bi-phasic, early non-responders, Paradoxical worsening, and Non-responders. A null hypothesis of no difference in the change in slope over time was tested using linear mixed modelling. The difference between pairs of slopes was determined using an interaction term. SAS Enterprise Guide 7.15 (SAS Institute Inc., Cary, NC, USA) was used to fit the linear mixed models.

Results

Of 175 individuals screened, 50 (29%) were screen failures (Figure 1B, Supplementary Table 2). Sputa from 45 (26%)

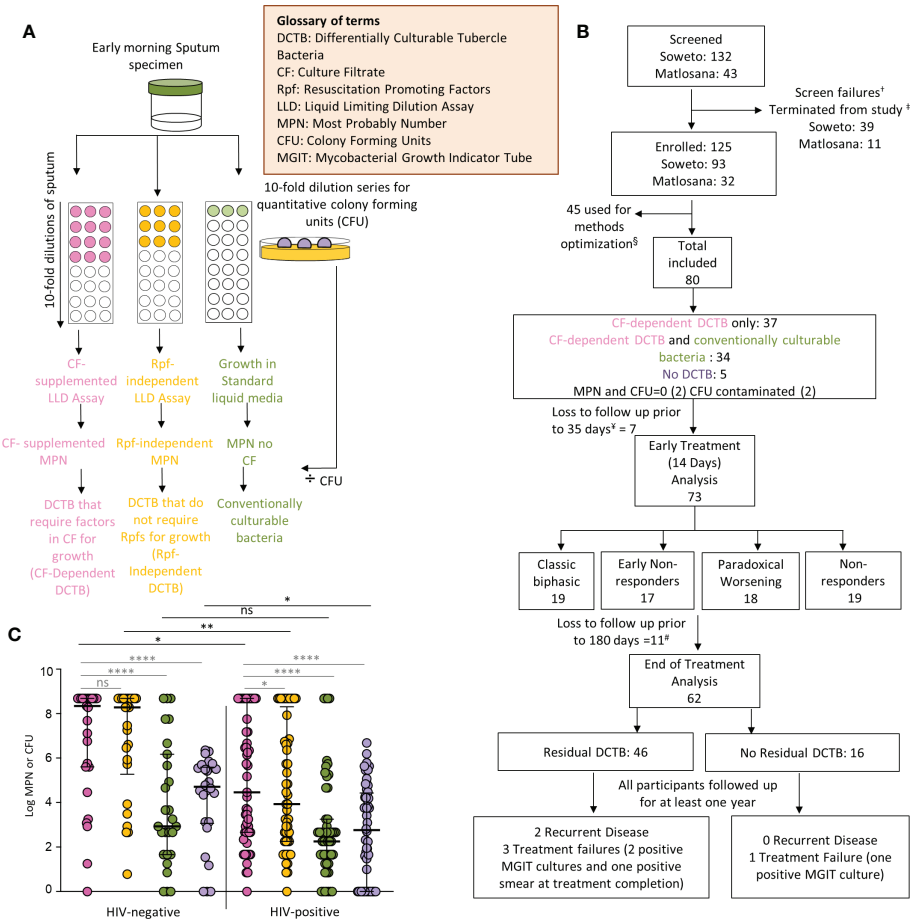


FIGURE 1
Participant disposition and enrollment microbiology. **(A)** Liquid limiting dilution assays (LLDs) to detect distinct differentially culturable populations in sputum samples. **(B)** Participant disposition flow chart. A total of 175 potential participants were screened for this study from primary health care clinics in the Soweto and Matlosana regions. From each cohort, the arrows depict participants who were removed from the study for various clinical and laboratory reasons. †Screen failures (18 from Soweto, 8 from Matlosana) include potential participants who were initially suitable for the study and then found to not meet the requirements. ‡Participants were terminated from the study (21 from Soweto, 3 from Matlosana) and reasons for their removal are documented in the supplementary information. Methods optimization denoted by (§) include LLDs performed for sputum samples with different plates and plate-sealing methods to address emerging evaporation of cultures and contamination issues. This aspect is further discussed in the supplementary material. ¥Loss to follow up and one participant was excluded as no sputum sample was received for baseline LLD and CFU data. # One participant missed the day 180 time point but returned thereafter, the individual was excluded from the post 180 day analysis. Data from a total of 80 participants were available for baseline analysis. Additional participants were lost to follow up at various time points throughout the study. Those lost before or after day 35 were classified as early and late dropouts respectively. Data from 73 participants were available for early treatment analysis (sputum samples received up to day 14 following treatment initiation) and data from 62 participants were available for end of treatment analysis (sputum samples received up to 180 days following treatment initiation). **(C)** Bacterial yield using CF-supplemented LLDs (pink), Rpf ⁻ CF-supplemented LLDs (orange), un-supplemented LLDs (green) and colony forming units (purple) in enrollment sputum from TB and TB-HIV infected individuals. *p<0.05, **p<0.01, ****p<0.0001. ns = Not significant.

participants were used to optimize LLD culture conditions, which were conducted in real time as sputum was received at the laboratory (Supplementary Figure 3 and Supplementary Figure 4). Therefore, 80/175 (46%) participants in total were included in the baseline analysis, 48 (60%) from Soweto and 32 (40%) from Matlosana. In this cohort, 55/80 (68%) were men, 53/80 (66%) were HIV-positive with a median CD4 T cell count of 167 (IQR 93 – 310) cells/mm^3 and 21/80 (26%) were +++ smear positive on the World Health Organization grading scale (Table 1, which gives statistical results of tests comparing the association between HIV status and demographic, clinical, immunological, microbiological and diagnostic variables). HIV-positive individuals with TB had lower sputum bacterial loads compared to their HIV-negative counterparts as measured by smear status, GeneXpert Cycle threshold, and MGIT culture time to positivity (Table 1).

At enrollment, CF-supplemented LLD assays yielded a statistically significant higher bacterial count when compared

10.3389/fcimb.2022.1064148

TABLE 1 Demographics, immunology, microbiological and diagnostic data for tuberculosis patients categorized by HIV-1 infection status and C4 T-cell counts.

Variable	Overall (n = 80)	HIV-negative (n = 27)	HIV-positive (n = 53)	p-value
Demographics				
Male, n (%)	55 (68·8)	22 (81.5)	33 (61·8)	0.125*
Female, n (%)	25 (31·3)	5 (18.5)	20 (38·1)	
Age, yr, median (IQR)	36·0 (27·0- 44·0)	27·0 (23 -48·0)	38·5 (31·0 – 43·8)	0.016
BMI				
Median at baseline (IQR), kg/m^2	19·2 (17·9 – 21·6)	18.8 (17·7 – 20.6)	19.4 (18.1 – 22.1)	0.151
CD4 T-cell count Median cells/mm3 (IQR)	NA	NA	167·0 (95·5 – 315.5)	
Conventional tuberculosis diagnosis, n (%)				
Auramine smear				
Smear grade positive‡	45 (56.3)	20 (74.1)	25 (47.1)	0.007Ψ
Smear grade negative	25 (31.3)	3 (11.1)	22 (41.5)	
Scanty	6 (7.5)	2 (7.4)	4 (7.5)	
Unknown	4 (5)	2 (7.4)	2 (3.8)	
+	13·0 (16.3)	3 (11.1)	10·0 (18·9)	
++	11·0 (13·8)	9 (33.3)	2·0 (3.8)	
+++	21·0 (26.3)	8 (29.6)	13 (24.5)	
GeneXpert result §				
Median GeneXpert cycle threshold (IQR)	19.9 (13.9 – 24.0)	16.5 (13.5·0 – 20·9)	22·3 (14.3 – 26·8)	0.016
High, n (%)	23·0 (28.8)	11·0 (40.7)	12·0 (22·6)	–
Medium, n (%)	16·0 (20)	9·0 (33.3)	7·0 (13.2)	
Low, n (%)	16·0 (20)	3·0 (11.1)	13·0 (24.5)	
Very low, n (%)	8·0 (10)	1·0 (3.7)	7·0 (13.2)	
MTB not detected, n (%)	12·0 (15)	1 (3.7)	11·0 (20.8)	
Unknown	5 (6.3)	2 (7.4)	3 (5.7)	
Median MGIT time to positivity days (IQR)	6.5 (4·0 – 14·3)	5·0 (3·0 – 8.0)	9·0 (4·0 – 17·0)	0.018
Proportion positive MGIT cultures (%)#	63 (90)	22 (96)	41 (87)	

BMI = body mass index; IQR = interquartile range; MGIT = mycobacterial growth indicator tube; NA = not applicable.
* Using the Chi-squared test.
‡ Includes scanty, +, ++ and +++.
Ψ Using Fishers Exact test (2X2 table).
§ GeneXpert: M· tuberculosis was not detected in five patients.
# 70 MGIT culture results were available, 23 for HIV uninfected and 47 for HIV infected.

to CFUs [log median (IQR) of the CF supplemented LLD = 6.2 (2.9 – 8.7) compared to log median (IQR) CFU = 3.8 (0.0 – 5.1), p<0.0001, (Figure 1C)]. LLD assays with standard media yielded a lower bacterial count when compared to CF-supplemented LLDs [LLD without CF: log median (IQR) = 2.7 (1.7 – 4.7), p<0.0001 vs CF+ LLD: log median (IQR) = 6.2 (2.9 – 8.7), Figure 1C]. HIV-positive participants had lower CF-supplemented MPN and CFU counts compared to HIV-negative counterparts (Figure 1C). In both HIV-positive and HIV-negative individuals, the addition of CF to LLD assays resulted in a statistically significant increase in bacterial yield compared to the CFUs and MPN count without CF-supplementation (Figure 1C). CF-dependent DCTB were recovered from 37/80 (46%) baseline specimens, with no

bacteria emerging from the same specimens in standard media (Supplementary Figure 5B). The quantum of CF-dependent DCTB recovered varied between specimens. In 34/80 (43%) specimens, both CF-dependent DCTB and conventionally culturable organisms were recovered and, in most cases, supplementation of LLD assays with CF yielded higher bacterial counts [CF-supplemented MPN, log median (IQR) = 2.9 (1.9 – 3.4) compared to MPN count with no CF, log median (IQR) = 1.8 (0.8 – 2.7), p=0.0047]. In 5/80 (6.3%) sputum specimens, no DCTB were recovered, with the CFU count yielding higher bacterial loads compared to CF-supplemented and un-supplemented LLD assays (Supplementary Figure 5B). In 4/80 (5%) specimens, the MPN count or CFU was negative or the CFU was contaminated. Overall, 71/80 (89%) specimens demonstrated the presence of DCTB at baseline, suggesting a substantive increase in bacterial yield from sputum culture through supplementation of liquid growth assays with CF.

To assess whether the yield of DCTB required the presence of Rpfs in CF, baseline sputum LLDs supplemented with CF were compared with those containing CF from an Rpf-deficient mutant of *M. tuberculosis*. In all specimens, removal of Rpfs from the CF in LLDs yielded a 0.5 log decrease in bacterial count when compared to assays with CF containing Rpfs, suggesting that inclusion of Rpfs in the CF confers a benefit in determining bacterial yield prior to treatment. This effect was statistically significant in HIV-infected individuals only (Figure 1C) and given this, we focused on analyzing trends for the LLDs supplemented with CF containing Rpfs in the remainder of this analysis.

Comparing bacterial clearance from serial sputum sampling over the first 14 days of TB treatment revealed a slower rate of bacterial clearance for DCTB than that reported by CFUs or Mycobacterial Growth Indicator Tube Time to Positivity (MGIT-TTP) (Figure 2A, p=0.0735 and p=0.0038 respectively, Supplementary Table 6 and Supplementary Figure 6). Upon visual inspection of treatment response patterns, based on raw MPN counts, four patterns emerged (detailed in Supplementary Table 5; individual patient graphs are given in the Supplementary Figure 11). The first pattern (n=19), termed "Classic bi-phasic", was defined by a rapid initial decline in both MPN and CFU counts, during the first week, followed by a slower rate of bacterial clearance thereafter (Figure 2B). LOESS curves and linear mixed modelling indicated that DCTB and CFUs cleared at equivalent rates, but there were significant differences in clearance between DCTB and standard liquid culture (Supplementary Table 6). The second pattern (n=17), termed "Early non-responders", was defined by no substantial change (< 0.5 log) in MPN count between the enrolment sample and either at day 3 or at day 7 of treatment, followed by a steady decline up to day 14 of treatment (Figure 2C). CFUs and DCTB declined in comparable manner in this group (Supplementary Table 6). The third pattern (n=18), termed "Paradoxical worsening", was characterized by an increase in the bacterial load between enrolment and the 3rd or 7th day of treatment (Figure 2D). During the same period, MGIT TTP reported a decline in bacterial counts. The rate of DCTB decline was slower compared to CFU, but this difference was not significant (Supplementary Table 6). A fourth group (n=19), termed "non-responders", was characterized by low initial bacterial counts and yielded non-responsive patterns showing negligible changes in DCTB counts during early treatment (Figure 2E and Supplementary Table 6). We also conducted a correlation analysis of CF-supplemented MPNs versus CFU counts for all participants, at days 0, 3, 7 and 14. The correlation values are generally low or poor when comparing data collected on the same day (Day 0 vs Day 0, Day 3 vs Day 3 etc) except for CFU and CF-Supplemented MPNs at day 14 (r=-0.34) where the correlation is negative and moderate (Supplementary Figure 7). These data indicate that CF-supplemented LLD assays detect populations of bacteria that are distinct from those detected by the CFU assay.

To investigate if clinical or diagnostic characteristics were associated with these four patterns of response, we compared clinical and microbiological measurements between these groups (Supplementary Table 7). Non-responder participants had a larger proportion of HIV-positive individuals, were older than the other groups and had more smokers. No differences in BMIs were noted. There were significant differences for both GeneXpert and MGIT TTP at enrolment between the four groups, with the Classic bi-phasic and Early non-responder groups displaying higher bacterial loads prior to treatment initiation. A caveat is that our sample size was small, and that these patterns were visually identified, suggesting that further interpretation of these data requires caution.

In addition, within these four patterns, we also noted participants (n=18) in whom no CFUs were recorded across all time points (or there was only 1 time point where there was a readable CFU) within the first two weeks of treatment. In these individuals, MPN counts and MGIT TTP was able to measure the response to TB therapy (Figure 2F, graphs for individual patients in Supplementary Figure 8).

At the end of 6 months of treatment, 41/44 (93.2%) available, non-contaminated MGIT cultures, from early morning sputum were negative for *M. tuberculosis*. The LLD assay (with and without CF) however, identified 46/62 (74.1%) patients whose sputum specimens at treatment completion still contained what appeared to be residual bacteria (Figure 3A). In contrast to enrolment data, there were no differences in recovery of DCTB from HIV-infected versus their uninfected counterparts (Figure 3B). Within our four categories, the Classic bi-phasic, Paradoxical worsening and early non-responder groups had similar proportions of participants with positive LLD cultures at the end of treatment (6/12 [50%], 11/16 [68.8%], 11/17 [64.7%], respectively). The Non-responder group had a higher proportion of positive LLD cultures (14/17 [82.4%]) upon treatment completion but this difference was not statistically

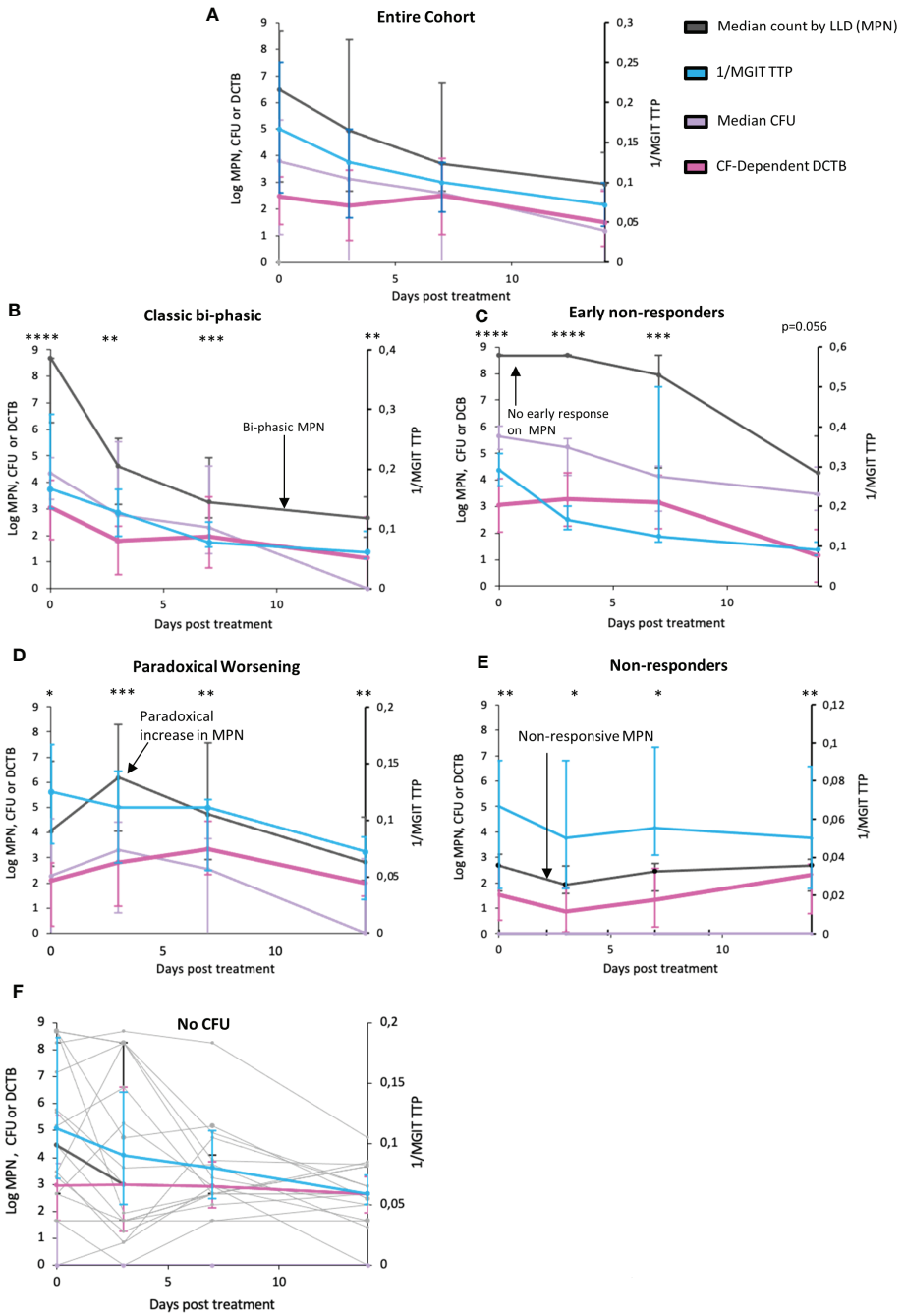


FIGURE 2
Trends in bacterial clearance over time during standard TB treatment using various measure of bacterial load in sputum. **(A)** Trendlines for bacterial clearance in all sputum specimens. **(B–E)** Bacterial clearance patterns for subcategories including Classic bi-phasic, Early non-responders, Paradoxical worsening, and Non–responsive groups, based on the median trend line of the LLDs. Error bars represent the interquartile range. **(F)** Trends in bacterial clearance in individuals where all no CFUs were recorded across all time points (or there was only 1 time point where there was a readable CFU) within the first two weeks of treatment. Light grey bars represent individual participant MPN values. MPN indicates the most probable number obtained from LLDs assays. *: p<0.05; **: p<0.01; ***p<0.001; ****p<0.0001.

significant. There was no difference in the amount of DCTB recovered between categories (Supplementary Figure 9). To assess viability of organisms obtained in the end of treatment LLD assay, we used a fluorescent derivative of trehalose carrying a 4-*N*,*N*-dimethylamino-1,8-napthalimide (DMN) fluorophore, termed DMN-Tre, as a probe for active metabolism (Kamariza et al., 2018). DMN-Tre has a high degree of specificity for mycobacteria and the stain only provides a signal in

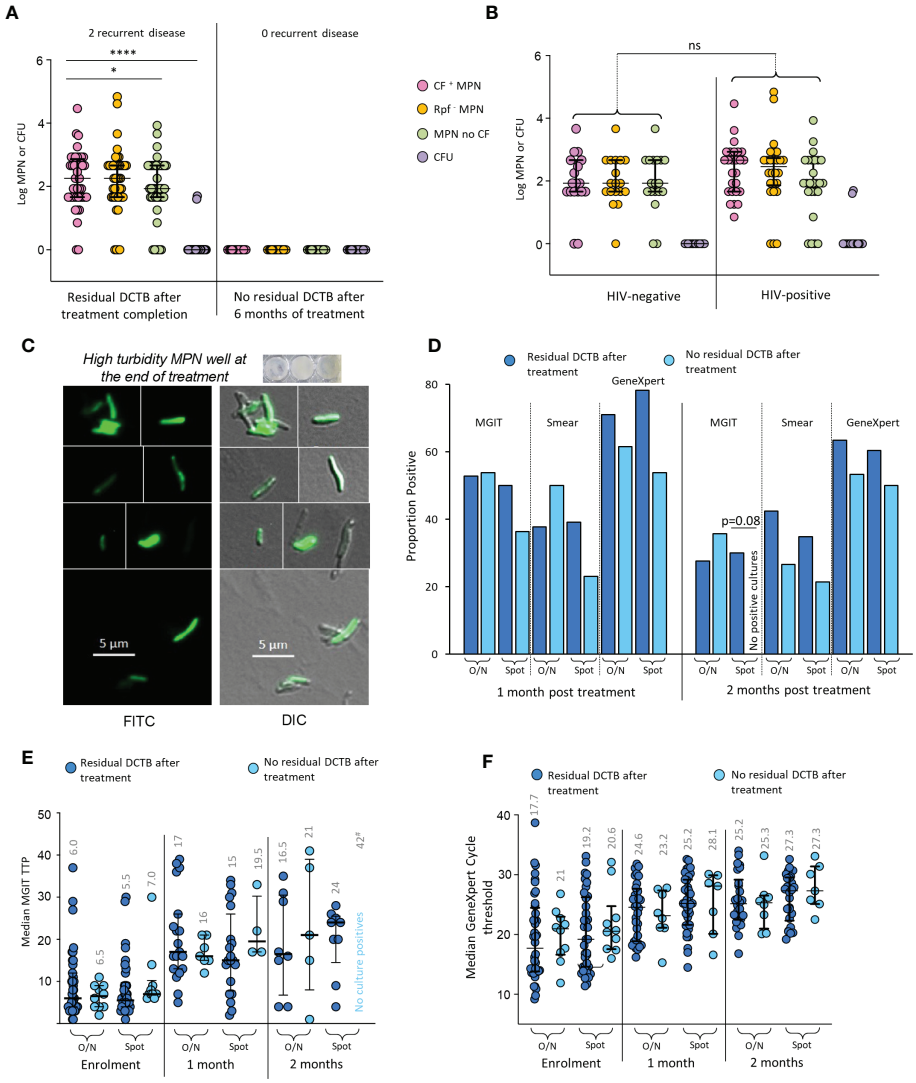


FIGURE 3
Residual DCTB at the end of treatment. **(A)** Shown is a scattergram of MPN and CFU values in individuals with and without residual DCTB after treatment completion. **(B)** Scattergram of MPN and CFU values for specimens from individuals with residual DCTB after treatment completion stratified by HIV-infection status. Shown are bacterial yields from CF-supplemented LLDs (pink), Rpf $^{-}$ CF-supplemented LLDs (orange), un-supplemented LLDs (green) and colony forming units (purple). **(C)** Staining of bacteria from end of treatment LLDs using the DMN-Trehalose viability stain, shown is a representative from LLD wells with high turbidity. **(D)** Proportion MGIT, smear and GeneXpert positivity for sputum specimens from individuals with or without residual DCTB after treatment completion. **(E)** Median MGIT time to positivity (TTP) for sputum specimens from individuals with or without residual DCTB after treatment completion. Numbers represent median TTP. **(F)** Median GeneXpert Cycle threshold (CT) for sputum specimens from individuals with or without residual DCTB after treatment completion. Numbers represent median CT. O/N: Overnight specimen, Spot: Spot specimen. * p<0.05; **** p<0.0001. ns = Not significant.

metabolically active bacilli when incorporated into the mycolic acid layer of viable mycobacterial cells (Kamariza et al., 2018). In 6 randomly selected specimens tested for DMN-Tre staining, rod-shaped organisms were observed in samples derived from CF supplemented LLDs with diverse levels of turbidity, confirming the presence of residual metabolically active *M. tuberculosis* in patient sputum at the end of treatment (Figure 3C and Supplementary Figure 10).

In individuals with residual DCTB at the end of treatment, a higher proportion had positive MGIT cultures, smears and GeneXpert tests at 2 months after treatment when compared to those without residual DCTB but most of these differences were not statistically significant (Figure 3D). We did note a significant difference in MGIT culture positivity with spot specimens where 30% of specimens with DCTB at the end of treatment were positive on MGIT at 2 months versus 0%

positivity in specimens with no DCTB. Similarly, median MGIT TTPs at 2 months post treatment were lower (suggesting higher bacterial load) in individuals with residual DCTB after treatment completion (Figure 3E). At this time point, there were no differences in GeneXpert cycle threshold, presumably as the DNA of DCTB still gets detected in molecular diagnostics (Figure 3F). In the group with residual DCTB after treatment, we recorded 2 recurrent disease episodes within 12 months of treatment completion.

Discussion

In this prospective cohort of drug susceptible TB patients, we assessed the utility of measuring bacterial load in sputum using LLD assays supplemented with CF as a source of growth stimulatory factors. In most sputum specimens prior to treatment initiation, CF-supplemented LLD assays yielded higher bacterial yield compared to un-supplemented LLD assays and CFUs in both HIV-positive and HIV-negative individuals. In addition to DCTB that required CF to grow, we also found conventionally culturable organisms in sputum as expected. In most cases, these organisms occurred as a mixture of CF-dependent DCTB and conventionally culturable bacteria, underscoring the inherent complexity of bacterial populations in sputum prior to treatment.

Rpfs have been implicated in resuscitation of DCTB in sputum and we observed this in our prior work, however, we also noted the presence of Rpf-independent DCTB (Mukamolova et al., 2010; Chengalroyen et al., 2016). We probed this further in our current study by comparing the yield of DCTB between LLD assays that contained Rpfs to those without. Prior to treatment initiation, a significant requirement for Rpfs to recover DCTB in HIV-infected individuals was noted. These participants had lower bacterial loads than their HIV-uninfected counterparts, suggesting that Rpfs may exert their effects maximally in cases where the bacterial load in sputum is low, an effect that can be exploited to develop better diagnostic tests, particularly for vulnerable groups of individuals. In addition to Rpfs, CF likely contains other molecules that are equally potent in resuscitating DCTB from sputum. Such molecules, including by-products of cell wall cleavage, have been described in literature (Nikitushkin et al., 2012; Shleeva et al., 2014; Nikitushkin et al., 2015). Our prior analysis suggests that the growth stimulatory effect observed with CF is most likely the result of a combination of factors (Gordhan et al., 2021).

During the first 14 days of treatment, CF-supplemented LLDs yielded a significantly greater bacterial count when compared to CFUs. This indicated that in addition to enrolment sputum specimens, sputum from individuals on treatment harbor notable DCTB populations, the detection of which may enable better evaluation of TB treatment response (Zainabadi et al., 2021). This could have important implications for assessing early bactericidal activity (EBA) during the first two weeks of treatment (Diacon and Donald, 2014; Evangelopoulos et al., 2022). EBA studies are the current standard to measure the bactericidal drug activity of new anti-TB drugs or the efficacy of perturbations in current regimens before large phase 3 trials. However, the limitations of EBA to monitor the activity of drugs that have excellent sterilizing capacity but limited bactericidal activity early in treatment are acknowledged (O'brien, 2002; Evangelopoulos et al., 2022). Whilst attempts have been made to improve EBA-type approaches through data modelling, or the use of DNA metrics (Heyckendorf et al., 2017; Gillespie et al., 2002) an alternative assay which describes the ability of drugs to eradicate bacterial persisters has not emerged and remains an urgent priority. MGIT cultures have also been utilized for EBA analyses, however, the four patterns that we have noted in LLD responsive organisms suggests that the standard CFU assay and MGIT culture have limited ability to report on elimination of drug tolerant bacteria such as DCTB. This is further corroborated by the observation that serial sputum specimens from numerous participants in our cohort yielded no CFUs yet had detectable levels of bacteria using LLDs. In cases where patterns of bacterial clearance recorded by LLDs were concordant with those observed by CFUs, the former still yielded a higher bacterial count. In addition, patterns of bacterial decline as measured by LLDs for the Non-responsive and Early non-responder LLD groups differed to those measured by standard MGIT TTP indicating that CF-supplemented LLD assays detect bacterial populations that are missed by standard methods used in the clinical setting. These collective observations could form the basis of development of a new assay to monitor drug activity in individuals who either respond slowly or are unresponsive to therapy.

Using LLDs, residual bacterial growth could be detected in two-thirds of participants at the end of treatment; we confirmed viability of these mycobacteria in a randomly selected subset of specimens using a metabolic probe. As our sample size did not allow for a robust statistical analysis, this observation merits further study. Given that only 2 recurrences were noted in the group of individuals with residual DCTB, it is unlikely that DCTB positivity upon treatment completion is predictive of relapse. Rather, it appears that TB treatment does not eradicate all bacteria and at treatment completion some residual organisms remain. Continued immunological containment of these bacteria would be important to prevent relapse.

Other limitations of our work include the lack of anti-TB drug plasma concentrations to confirm adherence. Whilst directly observed therapy was conducted on days preceding sampling time points, together with an oral report of adherence, other measures of adherence were not done. That said, the majority of patients achieved clinical cure, suggesting that treatment adherence was not a confounder in our study. Furthermore, the use of antiretroviral therapy in HIV-infected participants may have affected bacterial clearance. As we did not

collect detailed information on this, no analyses could be done. Also, our separation of treatment response patterns was done using a visual inspection of bacterial clearance patterns. This approach does not exclude the possibility that the groupings arise by random chance, hence we have been cautious in deriving any interpretations from this analysis.

Our study provides an in-depth analysis of DCTB in sputum over the course of TB treatment with findings that may enable the development of new clinical endpoints for measuring drug effectiveness. The superior performance of the DCTB assays in individuals with either low bacterial loads or who respond poorly to treatment underscores the importance of using supplemented liquid cultures for diagnosis of TB, particularly in HIV-endemic settings. This analysis suggests that DCTB assays may be used as a novel measure of treatment response in EBA assays to simultaneously assess both drug tolerant and drug sensitive populations.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Human Research Ethics Committee, University of the Witwatersrand. The patients/participants provided their written informed consent to participate in this study.

Author contributions

BK, JP and AM designed experiments, performed experiments, wrote and edited the manuscript with assistance from NM, CB and TS. AP and TM provided technical assistance, BG managed the study, ZW, ML and NM managed the clinical site operations at the PHRU. KO, NM and TS assisted with statistical analysis. CB and MK aided with metabolic probes and associated data analysis. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by grants from the Bill and Melinda Gates Research Foundation (OPP1100182), South African Medical Research Council with funds from the Department of Health, the Department of Science and Innovation, the South African National Research Foundation and the Howard Hughes Medical Institute.

Acknowledgments

The authors thank Clinical Laboratory Services and PHRU staff for diagnostic sample processing and assistance with patient recruitment respectively. The authors also thank the members of the Centre of Excellence for Biomedical TB research for insightful comments and technical advice. We thank Christopher Ealand for assistance with microscopy analysis. We acknowledge and thank all participants of this study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.1064148/full#supplementary-material

References

Chengalroyen, M. D., Beukes, G. M., Gordhan, B. G., Streicher, E. M., Churchyard, G., Hafner, R., et al. (2016). Detection and quantification of differentially culturable tubercle bacteria in sputum from patients with tuberculosis. *Am. J. Respir. Crit. Care Med.* 194, 1532–1540. doi: 10.1164/rccm.201604-0769OC

Dhillon, J., Fourie, P. B., and Mitchison, D. A. (2013). Persister populations of mycobacterium tuberculosis in sputum that grow in liquid but not on solid culture media. *J. Antimicrob. Chemother.* 69, 437–440. doi: 10.1093/jac/dkt357

Diacon, A. H., and Donald, P. R. (2014). The early bactericidal activity of antituberculosis drugs. *Expert Rev. Anti Infect. Ther.* 12, 223–237. doi: 10.1586/14787210.2014.870884

Dusthackeer, A., Balasubramanian, M., Shanmugam, G., Priya, S., Nirmal, C. R., Sam Ebenezer, R., et al. (2019). Differential culturability of mycobacterium tuberculosis in culture-negative sputum of patients with pulmonary tuberculosis and in a simulated model of dormancy. *Front. Microbiol.* 10. doi: 10.3389/fmicb.2019.02381

Evangelopoulos, D., Shoen, C. M., Honeyborne, I., Clark, S., Williams, A., Mukamolova, G. V., et al. (2022). Culture-free enumeration of mycobacterium tuberculosis in mouse tissues using the molecular bacterial load assay for preclinical drug development. *Microorganisms* 10(2),420. doi: 10.3390/microorganisms 10020460

Gillespie, S. H., Gosling, R. D., and Charalambous, B. M. (2002). A reiterative method for calculating the early bactericidal activity of antituberculosis drugs. *Am. J. Respir. Crit. Care Med.* 166, 31–35. doi: 10.1164/rccm.2112077

Gold, B., and Nathan, C. (2017). Targeting phenotypically tolerant mycobacterium tuberculosis. *Microbiol. Spectr.* 5(1),10.1128. doi: 10.1128/microbiolspec.TBTB2-0031-2016

Gordhan, B. G., Peters, J. S., Mcivor, A., Machowski, E. E., Ealand, C., Waja, Z., et al. (2021). Detection of differentially culturable tubercle bacteria in sputum using mycobacterial culture filtrates. *Sci. Rep.* 11, 6493. doi: 10.1038/s41598-021-86054-z

Heyckendorf, J., Labugger, I., van der Merwe, L., Garcia-Basteiro, A. L., Diacon, A. H., and Lange, C. (2017). Serial measurements of transrenal mycobacterial DNA as indicators of the early bactericidal activity (EBA) of antituberculosis drugs. *Tuberculosis (Edinb)* 102, 31–33. doi: 10.1016/j.tube.2016.10.005

Hu, Y., Liu, A., Ortega-Muro, F., Alameda-Martin, L., Mitchison, D., and Coates, A. (2015). High-dose rifampicin kills persisters, shortens treatment duration, and reduces relapse rate *in vitro* and *in vivo*. *Front. Microbiol.* 6. doi: 10.3389/fmicb.2015.00641

Kamariza, M., Shieh, P., Ealand, C. S., Peters, J. S., Chu, B., Rodriguez-Rivera, F. P., et al. (2018). Rapid detection of mycobacterium tuberculosis in sputum with a solvatochromic trehalose probe. *Sci. Transl. Med* 10(430),eaam6310. doi: 10.1126/scitranslmed.aam6310

Kana, B. D., Gordhan, B. G., Downing, K. J., Sung, N., Vostroktunova, G., Machowski, E. E., et al. (2008). The resuscitation-promoting factors of mycobacterium tuberculosis are required for virulence and resuscitation from dormancy but are collectively dispensable for growth *in vitro*. *Mol. Microbiol.* 67, 672–684. doi: 10.1111/j.1365-2958.2007.06078.x

Kolwijck, E., Friedrich, S. O., Karinja, M. N., Van Ingen, J., Warren, R. M., and Diacon, A. H. (2014). Early stationary phase culture supernatant accelerates growth of sputum cultures collected after initiation of anti-tuberculosis treatment. *Clin. Microbiol. Infect.* 20, O418–O420. doi: 10.1111/1469-0691.12441

Liu, J., Gefen, O., Ronin, I., Bar-Meir, M., and Balaban, N. Q. (2020). Effect of tolerance on the evolution of antibiotic resistance under drug combinations. *Science* 367, 200–204. doi: 10.1126/science.aay3041

Mcaulay, K., Saito, K., Warrier, T., Walsh, K. F., Mathurin, L. D., Royal-Mardi, G., et al. (2018). Differentially detectable mycobacterium tuberculosis cells in sputum from treatment-naive subjects in Haiti and their proportionate increase after initiation of treatment. *MBio* 9(6),e02192-18. doi: 10.1128/mBio.02192-18

Mcivor, A., Gordhan, B. G., Waja, Z., Otwombe, K., Martinson, N. A., and Kana, B. D. (2021). Supplementation of sputum cultures with culture filtrate to detect tuberculosis in a cross-sectional study of HIV-infected individuals. *Tuberculosis (Edinb)* 129, 102103. doi: 10.1016/j.tube.2021.102103

Mesman, A. W., Baek, S. H., Huang, C. C., Kim, Y. M., Cho, S. N., Ioerger, T. R., et al. (2021). Characterization of drug-resistant lipid-dependent differentially detectable mycobacterium tuberculosis. *J. Clin. Med* 10(15):3249. doi: 10.3390/jcm10153249

Mukamolova, G. V., Turapov, O., Malkin, J., Woltmann, G., and Barer, M. R. (2010). Resuscitation-promoting factors reveal an occult population of tubercle bacilli in sputum. *Am. J. Respir. Crit. Care Med.* 181, 174–180. doi: 10.1164/rccm.200905-0661OC

Nikitushkin, V. D., Demina, G. R., Shleeva, M. O., Guryanova, S. V., Ruggiero, A., Berisio, R., et al. (2015). A product of RpfB and RipA joint enzymatic action promotes the resuscitation of dormant mycobacteria. *FEBS J.* 282, 2500–2511. doi: 10.1111/febs.13292

Nikitushkin, V. D., Demina, G. R., Shleeva, M. O., and Kaprelyants, A. S. (2012). Peptidoglycan fragments stimulate resuscitation of "non-culturable" mycobacteria. *Antonie Van Leeuwenhoek* 103(1),37-46. doi: 10.1007/s10482-012-9784-1

O'brien, R. J. (2002). Studies of the early bactericidal activity of new drugs for tuberculosis: A help or a hindrance to antituberculosis drug development? *Am. J. Respir. Crit. Care Med.* 166, 3–4. doi: 10.1164/rccm.2205007

Rosser, A., Pareek, M., Turapov, O., Wiselka, M. J., and Mukamolova, G. V. (2018). Differentially culturable tubercule bacilli are generated during nonpulmonary tuberculosis infection. *Am. J. Respir. Crit. Care Med.* 197, 818–821. doi: 10.1164/rccm.201705-1048LE

Saito, K., Mishra, S., Warrier, T., Cicchetti, N., Mi, J., Weber, E., et al. (2021). Oxidative damage and delayed replication allow viable mycobacterium tuberculosis to go undetected. *Sci. Transl. Med.* 13, eabg2612. doi: 10.1126/scitranslmed.abg2612

Saito, K., Warrier, T., Somersan-Karakaya, S., Kaminski, L., Mi, J., Jiang, X., et al. (2017). Rifamycin action on RNA polymerase in antibiotic-tolerant mycobacterium tuberculosis results in differentially detectable populations. *Proc. Natl. Acad. Sci. U.S.A.* 114, E4832–E4840. doi: 10.1073/pnas.1705385114

Shleeva, M., Goncharenko, A., Kudykina, Y., Young, D., Young, M., and Kaprelyants, A. (2014). Cyclic amp-dependent resuscitation of dormant mycobacteria by exogenous free fatty acids. *PloS One* 8, e82914. doi: 10.1371/journal.pone.0082914

Turapov, O., Glenn, S., Kana, B., Makarov, V., Andrew, P. W., and Mukamolova, G. V. (2014). The *in vivo* environment accelerates generation of resuscitation-promoting factor-dependent mycobacteria. *Am. J. Respir. Crit. Care Med.* 190, 1455–1457. doi: 10.1164/rccm.201407-1289LE

Turapov, O., O'connor, B. D., Sarybaeva, A. A., Williams, C., Patel, H., Kadyrov, A. S., et al. (2016). Phenotypically adapted mycobacterium tuberculosis populations from sputum are tolerant to first-line drugs. *Antimicrob. Agents Chemother.* 60, 2476–2483. doi: 10.1128/AAC.01380-15

Zainabadi, K., Walsh, K. F., Vilbrun, S. C., Mathurin, L. D., Lee, M. H., Saito, K., et al. (2021). Characterization of differentially detectable mycobacterium tuberculosis in the sputum of subjects with drug-sensitive or drug-resistant tuberculosis before and after two months of therapy. *Antimicrob. Agents Chemother.* 65, e0060821. doi: 10.1128/AAC.00608-21

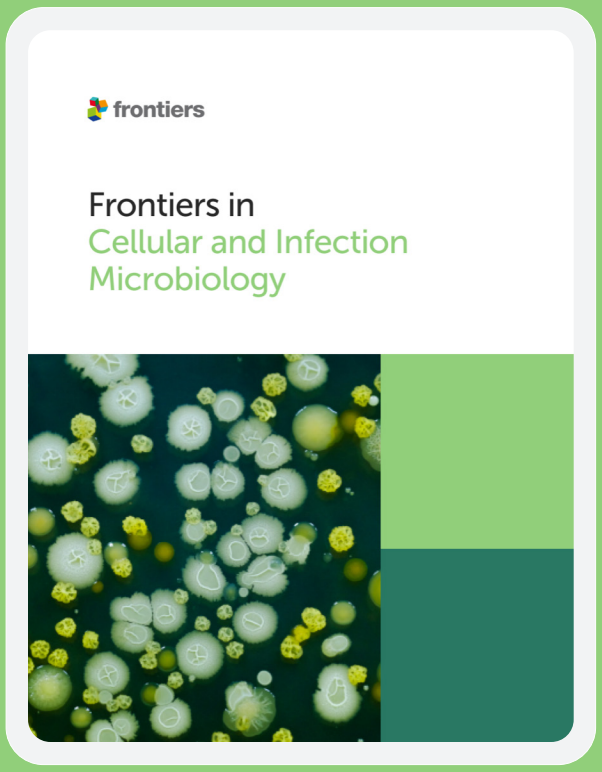
Frontiers in
Cellular and Infection Microbiology

Investigates how microorganisms interact with their hosts

Explores bacteria, fungi, parasites, viruses, endosymbionts, prions and all microbial pathogens as well as the microbiota and its effect on health and disease in various hosts.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact