PETROLEUM MICROBIAL BIOTECHNOLOGY: CHALLENGES AND PROSPECTS

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PETROLEUM MICROBIAL BIOTECHNOLOGY: CHALLENGES AND PROSPECTS

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Petroleum hydrocarbons are both a product of, and rich substrate for, microorganisms from across all Domains of life. Rooted deeply in the history of microbiology, hydrocarbons have been studied as sources of carbon and energy for microorganisms for over a century. As global demand for petroleum and its refined products continues to rise, so do challenges associated with environmental pollution, oil well souring, infrastructure corrosion, oil recovery, transport, refining, and upgrading of heavy crude oils and bitumens. Advances in genomics, synthetic biology and metabolic engineering has invigorated interest in petroleum microbial biotechnology as interest grows in technologies for in situ methane production, biodesulfurization and biodenitrogenation, bio-upgrading of heavy crudes, microbial enhanced oil recovery, corrosion control, and biocatalysts for generating value-added products. Given the complexity of the global petroleum industry and the harsh conditions in which it operates, a deeper understanding of the ecophysiology of aerobic and anaerobic microbial communities that have associations with petroleum hydrocarbons is needed if robust technologies are to be deployed successfully. This research topic highlights recent advances in microbial enhanced oil recovery, methanogenic hydrocarbon metabolism and carbon dioxide sequestration, bioremediation, microbiologically influenced corrosion, biodesulfurization, and the application of metagenomics to better understand microbial communities associated with petroleum hydrocarbons.

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Editorial: Petroleum Microbial Biotechnology: Challenges and Prospects

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Editorial on the Research Topic

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It has become evident that fossil fuels such as petroleum will continue to contribute a major fraction of the energy portfolio worldwide for the coming decades. The United States Energy Information Administration anticipates a growth of global oil demand up to 123 million barrels per day by 2025 (Sahu et al., 2015). Accordingly, oil production and processing operations are expanding continuously to meet the accelerated growth in global energy demand.

Unfortunately, serious environmental pollution issues are associated with petroleum recovery, transportation, and refining. In addition, these processes are energy-intensive, costly, and, in some cases, not sufficiently efficient (Gray, 1994; Kilbane, 2006; Ramirez-Corredores and Borole, 2006). Furthermore, the increased demand for fossil fuels will inevitably force the oil industry to produce and refine increasing amounts of unconventional resources such as heavy and extra-heavy crudes as well as bitumen. This will lead to even more environmental issues in addition to technical challenges for the oil industry (Ramirez-Corredores and Borole, 2006; Speight, 2013).

The continuously rising global demand for cleaner fuels, together with the depletion of light crude oil resources and strict environmental regulations, have provoked the need for alternative or complementary novel technologies for oil production and refining. The interaction between microorganisms and petroleum hydrocarbons has been well recognized and the intimate contact between them starts in oil-bearing subsurface formations (Ehrlich et al., 2016). This constituted the basis from which petroleum biotechnology has emerged. Petroleum biotechnology exploits the astonishing metabolic and adaptive capabilities of dedicated hydrocarbon-degrading/transforming microorganisms (Van Hamme et al., 2003; Mbadinga et al., 2011). As compared to conventional thermochemical and physical approaches, biotechnology-based processes are generally environmentally friendly, economic, and are characterized by high selectivity (Le Borgne and Quintero, 2003; Kilbane, 2006).

Petroleum biotechnology has been applied for environmental cleanup of oil spills and biological treatment of refinery wastes (bioremediation). Other emerging applications include oil exploration, microbial enhanced oil recovery (MEOR), biodesulfurization and biodenitrogenation of distillates, biodemetallation, bioupgrading of heavy crudes and refining residues, valorization of refining wastes, bioconversion of residual oil to methane, control of oil field souring and corrosion, formulation of petrochemicals, etc. (Vazquez-Duhalt and Quintero-Ramierez, 2004; Morales et al., 2010; Mbadinga et al., 2012; Wang et al., 2012, 2014; Zhou et al., 2012, 2016; Bachmann et al., 2014; Bian et al., 2015; Head and Gray, 2016).

With the exception of bioremediation and enhanced oil recovery, most of these applications are still in the research and development phase in laboratories (Morales et al., 2010; Bachmann et al., 2014). For commercial application, intensive research and development work is still needed to thoroughly understand the structure, function and ecophysiology of microbial communities inhabiting oilfields, refineries as well as oil- and hydrocarbon-impacted ecosystems. This direction of research has been greatly advanced by both culture-independent and conventional enrichment approaches. Currently, the power of metagenomics afforded by rapidly evolving advanced sequencing techniques can be harnessed for comprehensive structural and functional characterization of microbial communities to advance knowledge on the active microbial community members and the functional genes expressed in subsurface ecosystems and other environments (Joshi et al., 2014; Tan et al., 2015).

A major challenge that impedes the development of further biotechnological applications for the petroleum industry is the complex, heterogeneous, and hazardous nature of crude oil and various distillates, residues and wastes. Successful application of petroleum biotechnology is based on the development of robust microbial biocatalysts that can adapt to and tolerate the various hydrocarbon substrates, and perform the desired biodegradation or biotransformation reactions at commercially viable rates. Recent advances in the field of hydrocarbon activation under anoxic conditions have enriched the scientific knowledge on new biochemical processes, and the fumarate-addition pathway is an example as evidenced in enrichment cultures and oilfields (Mbadinga et al., 2011; Bian et al., 2015; Zhou et al., 2016). In this context, microbial consortia, naturally occurring or reconstituted, offer many advantages as compared to axenic strains for energy recovery or production of value-added intermediate chemicals (McGenity et al., 2012; Mikesková et al., 2012).

In this research topic, we received 19 articles that cover 6 applications on different aspects of petroleum biotechnology. Three papers focus on microbial enhanced oil recovery, 3 papers address methanogenic degradation of hydrocarbons and CO_2 sequestration, 3 papers investigate bioremediation, 4 articles characterize microbial communities, 4 articles discuss microbiologically influenced corrosion, 1 article addresses biodesulfurization, and 1 is an opinion article. In the following, we briefly describe these articles in chronological order starting with the most recent.

Dong et al. investigated microbial enhanced oil recovery using rhamnolipid biosurfactants produced by a novel strain of *Acinetobacter junii*. They show significant increase in oil recovery due to reduction of interfacial tension, alteration of wettability and mobility of microorganisms. Liang et al. performed functional and structural characterization of alkane-degrading methanogenic enrichment cultures, employing long-term incubation to eliminate inactive members and accumulate the key players. They also analyzed the degradation process and the functional genes involved. Nie et al. highlight factors that shape taxonomic and functional composition of microbial communities in oil reservoirs using shotgun sequencing of metagenomes from geographically distant oil reservoirs.

Obi et al. adopted Illumina sequencing of 16S rRNA genes to explore the impact of pollutant type and level on microbial community structure and function in estuarine sediments of Lagos lagoon (Nigeria) to identify key hydrocarbon degraders and those affected by the pollution. In a study on microbially influenced corrosion, Liang et al. establish the role of the halophilic *Halanaerobium* as a corrosion-causing bacterium in a gas production field. They also report that the dominant *Halanaerobium* strain degrades guar gum, which is used in fracture fluids to produce acetate and sulfide. The impact of substrate complexity on the community structure of methanogenic hydrocarbon-degrading enrichment is described by Fowler et al. Their findings highlight the stability of syntrophic interactions regardless of substrate diversity and show the importance of syntrophic interactions amongst the members of complex methanogenic communities.

In another work on corrosion, Voordouw et al. used uniformly sized carbon steel beads for monitoring corrosion in oil fields. For bioremediation of desert soil, Al-Kindi and Abed applied biostimulation to oil-contaminated desert soil with sewage sludge, wheat straw, and soybean meal. They presented data on oil degradation and shifts in microbial community composition. The biodesulfurization potential of mixed cultures is addressed by Ismail et al., with the authors showing how the sulfur source affects the structure of a biodesulfurizing mixed culture. They also studied the biodesulfurization spectrum and catalytic activity. Gao et al. shed light on stimulation of microbial enhanced oil recovery using a lipopeptide-producing *Bacillus subtilis* strain that is added during water flooding of oil reservoirs.

Aüllo et al. describe two *Desulfotomaculum* populations as key degraders of monoaromatic compounds in a deep subterranean gas storage aquifer. They also present carbon and hydrogen isotopic fractionation data that highlight the mechanism of anaerobic benzene activation under sulfate-reducing conditions. In his opinion article, Kilbane reviews the potential contributions of biotechnology to the future energy industry and discusses the underlying challenges. Muhr et al. developed a fluorescent bioreporter for acetophenone using the acetophenone catabolic operon as a scaffold. They show that the mCherry-based bioreporter is specific to acetophenone and two enantiomers of 1-phenylethanol, which are transformed to acetophenone and has potential application in environmental monitoring and petroleum exploration.

An et al. applied metagenomics to analyze sodium bisulfite impact on corrosion-provoking microbial communities in bitumen production pipelines. They show increased abundance of hydrogen-utilizing delta and epsilonproteobacteria after excess application of sodium bisulfite as an oxygen scavenger. Mand et al. investigated how methanogens and acetogens play a role in microbial influenced corrosion in low-temperature oil reservoirs using carbon steel organics as an electron donor. Xue and Voordouw studied the combination of biocides and nitrate for control of oil reservoir souring. Elshafie et al. characterized sophorolipid biosurfactants produced by *Candida bombicola* and explored their applicability for microbial enhanced oil recovery.

Ren et al. draw our attention to the impact of allochthonous bacteria, introduced into oil reservoirs during water flooding, on indigenous microbial communities. They present permeability of the strata and reservoir conditions as determinants for the abundance of the bacteria injected in the production well. Liu et al. performed functional characterization of microbial communities in CO_2-flooded oil reservoirs and highlight the potential conversion of CO_2 into methane using functional genes of methanogens and hydrogenases as biomarkers.

The collection of research results compiled in this research topic is by no means exhaustive. It merely serves to showcase current understanding of some petroleum biotechnology applications from active researchers around the world. Though very challenging, the potential of biotechnology in the fossil fuel industry is immense and the coming decades will witness considerable progress in the field. This will be fueled by advances in relevant disciplines such as metabolic engineering, bioprocess technology, biochemical engineering, as well as biocatalysis. In particular, the field of artificial metalloenzymes has turned into a vibrant research area, which can be exploited for development of novel hydrocarbon biotransformations. New knowledge on hydrocarbon biodegradation and biotransformation has been derived from recent research, especially from studies on specific biochemical processes and the underlying genes and microorganisms. Recent metagenomics techniques coupled with stable isotope probing, single cell sequencing, metaproteomics and metabolomics will illuminate new insights into microbial ecophysiology in subsurface ecosystems. At the same time, synthetic biology will play an important role in the optimization of the assembly of selected functions into engineered organisms or consortia to realize purpose-directed development of efficient biocatalysts. In parallel with scientific advances, extensive collaboration between academia and the fossil fuel industry is essential to ensure successful development and implementation of biotechnology applications that tackle specific technical, economic, and environmental issues. Overall, while petroleum has deep roots in the history of microbiology, it is clear that the subject remains critically important for industry and the environment and will remain an active area of fundamental and applied research for decades to come.

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

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OPINION
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Future Applications of Biotechnology to the Energy Industry

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There has been a dramatic growth in the production of biofuels in recent times. Global biofuel production tripled between 2000 and 2007, and biofuels accounted for about 1.6% of global transportation fuel in 2012 (International Energy Agency). At the time of this writing, 2015, ethanol production is by far the greatest contribution by biotechnology to energy production, with revenue accounting for $40.9 billion worldwide in 2014 vs. $3.8 billion for biodiesel and $0.019 billion for bio-methane. It is logical to imagine the future contribution of biotechnology to world energy production may increase not only in the area of biofuel production, but also in petroleum production, petroleum upgrading, biogas production, chemical production, crop improvement, bioremediation, microbiologically influenced corrosion, space travel, and other topics. However, the future contributions of biotechnology to the energy industry are not only influenced by technical advances in biotechnology, but also by the price of fossil fuels, the development of renewable energy generally, politics, global population growth, and other factors. Concerns about the use of crops for food versus fuel production, environmental effects of land use related to biofuel production, decreased oil prices, ever-increasing advances in the generation and use of wind and solar energy, and political will to promote/subsidize the development of alternative energy are also influencing factors.

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BIOTECHNOLOGY AND THE FOSSIL FUEL INDUSTRY

The contributions of biotechnology to the energy industry are not restricted to the production of biofuels, and the microbial production of methane may well be the largest contribution in the future. From 60 to 80% of oil in geological deposits is left in place by the oil industry as it is considered to be technically and/or economically non-recoverable (Muggeridge et al., 2014). However, microbial conversion of hydrocarbons to methane could dramatically increase the amount of energy recovered. Quantification of the relative abundance of stable isotopes of carbon and hydrogen can reveal the origin of methane in geological deposits because chemical and biochemical pathways for the formation of methane have different reactivities/preferences for different isotopes. It is estimated that 20–40% of methane in oil and gas reservoirs is of microbial origin (Katz, 2011) and most of that is derived from the conversion of carbon dioxide into methane. Similarly, the presence of biologically produced methane in coal deposits demonstrates that biotechnology can also aid in the recovery of energy from coal (Cheung et al., 2010). If depleted/uneconomical oil and coal deposits were treated in an appropriate manner it is conceivable that the residual hydrocarbon value in those deposits could be recovered at an accelerated rate through the use of CO2 injection and biomethanation. This approach would allow multiple cycles of CO2 injection and methane harvest to occur, rather than a one-time injection and disposal of CO2.

Therefore, the potential exists to employ biotechnology to convert the residual hydrocarbons in depleted oil wells and coal deposits into methane and recover a far greater percentage of the energy content in a reasonable time frame while simultaneously reducing the amount of CO2 released to the atmosphere (Geig et al., 2008).

Biotechnology can be used to upgrade petroleum and coal by removing undesirable elements/components such as sulfur, nitrogen, metals, and ash and by reducing viscosity. Bioprocessing can make oil easier/less expensive to refine and can reduce the production of air polluting gases resulting from the combustion of oil and coal (Youssef et al., 2009; Bachmann et al., 2014). However, these applications of biotechnology to the energy industry have not yet been implemented on a commercial scale, so it remains to be seen if future developments can overcome current obstacles. The chief obstacle for the implementation of any technology is cost. The biotechnology industry has previously been dominated by the production of low volumes of high value products, while the production of biofuels such as ethanol and biodiesel seek to make large volumes of products at the lowest possible cost. It is conceivable that the greatest impact of the development of biofuels will be to transform the biotechnology industry. Experience gained in the production of large volumes of low cost biofuels has the potential to dramatically increase the number and decrease the cost of products from the biotechnology industry worldwide.

BIOTECHNOLOGY IN SPACE

One of the most important crew members for interstellar travel will be the biotechnologist. Space travel for extended times creates nutritional, air and water quality, medical, and other issues that can be addressed through biotechnology. Methanogenic bacteria degrade organic matter, such as excrement, and produce methane. Recycling organic waste is crucially important in space travel where living space is limited and every available resource must be utilized. The biodegradation of organic waste creates methane as well as composted soil/nutrients that can be used to grow plants and/or photosynthetic microbes that can utilize sunlight and carbon dioxide to produce oxygen and food. Methanotrophs utilize methane for growth and have been demonstrated to be a nutritionally complete food source (Overland et al., 2010), and through the use of genetic engineering it is possible to use methanotrophs to produce nearly any biotechnology industry product without using carbon sources like sugars that can be used to feed people and animals (Sharpe et al., 2007). The rapid growth and small space requirements for the growth of methanotrophs (Gilman et al., 2015) will allow a diversity of products to be made in space, and is more practical than trying to stock a space ship with every pharmaceutical/bioproduct that may be needed.

RECYCLING ORGANIC WASTE

Just as the recycling of nutrients from waste material is important in space travel, nutrient recycling from all forms of waste will be increasingly important in the future for sustaining agricultural productivity on Earth. Sixty percent of the world's arable lands have mineral deficiencies or elemental toxicity problems (Fageria et al., 2008). Fertilizers increase the cost of food production and increasingly contribute to environmental pollution. Biotechnology, and engineering, can make great contributions to waste management to improve methane recovery from landfills and other waste, and to produce organic fertilizers to sustain agriculture.

The overwhelming majority of current ethanol production comes from sugar cane and corn that could be used for human and/or animal food. Biofuel production in the future will be increasingly derived from materials currently considered as waste. The goal of the ethanol industry is to shift to the use of agricultural wastes (lignocellulosic material) instead of sugar cane or corn for the production of ethanol (Voegle, 2013; Miller and Sorrell, 2014; Azad et al., 2015). While claims that the production of biofuel was a key cause in the doubling of the prices for rice, wheat and maize from 2005 to 2008 have been demonstrated to be false (Suzuki et al., 2015), it is crucial that the production of biofuels in the future should not compete (or seen to compete) with the production of food for people or animals, and that biofuel production should not be the cause of deforestation or any form of environmental damage.

While agricultural wastes are not food, it is first necessary to convert lignocellulosic material into simple sugars and only then can ethanol, butanol, and other biofuels be produced. Those simple sugars derived from agricultural waste could be used as human and/or animal food, but the fermentation industry is likely to need those sugars as the feedstocks to support the future production of pharmaceuticals, nutriceuticals, vitamins, enzymes, bio-plastics, enzymes, organic acids, and all the other products valued at $173 billion in 2013 made by the global biotechnology industry (Biotechnology Market Analysis and Segment Forecasts to 2020, ISBN: 978-1-68038-134-4, 2014). The same societal and political forces that influence the fuel ethanol industry to switch from the use of food crops to agricultural wastes will increasingly act on the biotechnology industry generally to make that same switch.

ENERGY, THE INTERNATIONAL BALANCE OF TRADE, AND RENEWABLE FUEL SOURCES IN THE FUTURE

Modern society is increasingly dependent on the abundant supply of energy. Traditionally, fossil fuels have supplied the vast majority of energy and the income from fossil fuel sales and the expense from fossil fuel purchases have been the largest contributors to the economic status of countries (Wiedmann et al., 2015). If a country has fossil fuel deposits these are valuable resources for sure, but they are a mixed blessing. The exploitation of these fossil fuel resources requires capital investment and technology that may be beyond the capabilities of some countries resulting in the involvement of foreign companies, banks, workers, and political agendas in bringing these fossil fuels to the market.

In these countries the development of these fossil fuel resources brings increased revenue, but often at the expense of increased corruption, income inequality, and foreign involvement generally without the promised benefits of increased employment, technology, manufacturing, and infrastructure development (Al-Kasim et al., 2013).

Therefore, when predicting the impact of biotechnology to the energy industry in the future it is a certainty that the production of biofuels from biomass resources will increasingly contribute to the global energy supply, and that the development of renewable energy will increasingly be promoted by those countries that lack fossil fuel resources. Currently countries/communities that do not possess fossil fuel resources must spend a high percentage of their gross domestic product to import energy. This results in a negative trade balance and a huge source of debt. However, if biomass resources are available, then modern and increasingly efficient technologies can be applied to convert biomass and/or organic waste resources to energy with modest capital investment as compared with capital investments needed to produce fossil fuels (Al-Kasim et al., 2013). The investment in the development of renewable fuels from biomass and organic waste almost exclusively results in the creation of jobs in the local economy (http://www.irena.org/News/Description.aspx?NType=A&mnu=cat&PriMenuID=16&CatID=84&News_ID=407). In contrast, the development of fossil fuel resources or the production of solar or wind energy creates fewer jobs than biofuel production and frequently the fossil/solar/wind jobs are not a part of the local economy and instead usually results in a disproportionate creation of jobs in technologically affluent countries at the expense of technologically deficient countries.

The rapid development of improved technologies for the production of biofuels from readily available biomass resources, and the relatively low cost of constructing biofuel production facilities as compared with fossil fuels (Al-Kasim et al., 2013), will make biofuels highly attractive for implementation in economically disadvantaged parts of the world. Biotechnology can provide much of the power to support a modern industrial society, using readily available and easily implemented technology (Cremonez et al., 2015; Wiedmann et al., 2015). Therefore, the current international imbalance of trade will be increasingly rebalanced in the future by the production of biofuels, and other forms of renewable energy.

CONCLUSIONS

Biotechnology can contribute to the fossil fuel industry by assisting the production of fossil fuels, upgrading fuels, bioremediation of water, soil, and air, and in the control of microbiologically influenced corrosion (MIC; Youssef et al., 2009; Bachmann et al., 2014). The application of biotechnology to increase the production of fossil fuels is mostly experimental, but the potential growth of this area is immense. Increasing the recovery of energy from depleted/uneconomical petroleum and coal deposits, particularly in combination with CO2 utilization, could be a major component of the biotechnology industry in the future. The production of liquid biofuels and methane from organic wastes has increased dramatically in recent years, but the worldwide use of these technologies has barely begun so the future will undoubtedly see exciting growth in this area. It would seem clear that biotechnology can make even greater contributions to the energy industry in the future, but some analysts conclude that the entirety of global energy can be supplied in the future using wind, water, and solar power without the use of biotechnology (Jacobson and Delucchi, 2011), so the challenge to the biotechnology industry is to continue to demonstrate relevance to the energy industry.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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An Exogenous Surfactant-Producing *Bacillus subtilis* Facilitates Indigenous Microbial Enhanced Oil Recovery

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This study used an exogenous lipopeptide-producing *Bacillus subtilis* to strengthen the indigenous microbial enhanced oil recovery (IMEOR) process in a water-flooded reservoir in the laboratory. The microbial processes and driving mechanisms were investigated in terms of the changes in oil properties and the interplay between the exogenous *B. subtilis* and indigenous microbial populations. The exogenous *B. subtilis* is a lipopeptide producer, with a short growth cycle and no oil-degrading ability. The *B. subtilis* facilitates the IMEOR process through improving oil emulsification and accelerating microbial growth with oil as the carbon source. Microbial community studies using quantitative PCR and high-throughput sequencing revealed that the exogenous *B. subtilis* could live together with reservoir microbial populations, and did not exert an observable inhibitory effect on the indigenous microbial populations during nutrient stimulation. Core-flooding tests showed that the combined exogenous and indigenous microbial flooding increased oil displacement efficiency by 16.71%, compared with 7.59% in the control where only nutrients were added, demonstrating the application potential in enhanced oil recovery in water-flooded reservoirs, in particular, for reservoirs where IMEOR treatment cannot effectively improve oil recovery.

Keywords: microbial enhanced oil recovery (MEOR), *Bacillus subtilis*, surfactants, microbial community, stimulation, high-throughput sequencing

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INTRODUCTION

With the increasing global energy demand and depletion of oil reserves, oil recovery by microbial flooding is currently under intensive development, and has been shown to be economically feasible by laboratory and field trials (Belyaev et al., 1998; Gao and Zekri, 2011; Xiao et al., 2013; Li et al., 2014; Shibulal et al., 2014; Dong et al., 2015; You et al., 2015). This technique is generally classified into exogenous and indigenous microbial enhanced oil recovery (MEOR). Exogenous MEOR includes injection of exogenous microorganisms and *ex situ*-produced products into reservoirs to enhance oil recovery (Zobell, 1947). This is an effective way to quickly establish the appropriate activity in reservoirs (Youssef et al., 2009, 2013). The drawback is that these microorganisms must be able to grow in the presence of competing indigenous populations (Bryant, 1991). Furthermore, because of the sieve effect of strata on microbial cells, the injected microorganisms are generally

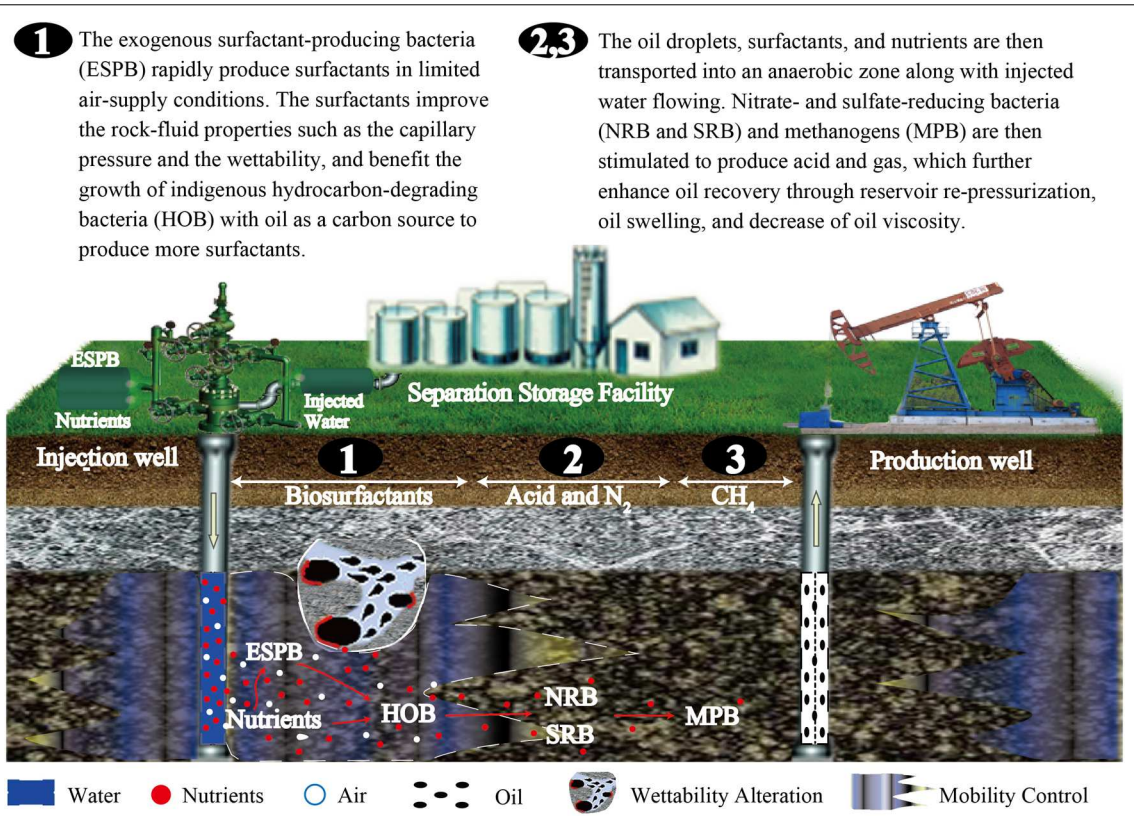

FIGURE 1 | Schematic showing how exogenous surfactant-producing bacteria facilitate indigenous microbial enhanced oil recovery.

difficult to migrate into reservoir strata to reach the production wells (Youssef et al., 2009; Gao et al., 2015a; Ren et al., 2015). Indigenous microbial flooding is another promising oil-recovery technique that has been successfully applied in the petroleum industry (Belyaev et al., 1998; Liu et al., 2005; Li et al., 2014; Wang et al., 2014b; Le et al., 2015). This technique improves oil recovery by stimulating reservoir microorganisms through introducing air and nutrients into reservoir strata. However, this technique generally needs long incubation times before indigenous microorganisms grow in large numbers and produce sufficient metabolites, which means that the limited air supply will be exhausted by saprophytic bacteria, leading to an inefficient surfactant-producing process.

Surfactant production is thought to be the initial critical stage of indigenous microbial flooding, and is beneficial for the stimulation of other anaerobic populations that follow in the reservoir ecosystem (**Figure 1**). Thus, adding exogenous surfactant-producing bacteria may be an appropriate way to improve this process during indigenous microbial flooding. The injected exogenous bacteria will grow and colonize the aerobic zone of injection wells, and rapidly produce larger amounts of surfactants, which not only reduces the interfacial tension between brines and the oil phase, but also emulsifies oil into droplets. The oil droplets further enhance the growth of indigenous hydrocarbon-degrading bacteria with oil as the carbon source to produce more surfactants. The partially emulsified oil, surface-active agents, and nutrients are then transported into the reservoir strata (an anaerobic zone) along with injected water. Then, nitrate- and sulfate-reducing bacteria and methanogens are stimulated to produce acid, alcohol, and gas, which further enhances oil recovery through reservoir re-pressurization, oil swelling, and decrease of oil viscosity.

To date, several surfactant-producing populations, in particular *Pseudomonas* and *Bacillus*, have been injected into reservoir strata to improve oil production (Simpson et al., 2011; Youssef et al., 2013; You et al., 2015). These studies also showed the feasibility of *in situ* biosurfactant production and its potential to improve oil production. However, the microbiological processes that take place in the MEOR process have not yet been elucidated. How do the injected microbial populations strengthen and improve the indigenous microbial flooding process? What is the interplay process taking place between the injected microbial populations and the indigenous microorganisms? To explore these issues, we used an exogenous *Bacillus subtilis* strain M15-10-1 to strengthen the indigenous microbial enhanced oil recovery process. The microbial processes and driving mechanisms were investigated by analyzing the changes in oil properties and the interplay between the exogenous *B. subtilis* and indigenous microbial populations in microcosms stimulated with different kinds of nutrients. The application potential for enhancing oil recovery was verified by a core oil-displacement test.

MATERIALS AND METHODS

Water Samples Collection and Nutrients Selection

Water samples were taken through sampling valves located at the wellhead of production wells in the Lu water-flooded petroleum reservoir in Xinjiang Oil Field, China. This reservoir is a sandstone reservoir that has been subjected to water flooding since 2001. The depth of the oil-bearing strata is less than 1200 m with a temperature of 37°C and a formation pressure of 10.2 MPa. The porosity of the reservoir is 29.9%, with an average permeability of 522×10^{-3} μm^2. The viscosity of the crude oil is 18 mPa-s with a density of 0.846 g cm^{-3}. The physicochemical characteristics of the formation brines indicated extremely low levels of phosphate and nitrate sources (Supplementary Table S1). Based on the nutrient deficiency in the formation brines, nutrient compounds containing carbon, nitrogen, and phosphorus were selected for the following microbial stimulation.

Strain M15-10-1 and Its Metabolic Characteristics

Strain M15-10-1 was isolated from the produced water of a mesothermal water-flooded petroleum reservoir. The 16S rRNA sequence and the lipopeptide-producing gene *srfA* were amplified using the primer sets 27F-1541R and srfAF-srfAR (**Table 1**). The obtained sequences were aligned with related species on the National Center for Biotechnology Information (NCBI) database, and were used to construct phylogenetic trees based on the neighbor-joining method with 1000 bootstrap replicates using MEGA 4 (Tamura et al., 2007). To test the ability of the strain to grow and produce surfactants on various carbon sources, water-soluble substrates and water-insoluble carbon sources, including glucose, sucrose, molasses, corn steep powder, ethanol, glycerol, sodium acetate (NaAc), peanut oil, bean oil, and hydrocarbons, were investigated. Each substrate was sterilized by filtration through a 0.45 μm filter before being added to heat-sterilized basic salts medium (BSM) for fermentation, which was performed at 40°C in a 250 mL flask containing 50 mL fermentation medium. BSM included the following nutrients: 10 g L^{-1} $NaH_2PO_4{\cdot}2H_2O$, 2 g L^{-1} $K_2HPO_4{\cdot}3H_2O$, 2 g L^{-1} NH_4NO_3, 0.2 g L^{-1} $MgSO_4{\cdot}7H_2O$, and 0.01 g L^{-1} yeast extract powder, with a pH of 7.2.

Microbial growth curves were measured by determining the optical density of the culture (OD_{600}). The surface tension was measured at room temperature using a digital tension meter (POWEREACH JK99B, China). The crude surfactant was separated from the culture medium using acid precipitation and the solvent extraction method (Liu et al., 2012). The collected brownish oily residue was ground with KBr powder, dispersed uniformly in a matrix of paraffin, and compressed to form an almost transparent disk for Fourier transform infrared spectrometry (FTIR) measurement in the frequency range of 4,000–500 cm^{-1}. The oil-degrading ability of the *B. subtilis* was investigated in a 250 mL flask containing 100 mL BSM with 2% crude oil. Cultivation was carried out at 40°C under aerobic conditions. The oil degradation was analyzed according to the following methods.

Growth of *Bacillus subtilis* M15-10-1 in an Artificial Microflora

To investigate the inhibitory effect of the *B. subtilis* on reservoir microbial populations, a microflora, including the *B. subtilis* M15-10-1, *Dietzia* sp. ZQ-4, *Rhodococcus* sp. M, and *Enterobacter cloacae* FY-07, was constructed. The strains were all isolated from oilfield environments. The four strains were first inoculated into 5 mL LB culture medium, and shaken overnight at 40°C on a rotary shaker at 180 rpm. Then, 10 μL cultures were inoculated into a 250 mL flask containing 100 mL LB or 100 mL BSM with 2% crude oil, and were shaken at 180 rpm at 40°C. The numbers of the four stains were determined at different time points by quantitative PCR (qPCR). The molecular markers and PCR conditions used to quantify the four stains are listed in **Table 1**. The reaction systems were denatured for 2 min at 95°C followed by 35 cycles at 94°C for 30 s, annealing for 30 s, and 72°C for 30 s. Fluorescence was determined at the end of every 72°C extension phase in a Bio-Rad iQ5 Sequence detection system. Gene copy numbers in unknown samples were determined based

TABLE 1 | Primers used in the present work for qPCR.

Target	Primer set	Sequence	PCR conditions
Bacillus subtilis M15-10-1	srfAF	5-CAAAATCGCAGCATACCACTTTGAG-3	56°C 30 cycles
	srfAR	5-AGCGGCACATATTGATGCGGCTC-3	
Dietzia sp. ZQ-4	algF	5-GTCCACCACGAAGCAGC-3	56°C 30 cycles
	algR	5-CCTACAACGGCATCAAACTG-3	
Rhodococcus sp. M	alkF	5-AGTGGGCGCTCGCCCCGTCGTTCTAC-3	56°C 30 cycles
	alkR	5-CACGAGATACGGCGCGATCGACAGAC-3	
Enterobacter cloacae FY-07	895F	5-GGCAGCGTGTCAAACTCAA-3	57°C 30 cycles
	895R	5-TTTACCGACGGCTCACAGAT-3	
Bacteria	27F	5-AGAGTTTGATCCTGGCTCAG-3	55°C 30 cycles
	1541R	5-AAGGAGGTGATCCAGCCGCA-3	
Bacteria	8F	5-AGAGTTTGATYMTGGCTC-3	55°C 30 cycles
	338R	5-GCTGCCTCCCGTAGGAGT-3	
Hydrocarbon degrader	alkBwF	5-AAYCANGCNCAYGARCTNGGVCAYAA-3	55°C 30 cycles
	alkBwR	5-GCRTGRTGRTCHGARTGNCGYTG-3	

on standard curves constructed from 10-fold serial dilutions of the standard as described previously (Li et al., 2014).

Stimulation with Nutrients or Combination with *Bacillus subtilis* M15-10-1

To investigate the influence of *B. subtilis* M15-10-1 on oil properties and indigenous microorganisms, stimulation with multiple nutrients or combination with *B. subtilis* was performed in 250 mL flasks containing 100 mL of produced water derived from the reservoir. The *B. subtilis* agent was first incubated for 16 h in a 250 mL flask containing 50 mL BSM with 0.2% glucose as the carbon source to prepare the microbial agent. Then, the fermentation broth was centrifuged at 10,000 *g* for 10 min to collect the microbial cells, which were suspended with BSM in the same volume to prepare the broth-free microbial agent. Considering the nutrient deficiency in the formation brines (Supplementary Table S1), the nutrient compounds contained 0.2% $(NH_4)_2HPO_4$, 0.4% $NaNO_3$, 2% crude oil, and/or 0.2% glucose (for industrial purposes, glucose ≥ 96%), 0.2% glycerol (for industrial purposes, glycerol ≥ 95%), 0.2% molasses (sucrose ≥ 35%, reducing sugar ≥ 16%, and nitrogen compounds ≥ 4.5%), and 0.2% corn steep powder (protein ≥ 43.0%, carbohydrate ≥ 8.0%). The inoculation quantity of the broth-free *B. subtilis* agent was determined according to the microbial concentration of the produced water of the reservoir. Cultivation was carried out at 40°C under aerobic and limited air supply conditions that were realized by sealing the flasks with rubber stoppers. The oil degradation and emulsification, microbial abundance, and community compositions were analyzed according to the following methods.

Analysis of Microbial Abundance and Community Compositions

Total genomic DNA was extracted using a bead shaker treatment and the AxyPrep^{TM} Genomic DNA Miniprep Kit (Axygen, USA). Briefly, microbial cells were resuspended with 1 mL TE buffer (80 mM Tris, 40 mM EDTA, pH 8.0), and then lysed using a mini bead-beater (BioSpec, USA) at 200 rpm for 1 min at room temperature with 0.1 mm glass beads. After bead beating, lysozyme was added (final concentration of 1 mg mL^{-1}), and the samples were incubated at 37°C for 1 h. Following the lysozyme treatment, 120 μL sodium-dodecyl sulfate (20% SDS, w/v) was added and the samples were incubated at 65°C for 60 min. Total genomic DNA was then extracted from the suspension solution using an AxyPrep^{TM} Genomic DNA Miniprep Kit (Axygen, USA) according to the manufacturer's instructions and finally stored at −80°C for subsequent analysis.

The universal primer set 515f (GTG CCA GCM GCC GCG GTAA) and 806r (GGA CTA CHV GGG TWT CTA AT) were used to amplify the microbial 16S rRNA gene V4 region (300–350 bp) according to the protocol described by Caporaso et al. (2011, 2012). Replicate PCR products of the same sample were mixed to remove PCR artifacts. Amplicon sequencing was conducted on an Illumina MiSeq platform at Novogene, Co., Beijing, China. Pairs of reads from the original DNA fragments were merged using fast length adjustment of short reads (FLASH; Magoc and Salzberg, 2011). Sequences were then demultiplexed and quality filtered using the default parameters of the Quantitative Insights into Microbial Ecology (QIIME) software package (Caporaso et al., 2010). The operational taxonomic unit (OTU) clustering pipeline UPARSE was used to select OTUs at 97% similarity (Edgar, 2013). The representative sequence sets were aligned and given a taxonomic classification using Ribosomal Database Project (RDP; Wang et al., 2007). The similarity among microbial communities was determined using histograms, UniFrac principal coordinates analysis (PCoA), and the unweighted pair-group method with arithmetic mean (UPGMA).

16S rRNA, and the *alkB* and *srfA* genes were used as molecular markers to quantify the total bacteria, hydrocarbon degraders, and the *B. subtilis*, respectively. qPCR of the bacterial 16S rRNA genes was performed with the primer set 8F and 338R as described by Schulz et al. (2010). Degenerate primers alkBwf and alkBwr were used to detect the *alkB* gene, which catalyzes the first step of the hydrocarbon-degradation process (Wang et al., 2010). The quantification of the *B. subtilis* was performed with the primer set srfAF and srfAR. The copy numbers and average copy numbers of the genes detected in the water samples stimulated by nutrients or in combination with *B. subtilis* M15-10-1 were calculated, and were compared using One Way Analysis of Variance (ANOVA) with Student–Newmnan–Keuls tests.

Analysis of Oil Degradation

Residual oil was extracted using a previously described protocol (Gao et al., 2013). Briefly, the residual oil was extracted twice with chloroform, and then separated into saturated hydrocarbon, aromatic hydrocarbon, asphaltene, and non-hydrocarbon fractions using a silica gel G (60–120 mesh) column (2 cm × 30 cm). The relative quantity of saturated hydrocarbons, aromatic hydrocarbons, asphaltene, and non-hydrocarbons in crude oil was calculated. The saturated hydrocarbons were analyzed according to the Chinese Standard SY/T 5779-2008 by gas chromatography–mass spectroscopy (GC–MS) using Agilent 7890–5975 machines equipped with HP-5MS capillary columns (60 m × 0.25 mm i.d., 0.25 mm thickness).

Core Oil-Displacement Test

The core oil-displacement test was performed to evaluate the potential application in improving oil recovery. The produced oil and water from Lu reservoir were used as the oleic and aqueous phases, respectively. The core models were 28.5–30.6 cm in length, 3.8 cm in diameter, had pore volumes of 60.3–62.1%, and water permeability of 1.97–2.15 μm^2 (**Table 2**). The oil-saturated core models were first flooded with injection water until the water cut in the effluent of the core models was higher than 98%, which means that the core reached its residual oil saturation. A total of 0.2 PV of prepared formation brine containing nutrients and 0.2 PV of an air package were then injected into the water-flooded cores. The prepared formation brine included 0.2%

TABLE 2 | Core-flooding test showing the application potential of an exogenous lipopeptide-producing *Bacillus subtilis* M15-10-1 in assisting enhancement of oil recovery based on nutrient stimulation.

Tested project	Pore volume (ml)	Oil saturation (ml)	Water permeability (μm^2)	Oil displacement efficiency (%)		
				First water flooding	Successive water flooding	oil displacement efficiency
Bacillus subtilis	59.5	48.5	2.06	45.65	48.27	2.62
Nutrients	61.6	49.2	2.15	46.25	53.84	7.59
Nutrients + *Bacillus subtilis*[a]	60.3	46.9	1.97	46.12	62.83	16.71
Nutrients + *Bacillus subtilis*[b]	62.1	48.9	2.09	47.58	58.54	10.96

The nutrients consisted of 0.2% $(NH_4)_2HPO_4$, 0.4% $NaNO_3$, and 0.2% corn steep powder.
The Bacillus subtilis inoculation quantity was 0.5%, which was determined according to the microbial concentration of the produced water of the reservoir.
[a]*Fermentation broth of Bacillus subtilis M15-10-1.*
[b]*Bacillus subtilis agent that was suspended with BSM.*

$(NH_4)_2HPO_4$, 0.4% $NaNO_3$, 0.2% corn steep powder and 0.5% fermentation broth of *B. subtilis* or 0.5% *B. subtilis* agent that were suspended with BSM. The *B. subtilis* inoculation quantity was selected according to the microbial concentrations of the produced water of production well Lu1039. The cores were sealed for 7 days at 40°C after nutrient injection. Water flooding was performed again until no further oil was observed in the effluent to calculate oil displacement efficiency. The controls with only air and nutrients, or air and *B. subtilis*, were performed under the same conditions to provide information on the background levels.

Nucleotide Sequence Accession Number

The raw reads obtained in this study were deposited in the NCBI Nucleotide Archive database under project identification number PRJNA269199 (http://www.ncbi.nlm.nih.gov/bioproject/PRJNA269199).

RESULTS

Metabolic Characteristics of *Bacillus subtilis* M15-10-1

The strain M15-10-1 showed highest 16S rDNA sequence similarity with *B. subtilis* (**Figure 2A**). Detection of a surfactant-producing gene revealed that the M15-10-1 strain has the lipopeptide-producing gene *srfA*, suggesting that this strain may produce lipopeptides (**Figure 2B**). The dynamic growth curves indicated that the strain reached the exponential phase in 4–5 h, and rapidly entered the stationary phase within a short time when grown with diverse agro-industrial substrates as carbon sources (**Figures 3A,B**). As a result of biosurfactant synthesis, the surface tension of the cultures grown on glycerol, glucose, molasses, corn steep powder, and vegetable oil was reduced to approximately

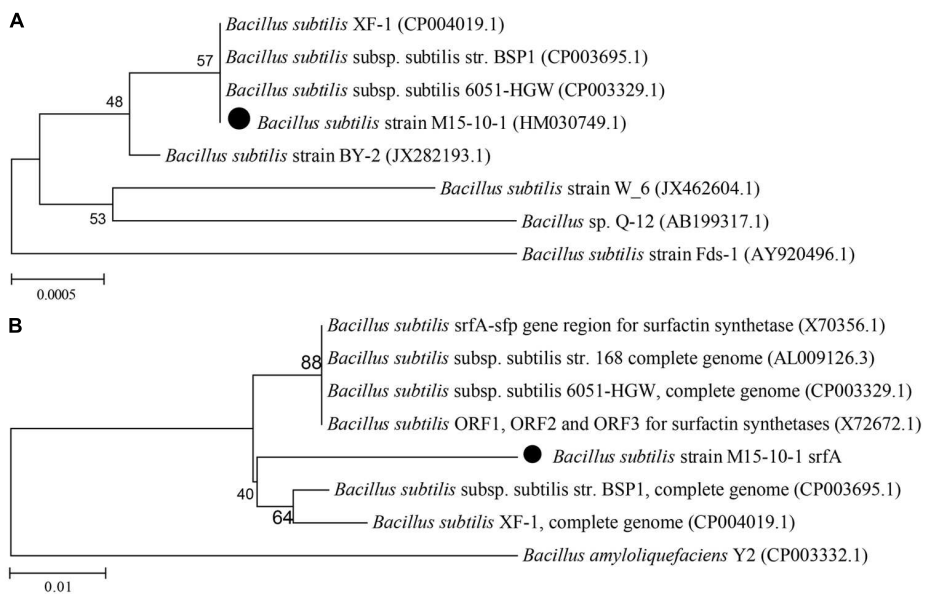

FIGURE 2 | Phylogenetic relationship based on (A) the 16S rDNA gene and (B) *srfA* gene sequences between the *Bacillus subtilis* M15-10-1 strain and related species.

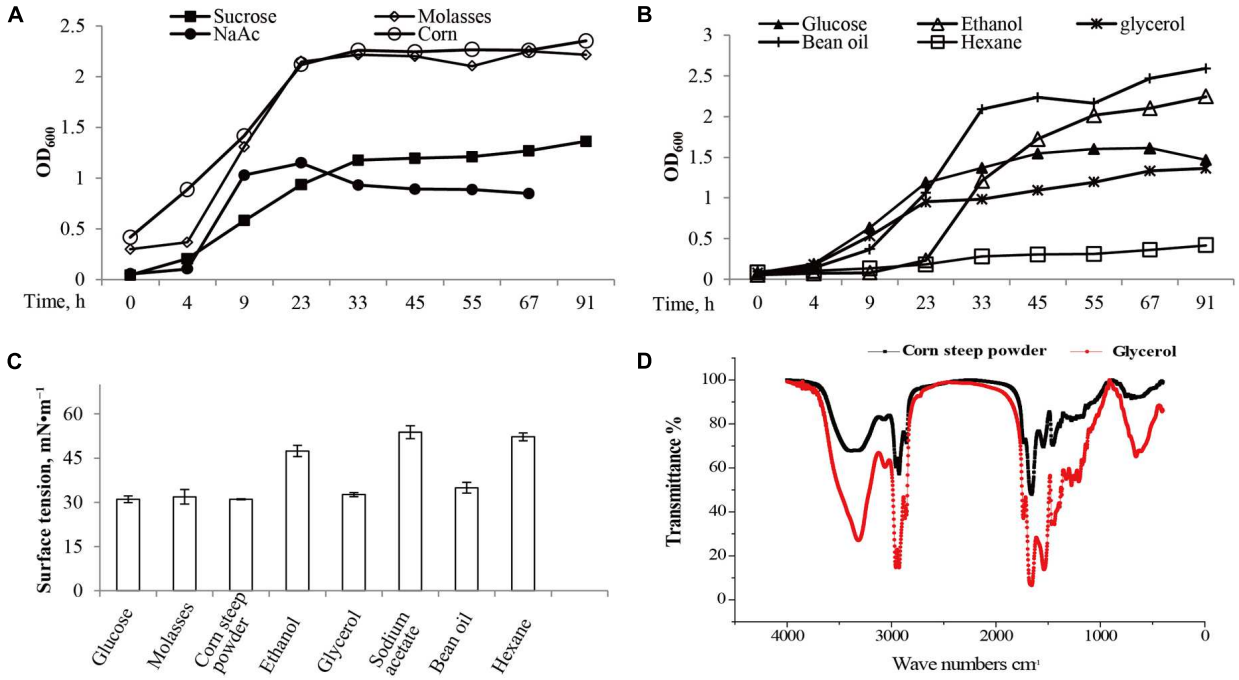

FIGURE 3 | Microbial growth and surfactant production of *Bacillus subtilis* M15-10-1 when grown with diverse carbon sources. The growth curves are measured by **(A,B)** optical density (OD_{600}), **(C)** the pH and surface tension of fermentation broth, and **(D)** the Fourier transform infrared spectrometry (FTIR) spectra of surfactants.

30 $mN \cdot m^{-1}$ (**Figure 3C**). The surfactant production by the strain was approximately 300–500 mg L^{-1}. The results indicated that various inexpensive nutrients could be used as substrates for M15-10-1 to produce surfactants. The lipopeptide-producing gene *srfA* detected in this strain and the FTIR spectra of the surfactant together revealed that *B. subtilis* M15-10-1 produced lipopeptides (**Figure 3D**). In addition, the *B. subtilis* cannot degrade crude oil (**Table 2**).

Inhibitory Effect of Strain M15-10-1 on Reservoir Microbial Populations

The inhibitory effect of the *B. subtilis* M15-10-1 on reservoir microbial populations in an artificial microflora was investigated using qPCR. As shown in **Figure 4A**, the *B. subtilis* had a similar growth curve whether cultured with other microbial populations or cultured alone in LB medium. The growth curves also indicated that the *B. subtilis* did not exert an observable inhibitory effect on the *Dietzia* sp. ZQ-4, *Rhodococcus* sp. M, and *E. cloacae* FY-07. Although the *B. subtilis* and *E. cloacae* cannot grow with oil as the carbon source, the number of the two species increased when cultured with the oil-degraders *Dietzia* sp. ZQ-4 and *Rhodococcus* sp. M in BSM crude oil medium (**Figure 4B**). This was clearly attributed to the growth of *Dietzia* and *Rhodococcus*, which are well-known for their oil-degradation ability and surfactant production.

Effect of Strain M15-10-1 on Oil Emulsification and Microbial Growth During Nutrients Stimulation

Both glucose and glycerol stimulated the growth of indigenous microorganisms, but did not improve oil emulsification in the process of stimulation of reservoir microorganisms. The results indicated that neither glucose nor glycerol stimulated indigenous microorganisms to produce enough surface-active agents to emulsify crude oil during stimulation. In contrast, molasses and corn steep powder not only stimulated microbial growth, but also improved oil emulsification (**Figure 5**). However, it was questionable which factors resulted in oil emulsification during stimulation. We therefore investigated the microbial concentration and oil degradation before and after nutrient stimulation. The qPCR results indicated that, even though there was no crude oil in the microcosms, the number of *alkB* genes reached 10^7–10^8 copies mL^{-1}. In the microcosms with glucose or glycerol, which could not stimulate reservoir microorganisms to emulsify crude oil, the number of *alkB* genes also reached 10^7–10^8 copies mL^{-1} (**Figure 6**). Unexpectedly, no oil degradation was clearly observed in these microcosms, with saturated hydrocarbons decreasing from 71.29 to 65.45% and 64.31% in the glucose and glycerol treatments, respectively (**Table 3**). These results suggest that the growth of hydrocarbon-degrading bacteria may not have a causal relationship with oil emulsification during stimulation, whereas the specific nutrient additions may be crucial for

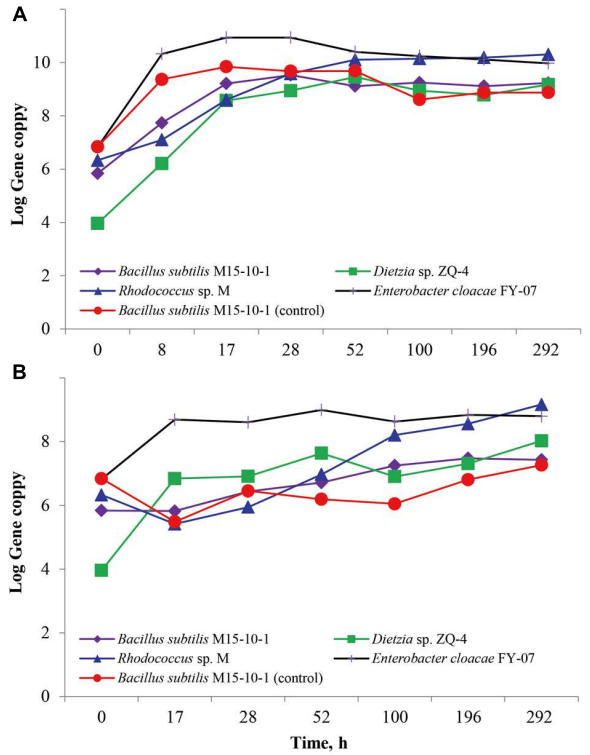

FIGURE 4 | Growth curves of *Bacillus subtilis* M15-10-1, *Dietzia* sp. ZQ-4, *Rhodococcus* sp. M, and *Enterobacter cloacae* FY-07 in an artificial microflora cultured on LB (A) and BSM (B) medium. *Bacillus subtilis* M15-10-1 (control): Growth curves of *Bacillus subtilis* M15-10-1 in LB **(A)** and BSM **(B)** medium.

stimulating reservoir microorganisms to produce surfactants to emulsify oil.

Although glucose and glycerol could not stimulate reservoir microorganisms to emulsify crude oil, the added *B. subtilis* M15-10-1 (the inoculation quantity was 0.5%, which was determined according to the indigenous microbial concentration) rapidly improved oil emulsification during stimulation. Interestingly, the exogenous *B. subtilis* M15-10-1 also accelerated oil degradation, with saturated hydrocarbons decreasing from 71.29 to 58.89% and 61.35% in the glucose and glycerol treatments, respectively (**Figure 7** and **Table 3**). Generally, pristane and phytane are not depleted by initial to moderate biodegradation, while 17α(H)-hopane is used as a conservative marker to calculate biodegradation rates (%) for saturated hydrocarbons. In this study, there was an obvious decrease in hydrocarbons and pristane and hydrocarbons and 17α(H)-hopane in microcosms with M15-10-1 compared with the control with only nutrient additions (**Figures 7C,D**). Furthermore, the ratios of hydrocarbons (C12–C35) all decreased in both microcosms with nutrient additions and strain M15-10-1. It is reasonable to deduce that the added *B. subtilis* M15-10-1 produced surfactants, which emulsified crude oil into droplets that further enhanced the growth of indigenous hydrocarbon-degrading bacteria with crude oil as the carbon source.

Response of Reservoir Microbial Community to the *Bacillus subtilis* During Stimulation

To elucidate the ecological influence of exogenous *B. subtilis* M15-10-1 on indigenous microbial communities, microbial abundance and community structures in the microcosms with nutrients, or nutrients and exogenous *B. subtilis* under limited air supply conditions, were investigated by qPCR and high-throughput sequencing. Compared with the microcosms stimulated with nutrients, the numbers of *srfA* genes reached 10^8 copies mL^{-1} in the microcosms with nutrients and *B. subtilis* M15-10-1 (**Figures 6A,B**). The results indicated that the added *B. subtilis* adapted to and grew in the new environments. However, it was questionable whether the growth of the *B. subtilis* exerted an inhibitory effect on the indigenous microorganisms. To explore the issue, community diversity and composition of each microcosm were analyzed. There was no obvious difference in the numbers of 16S RNA, *alkB*, and Shannon diversity index in microcosms with nutrients and exogenous *B. subtilis* compared with the microcosms with only nutrient additions (**Figure 6B** and Supplementary Figure S1). In microcosms with nutrients and the *B. subtilis*, the genus *Bacillus* only accounted for 2.2–6.6% of the communities (**Figure 8B**). The result is coincident with the quantification of the lipopeptide-producing gene *srfA*. These data indicated that the added exogenous *B. subtilis* and the produced surfactant did not exert an obvious inhibitory effect on the growth of the indigenous microorganisms during stimulation.

The phylogenetic variation between the stimulated microhabitats was further measured by UniFrac distances. Histograms, hierarchical clustering, and PCoA biplots were used to interpret the relative similarity of the microbial communities (**Figure 8**). The results indicated that the nutrient types exerted stronger influences on community structures than the added exogenous *B. subtilis*. As shown in **Figure 8**, the communities from microcosms with glucose and glycerol were classified as a group, while those of microcosms with molasses and corn steep powder were classified as another group (**Figures 8C,D**). Additionally, the microcosms with the *B. subtilis*, in particular, those stimulated by glucose and glycerol, have similar community structures to each other rather than those with only nutrient additions. The results may be related to the following facts: (1) compared with molasses and corn steep powder, glucose and glycerol have a single nutritional component, which may be only fit for the stimulation of several microbial populations, e.g., *Pseudomonas*; (2) the growth of the exogenous *B. subtilis* made the indigenous communities have a more similar community structure.

Core Oil-Displacement Test

The core-flooding test was designed to simulate the MEOR process. The results of the core-flooding tests are shown in **Table 2**. The results indicated that the brines with nutrients (0.2% $(NH_4)_2HPO_4$, 0.4% $NaNO_3$, and 0.2% corn steep powder) and 0.5% fermentation broth of *B. subtilis* M15-10-1 increased the oil displacement efficiency by 16.71%, while it increased by 10.96% with nutrients and 0.5% *B. subtilis* agent that was suspended

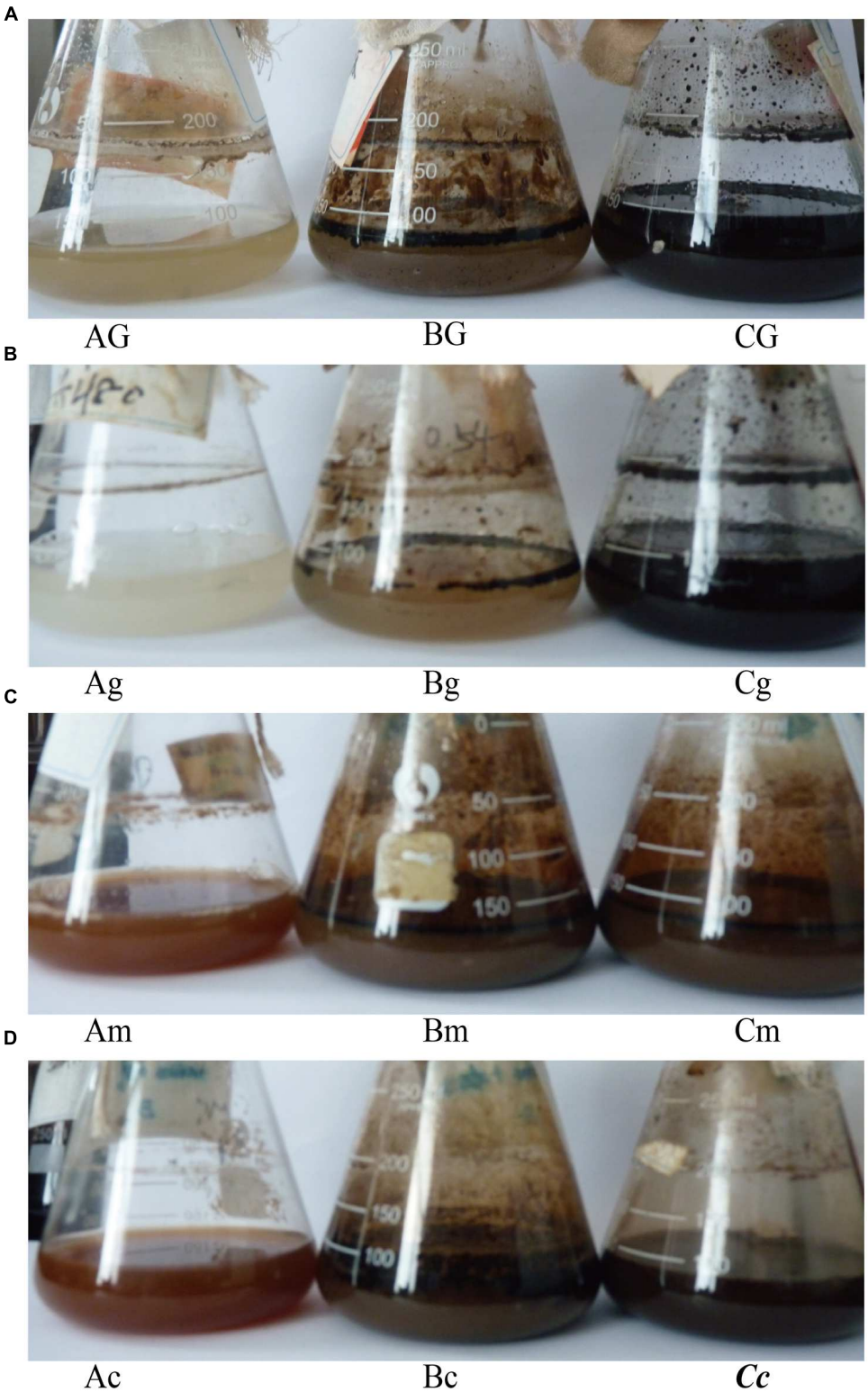

FIGURE 5 | Oil emulsification during stimulation with nutrients or in combination with *Bacillus subtilis* M15-10-1. Different combinations are presented in images **(A–D)**. A: microcosm containing nutrients; B: microcosm containing nutrients and crude oil; C: microcosm containing nutrients, crude oil, and *Bacillus subtilis* M15-10-1; G: glucose; g: glycerol; m: molasses; and c: corn steep powder.

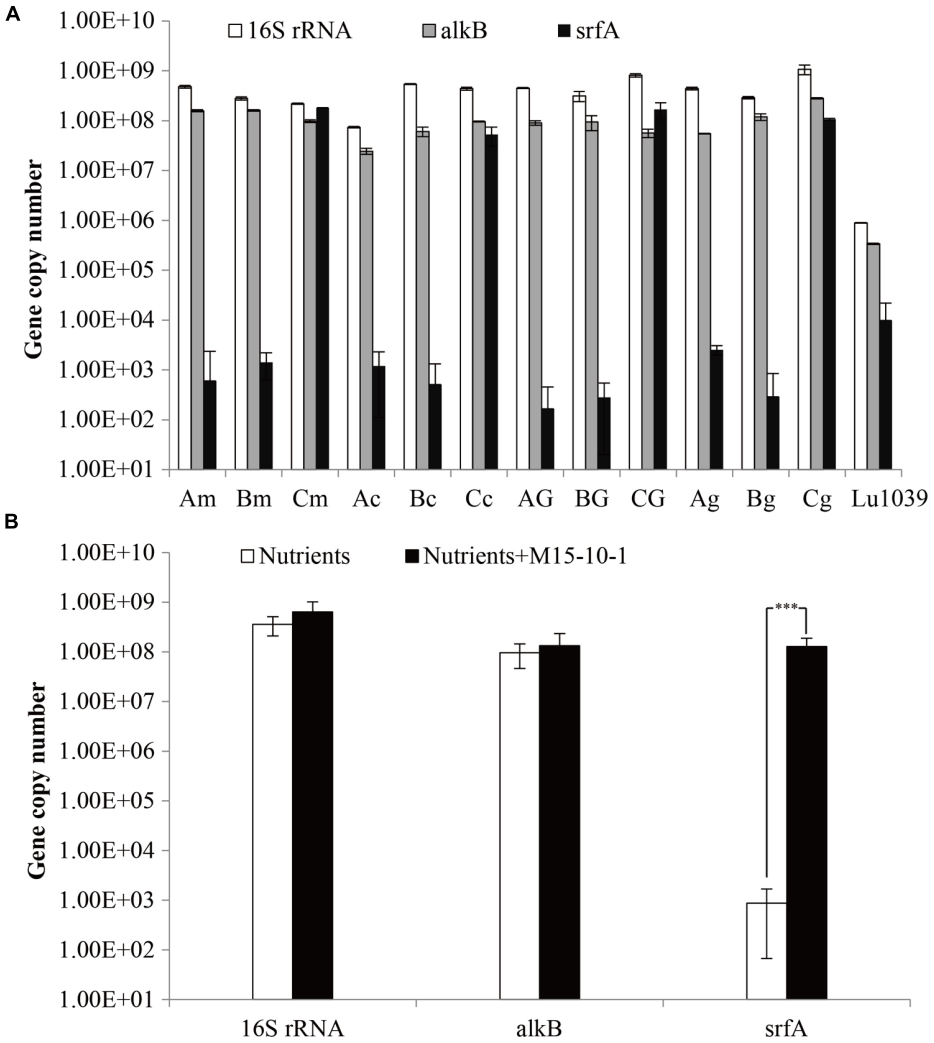

FIGURE 6 | Copy numbers (A) and average copy numbers (B) of 16S rRNA, *alkB* and *srf*A genes detected in the water samples stimulated by nutrients or in combination with *Bacillus subtilis* M15-10-1. A: microcosm containing nutrients; B: microcosm containing nutrients and crude oil; C: microcosm containing nutrients, crude oil, and *Bacillus subtilis* M15-10-1; G: glucose; g: glycerol; m: molasses; and c: corn steep powder. ***The abundance of the gene was significantly different among water samples stimulated by nutrients or in combination with *Bacillus subtilis* M15-10-1 ($P < 0.0001$).

with BSM. In contrast, it only increased by 7.59% with nutrient injection only, and by 2.62% with *B. subtilis* agent injection only.

DISCUSSION

Indigenous microbial enhanced oil recovery (IMEOR) is driven by the synthetic action of reservoir microorganisms and their metabolites, including surfactants, organic acids, CO_2, and CH_4 (Youssef et al., 2009; Voordouw, 2011). In IMEOR treatment, surfactant production is the first stage of nutrient stimulation (**Figure 1**), and is thought to be the most critical stage (Li et al., 2014). However, in most cases, it is difficult for reservoir microorganisms to produce enough surfactants to emulsify oil under limited air supply conditions. To improve this process, an exogenous lipopeptide-producing *B. subtilis* was used to facilitate the IMEOR process. The microbial processes and driving mechanisms were investigated by analyzing the changes in oil properties and the response of the indigenous microbial community when stimulated with nutrients or the exogenous *B. subtilis*. The results provided useful information to increase our understanding of the microbial processes occurring in the process of exogenous surfactant-producers strengthening IMEOR and for developing an efficient MEOR technique.

Along with the development of the MEOR technique, a large number of microbial populations, including commonly reported *Pseudomonas*, *Rhodococcus*, *Bacillus*, and *Dietzia*, have been detected and isolated from reservoir environments using culture-based methods and culture-independent techniques (Simpson et al., 2011; Xia et al., 2012; Wang et al., 2013, 2014a; Li et al., 2014). Most of these populations can produce surfactants when grown with crude oil as the carbon source. These properties

TABLE 3 | Relative quantities of saturated hydrocarbons, aromatic hydrocarbons, asphaltene, and non-hydrocarbons in crude oil before and after stimulation with nutrients or combination with *Bacillus subtilis* M15-10-1.

Experiments	Relative quantity of crude oil (%)			
	Saturated hydrocarbon	Aromatic hydrocarbon	Asphaltene	Non-hydrocarbon
Control	71.29	14.85	5.94	5.94
Control + M15-10-1	70.28	12.24	3.85	7.69
Glucose	65.45	16.97	8.48	5.45
Glucose + M15-10-1	58.89	22.22	2.96	12.59
Glycerol	64.31	18.73	2.12	9.19
Glycerol + M15-10-1	61.35	18.40	4.60	12.88
Molasses	55.38	18.88	9.38	15.13
Molasses + M15-10-1	54.69	22.92	3.65	14.58
Corn steep powder	51.52	24.24	13.13	9.09
Corn steep powder + M15-10-1	48.28	18.23	12.81	20.20

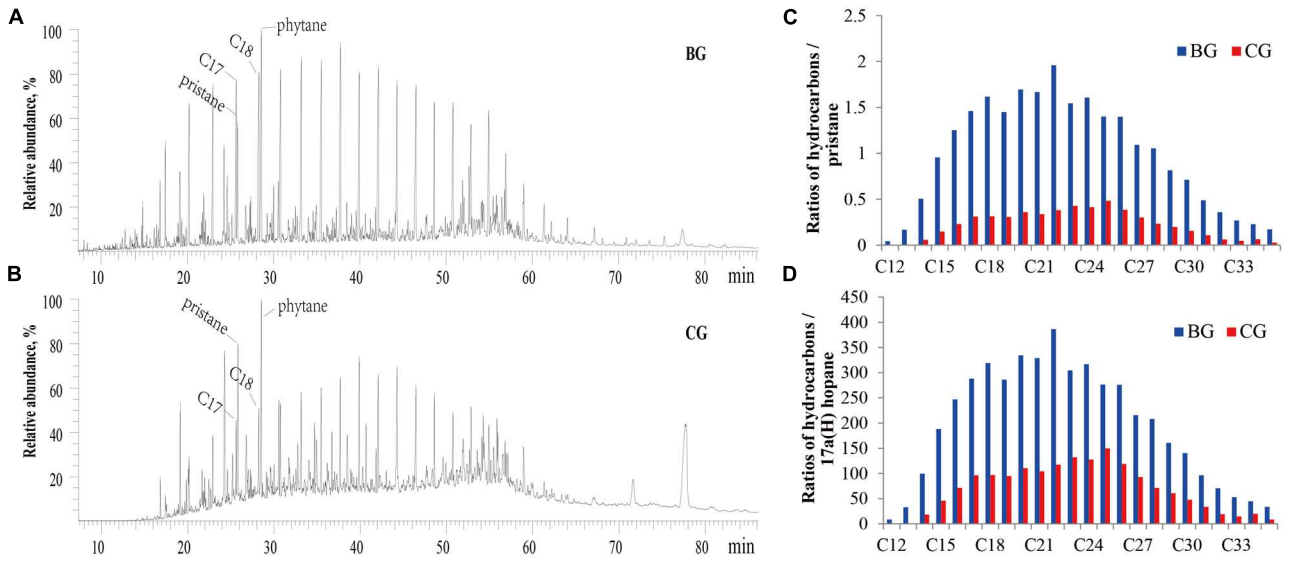

FIGURE 7 | Gas chromatography profile of total hydrocarbon (A,B) and the relative ratios of different chain-length hydrocarbons to pristine or 17α(H)-hopane in crude oil (C,D) during stimulation with nutrients or in combination with *Bacillus subtilis* M15-10-1. BG: microcosm containing glucose; CG: microcosm containing glucose and *Bacillus subtilis* M15-10-1.

have resulted in them receiving increasing attention from both researchers and oil companies. To date, *Pseudomonas* and *B. subtilis* have been widely used in the petroleum industry to aid oil recovery due to their ability to produce surfactants with highly desirable properties for oil recovery (Simpson et al., 2011; You et al., 2015). However, their oil-degrading ability makes it difficult to determine the influence of these populations when used to strengthen the IMEOR process. The *B. subtilis* M15-10-1 can grow and produce lipopeptides on various cheap carbon sources, except for crude oil, making it an ideal candidate for analyzing the role of exogenous microbial populations in the IMEOR process.

Water-flooded oil reservoirs contain diverse microbial populations. High-throughput sequencing has revealed that abundant potential surfactant-producing bacteria, in particular *Bacillus*, *Pseudomonas*, and *Dietzia*, inhabit the Lu water-flooded reservoir (Gao et al., 2015b). Most species of these populations have been isolated and demonstrated to be able to produce surfactants on multiple hydrocarbons and carbohydrates, e.g., crude oil, sugar, and grease (Xia et al., 2012; Gudina et al., 2013; Wang et al., 2013). Unexpectedly, it is hard to stimulate these microorganisms to emulsify crude oil when adding nutrients into the formation brines. The present study revealed that different kinds of nutrient compounds could stimulate the growth of indigenous microorganisms, but not all the nutrients improved oil emulsification in the stimulation process (**Figure 5**). This elucidated the reason why specific nutrient compounds were selected and used in different reservoirs (Belyaev et al., 1998; Liu et al., 2005; Bao et al., 2009; Li et al., 2014; Halim et al., 2015). However, it is easy to envisage that once these surfactant producers are domesticated in the laboratory, they may produce

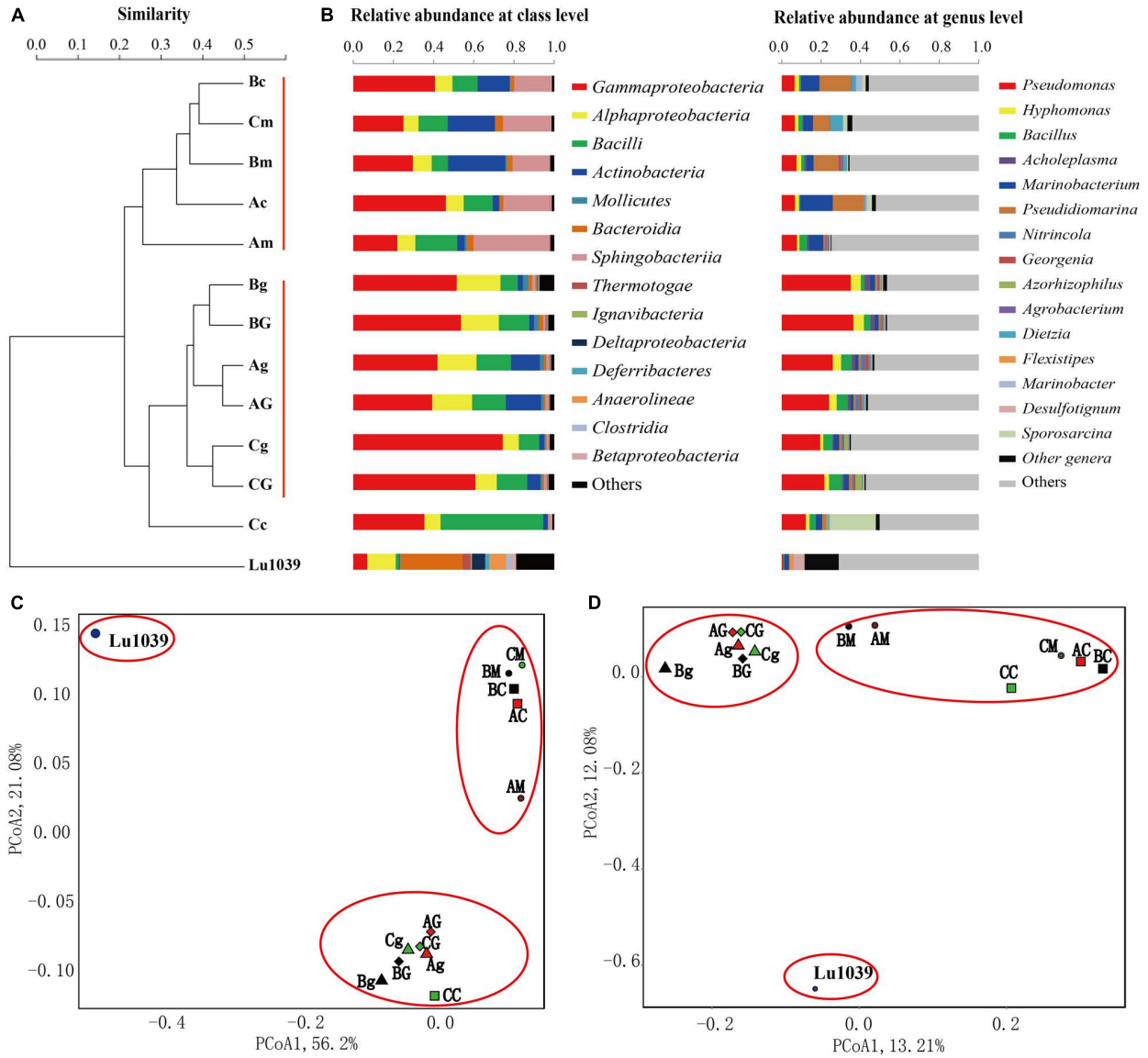

FIGURE 8 | Clustering (A), histogram (B), weighted Unifrac PCoA (C), and unweighted Unifrac PCoA (D) analysis of the microbial communities in water samples stimulated by nutrients or in combination with *Bacillus subtilis* M15-10-1. A: microcosm containing nutrients; B: microcosm containing nutrients and crude oil; C: microcosm containing nutrients, crude oil, and *Bacillus subtilis* M15-10-1; G: glucose; g: glycerol; m: molasses; and c: corn steep powder.

sufficient surfactants to emulsify crude oil when grown on multiple carbon sources. In the present study, glucose and glycerol did not stimulate the reservoir microorganisms to emulsify crude oil, but the added *B. subtilis* M15-10-1 rapidly improved oil emulsification. This phenomenon suggests that the exogenous *B. subtilis* can improve oil emulsification in the IMEOR process, in particular for the reservoirs where different kinds of nutrients cannot effectively improve oil recovery. Importantly, the exogenous *B. subtilis* also accelerated oil biodegradation, indicating that the emulsified oil may further enhance the growth of indigenous hydrocarbon-degrading bacteria with crude oil as the carbon source during stimulation (**Figure 7**).

Whether an exogenous MEOR treatment or exogenous population facilitates the IMEOR process, the added surfactant-producers must be able to grow in the presence of competing indigenous microorganisms. In an artificial microflora, we found that the exogenous *B. subtilis* M15-10-1 could live together with the ubiquitous reservoir microbial populations. It seems that the *B. subtilis* has no obvious inhibitory effect on these microorganisms. In addition, although the *B. subtilis* cannot grow with oil as the carbon source, the growth of the oil-degraders *Dietzia* and *Rhodococcus* improved the growth of the *B. subtilis* when cultured with crude oil as the sole carbon source (**Figure 4**). We further investigated the effect of the *B. subtilis* on the indigenous microbial community. The qPCR results indicated

that the added *B. subtilis* adapted to and grew in concert with the indigenous microbial populations (**Figure 6**). There was also no obvious difference in the numbers of 16S RNA, *alkB*, and alpha diversity in microcosms with exogenous *B. subtilis* compared with the microcosms with only nutrient additions (**Figure 6B** and Supplementary Figure S1).

Petroleum reservoirs represent special environments underground with low permeability. Owing to the sieve effect on microbial cells when injected fluid passes through a subsurface formation, the injected exogenous microorganisms may not migrate into the reservoir formation (Youssef et al., 2009; Gao et al., 2015a; Ren et al., 2015). Youssef et al. (2007) injected *B. licheniformis*, *B. subtilis* subsp. *Spizizenii*, and nutrients into two Viola formation wells, and improved oil recovery. They also pointed out that the injected exogenous microorganisms may only affect a radius of 2–4 m from the well in a follow-up study (Simpson et al., 2011). In this case, the injected exogenous bacteria will mainly grow and colonize the near-wellbore area. However, it may be suitable for an IMEOR treatment. When exogenous surfactant producers are injected into reservoir strata by a water injection well, most of these microorganisms may accumulate in the near-wellbore area of water injection well. The immobilized microorganisms may serve as a "seed bank." Therefore, once nutrients are injected through a water injection well, the "seed bank" will transport the surfactant producers into reservoirs continuously. Then, the surfactant producers will produce surfactants to strengthen the IMEOR process.

The core-flooding test is an economic model, and can simulate the oil-recovery operations usually conducted in reservoirs (Gudina et al., 2013). It was used to evaluate the application potential of the exogenous surfactant-producing *B. subtilis* in facilitating the IMEOR process. Previous investigations have shown that the stimulation of indigenous microorganisms enhances oil recovery by 3.7–9.14 % (Bao et al., 2009; Dong et al., 2015), and by 4.89–24 % in core-flooding tests with surfactant-producing bacteria and their metabolites (She et al., 2011; Castorena-Cortes et al., 2012; Xia et al., 2012; Gudina et al., 2013; Al-Wahaibi et al., 2014; Arora et al., 2014; Song et al., 2015). In this study, the brines with nutrients and fermentation broth of *B. subtilis* M15-10-1 increased the oil displacement efficiency by 16.71%, which is higher than the 7.59% with nutrient injection only. However, the *B. subtilis* can only grow and produce surfactants in the presence of oxygen, otherwise higher oil displacement efficiency may be realized. In this instance, facultative anaerobic surfactant-producing bacteria, such as *Geobacillus stearothermophilus*, may be an alternative candidate.

In summary, this study investigated the microbial processes and driving mechanisms in an exogenous *B. subtilis*-facilitated IMEOR process. The exogenous *B. subtilis* could live together with reservoir microbial populations, and did not show an obvious inhibitory effect on the indigenous microorganisms. The *B. subtilis* facilitated the IMEOR process by improving oil emulsification and accelerating microbial growth with oil as the carbon source. Core-flooding tests showed the great application potential for enhancing oil recovery by combining exogenous and indigenous microbial flooding technology. This oil recovery technique has obvious advantages: (1) the injected microbial populations may accumulate and form a "seed bank" in the near-wellbore area of water injection well; and (2) this technique is especially suited to reservoirs where IMEOR treatment cannot effectively improve oil recovery.

AUTHOR CONTRIBUTIONS

PG, GL, and TM conceived and proposed the idea. PG, Yan Li, Yanshu Li, HT, YW, and JZ carried out the experiments and conducted data analysis. PG and TM drafted the manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00186

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Rhamnolipids Produced by Indigenous *Acinetobacter junii* from Petroleum Reservoir and its Potential in Enhanced Oil Recovery

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Biosurfactant producers are crucial for incremental oil production in microbial enhanced oil recovery (MEOR) processes. The isolation of biosurfactant-producing bacteria from oil reservoirs is important because they are considered suitable for the extreme conditions of the reservoir. In this work, a novel biosurfactant-producing strain *Acinetobacter junii* BD was isolated from a reservoir to reduce surface tension and emulsify crude oil. The biosurfactants produced by the strain were purified and then identified via electrospray ionization-Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR-MS). The biosurfactants generated by the strain were concluded to be rhamnolipids, the dominant rhamnolipids were $C_{26}H_{48}O_9$, $C_{28}H_{52}O_9$, and $C_{32}H_{58}O_{13}$. The optimal carbon source and nitrogen source for biomass and biosurfactant production were $NaNO_3$ and soybean oil. The results showed that the content of acid components increased with the progress of crude oil biodegradation. A glass micromodel test demonstrated that the strain significantly increased oil recovery through interfacial tension reduction, wettability alteration and the mobility of microorganisms. In summary, the findings of this study indicate that the newly developed BD strain and its metabolites have great potential in MEOR.

Keywords: biosurfactant, *Acinetobacter junii*, ESI FT-ICR-MS, biodegradation, polar components, visualization micromodel, microbial enhanced oil recovery

INTRODUCTION

Water flooding is an efficient oil recovery technology employed worldwide. However, a large amount of petroleum still remains underground. To enhance oil recovery, various approaches have been developed to improve the oil recovery, such as chemical flooding, CO_2 foam flooding, and steam flooding. However, these technologies require substantial effort and have higher energy, economic and environmental costs. Therefore, microbial enhanced oil recovery (MEOR), an economically efficient and environmentally friendly technology, has gained increasing attention in

academic and industrial fields. MEOR technology is an economical and environmentally friendly tertiary recovery method that utilizes microorganisms and their metabolites in oil reservoirs to achieve increased oil recovery efficiency and longer exploitation of oil reservoirs (Sun et al., 2011; Xia et al., 2011; Gudiña et al., 2013). The suggested mechanisms of MEOR include: reduction of interfacial tension (IFT), wettability alteration, changes in flow pattern, gas production, oil emulsification, and oil viscosity reduction (Karimi et al., 2012; Nazina et al., 2013; Rabiei et al., 2013; Xiao et al., 2013; Li et al., 2014; Sarafzadeh et al., 2014). Among these mechanisms, two are thought to be the main mechanisms behind MEOR success. The first mechanism involves the reduction of the IFT induced by biosurfactants. Low liquid–liquid IFT is important in promoting emulsification and improving the mobility of crude oil. The second mechanism is wettability alteration (Afrapoli and Alipour, 2012; Rabiei et al., 2013; Chandankere et al., 2014). In general, biosurfactant producers are considered a key factor in enhancing oil recovery.

In the past decades, a number of studies have reported the capability of bacteria to utilize different carbon sources to produce biosurfactants and their application in the petroleum industry (Pereira et al., 2013; Nalini and Parthasarathi, 2014; Domingos et al., 2015; Marti et al., 2015). The biosurfactant producers are mainly categorized into two classes, low-molecular-weight biosurfactant producers and high-molecular-weight bioemulsifier producers (Rosenberg and Ron, 1999). Low-molecular-weight biosurfactant producers are mainly *Pseudomonas*, *Bacillus*, *Rhodococcus*, and *Nocardia* species, which can produce different kinds of glycolipids and lipopeptides (Cooper and Goldenberg, 1987; Xia et al., 2014; Liu et al., 2015). High-molecular-weight bioemulsifier producers are mainly *Acinetobacter*, *Bacillus*, and *Geobacillus* species, which can produce lipopolysaccharides and glycoproteins (Zheng et al., 2011; Yadav et al., 2012). These metabolic products play indispensable roles in multiple mechanisms for improving oil recovery (Dastgheib et al., 2008; Zheng et al., 2011). Indigenous microbes, which are better adapted to the oil reservoir environment, have been used in MEOR processes (Castorena-Cortés et al., 2012). Numerous indigenous species with the capability to degrade crude oil efficiently and produce low-molecular-weight biosurfactants, such as *Pseudomonas*, *Bacillus*, *Rhodococcus,* and *Arthrobacter* species, play a dominant role in enhancing oil recovery. Nevertheless, studies on *Acinetobacter* species isolated from reservoirs and its feasibility of MEOR are scarce, although it is a dominant species in many reservoirs (Zhang et al., 2010; Lenchi et al., 2013; You et al., 2016). Rhamnolipids are well-known glycolipid surfactants produced by *Pseudomonas* with a great quantity, exhibiting excellent performance in reducing surface (interfacial) tension and changing emulsification and wettability (Orathai et al., 2007). Successful applications of *Pseudomonas* species in the petroleum industry and environmental remediation have been widely documented (Pasumarthi et al., 2013; Xia et al., 2014; Zuo et al., 2015). However, some *Pseudomonas* species are opportunistic human or plant pathogens. Therefore, non-pathogenic and rhamnolipid-producing bacterial strains are still the targets of novel investigations in the academic and industrial community of petroleum.

Low-molecular-weight biosurfactants are usually generated during the utilization of hydrophobic feedstocks; therefore, the occurrence of crude oil biodegradation by indigenous microorganisms is generally detected during the MEOR process. The alteration of saturated and aromatic hydrocarbons during biodegradation has been reported (Zheng et al., 2011; Gudiña et al., 2013; Xia et al., 2014). However, studies on the alteration of non-hydrocarbons during MEOR processes have not been reported. According to Li et al. (2010) and Kilpatrick (2012), non-hydrocarbon compounds comprising a large percentage of crude oil are the main interfacial active components. Oil emulsification is closely related to non-hydrocarbon compounds (Pan et al., 2013). Oxygenated compounds are the most important polar group in the oil component and are closely related (Pan et al., 2013); additionally, oxygenated polar compounds have an important role in oil emulsification and wettability alteration in oil reservoirs (Kilpatrick, 2012). Nevertheless, the alteration of these compounds and its effects on the oil recovery during MEOR have not been extensively explored.

In the present study, a non-pathogenic, hydrocarbon-degrading bacterial strain, *Acinetobacter junii* BD, was isolated which has been demonstrated to produce biosurfactants with good potential for MEOR. This strain was able to emulsify oil in water by reducing the IFT between oil and water. The structural diversity of the produced biosurfactants was analyzed, and the strain was able to degrade crude oil and produce rhamnolipid surfactants. The biosurfactants and biomass yields were optimized. The recovery of residual oil was examined with a glass micromodel.

MATERIALS AND METHODS

Isolation and Identification of Microorganisms

The oil and formation water samples used in this study were collected from oil wells at Xinjiang Oilfield, which is located in Northwest China (45.41336°N, 85.04578°E). The depth of the petroleum reservoir was 1088 m, with a temperature of 32°C. Oil and formation water were collected at the wellheads after connection lines were flushed for 5 min prior to filling a 15 L sterilized plastic container. A 5-mL sample was then cultivated in 100 mL mineral enrichment medium (MEM) amended with 10 mL crude oil (from the same oil well). This was carried on under aerobic condition at 37°C for 7 days with agitation at 150 rpm. 100 μL of culture supernatant was then collected, spread on Luria Broth (LB) agar plates and grown for 3 days. The microbial colonies obtained were further purified by streaking on LB agar and cultivating the strains with ability of hydrocarbon emulsification and surface tension reduction. The emulsification activity of the biosurfactants was evaluated by emulsification index (E_{24}), which was quantified as the ratio of the emulsified volume to the total volume of a mixture containing 5 mL bioemulsifier solution and 5 mL of n-hexadecane after it was

vortexed for 5 min and then settled for 24 h (Zheng et al., 2011). The surface tension was determined using a surface tensiometer JK99B (POWEREACH, China). The strains BD was selected because of its superior performance in hydrocarbon emulsification and reduce surface tension, and its growth was assessed at wide ranges of temperatures, pH values and salinities. The MEM contained 6 g/L $NaNO_3$, 1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4$, 0.02 g/L $FeSO_4$, and 0.02 g/L Na_2MoO_4 at a pH of 7.0–7.2.

The morphological, physiological, and phylogenetic characteristics of the isolated strains were analyzed following Bergey's Manual of Systemic Bacteriology and SSU rRNA sequencing, as previously described by Chen et al. (2012).

Effects of Carbon and Nitrogen Sources on Biosurfactant Production

The growth of strain BD and biosurfactant production were evaluated using a mineral salt medium (MSM) with different carbon and nitrogen sources. The MSM contained 1.0 g/L K_2HPO_4, 1.0 g/L KH_2PO_4, 0.25 g/L $MgSO_4$, 0.02 g/L $Na_2Mo_2O_4$, and 0.02 g/L $FeSO_4$. Glucose, hexadecane, molasses, soybean oil, glycerol, paraffin, sucrose, ethanol, and diesel oil were evaluated as carbon sources while 8 g/L $NaNO_3$ was used as a nitrogen source. These carbon sources were added into the MSM at a concentration of 10 g/L. The following compounds were tested as nitrogen sources while soybean oil (10 g/L) was used as a carbon source: $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, beef extract, tryptone, and urea.

The optimization experiments were carried out in 250-mL flasks containing 100 mL of medium. Each flask was inoculated with 1% of a pre-culture grown in the same medium for 24 h. The flasks were incubated at 37°C for 120 h with agitation at 150 rpm. The cells were then harvested by centrifugation (at 10,000 rpm for 20 min), and the cell weight after drying at 105°C for 48 h was determined (Pereira et al., 2013). The cell-free supernatants were used to measure the biosurfactant yield. The biosurfactant yield was derived from a standard curve prepared with rhamnose and expressed as rhamnose equivalents (Vyas and Dave, 2011). All optimization experiments were conducted in triplicate, and the data are presented as the means.

Biosurfactant Extraction

The cell-free supernatant was obtained by centrifugation at 10,000 rpm for 20 min at 4°C. After the pH was adjusted to 2.0 with 1 mol/L HCl, 200 mL of the samples were extracted twice with equal volume of ethyl ether. The solvent was removed by vacuum distillation, to collect the dry product. The purified biosurfactants in chloroform were then spotted on a silica gel thin-layer chromatography (TLC) plate (Silica gel 60, Merck, Qingdao, China). The compounds were separated using a mobile phase containing chloroform, methanol, acetic acid, and water in volume ratios of 65:25:1:1, all of the solvent components being of analytical grade purity. The dry plates were sprayed with a phenol sulfate solution and incubated at 105°C for 5 min for glycolipid detection.

Purification and Structural Analysis of the Biosurfactants

The crude extracts of biosurfactants were dissolved in chloroform and were then loaded onto a column of silica gel (100–200 meshes; all silica separation materials used were from Qingdao Marine Chemical, Co. Ltd, Qingdao, China). The column was first washed with n-hexane and then with chloroform/methanol solvent systems to recover potential biosurfactants. The eluate was collected separately every 10 mL. The glycolipid was detected using phenol-sulfate colorimetry. The chemical composition of each component fractionated from the purified glycolipid was then preliminarily investigated via electrospray ionization (ESI) Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR-MS). Each mass typically yielded a unique molecular formula within a mass tolerance of 1.0 ppm with a ^{13}C-isotope because of high mass accuracy. Nevertheless, the molecular formulas must be validated using the rules described by Lin et al. (2014). The sum formulas of the true discriminant masses were calculated and validated through isotope pattern matching using Bruker Daltonics Data Analysis version 3.4. The molecular formulas were then validated using the ChemSpider database.

Biodegradation of Crude Oil by Strain BD

Bacterial cells from a 5-mL LB culture were harvested by centrifugation at 10,000 rpm for 2 min, washed in triplicate with sterilized saline and inoculated into 100 mL MEM (pH: 6.8–7.2) supplemented with crude oil in a concentration of 0.5% (w/v). One culture containing crude oil but without cells was used as control. The flasks were then separately incubated in a rotary shaker at 150 rpm for 2 or 14. The oil from the liquid culture was extracted three times with dichloromethane.

Analysis of Polar Components during Crude Oil Biodegradation

The polar components of the crude oil were examined using ESI FT-ICR-MS (Shi et al., 2010a). The extracted crude oil was dissolved in toluene and diluted to 0.2 mg/mL with toluene/methanol mixture (1:3, v/v). 15 μL ammonia solution (25%, HPLC grade) was then added into the solution to facilitate the deprotonation of the acid species and neutral nitrogen compounds to yield [M-H] ions. The analysis was performed on an Apex-ultra FT-ICR mass spectrometer (Bruker Daltonics, USA) equipped with an actively shielded 9.4-T magnet. Ions were generated with a negative-ion electrospray equipped with a 50-μL infused silica ESI needle. The samples were infused at a flow rate of 250 μL/h. The operating software was XMASS version 6.0 (Bruker Daltonics, USA). Each spectrum was composed of 64 scans.

Glass Micromodel Test

The setup of the micromodel is illustrated in **Figure 1**. The micromodel mainly consisted of a glass micromodel, a fluid injection system, a temperature controlling system, an optical and photography system and a monitoring computer. In this study, two glass micromodels (i.e., models A and B) were used in simulating oil recovery under reservoir conditions to examine

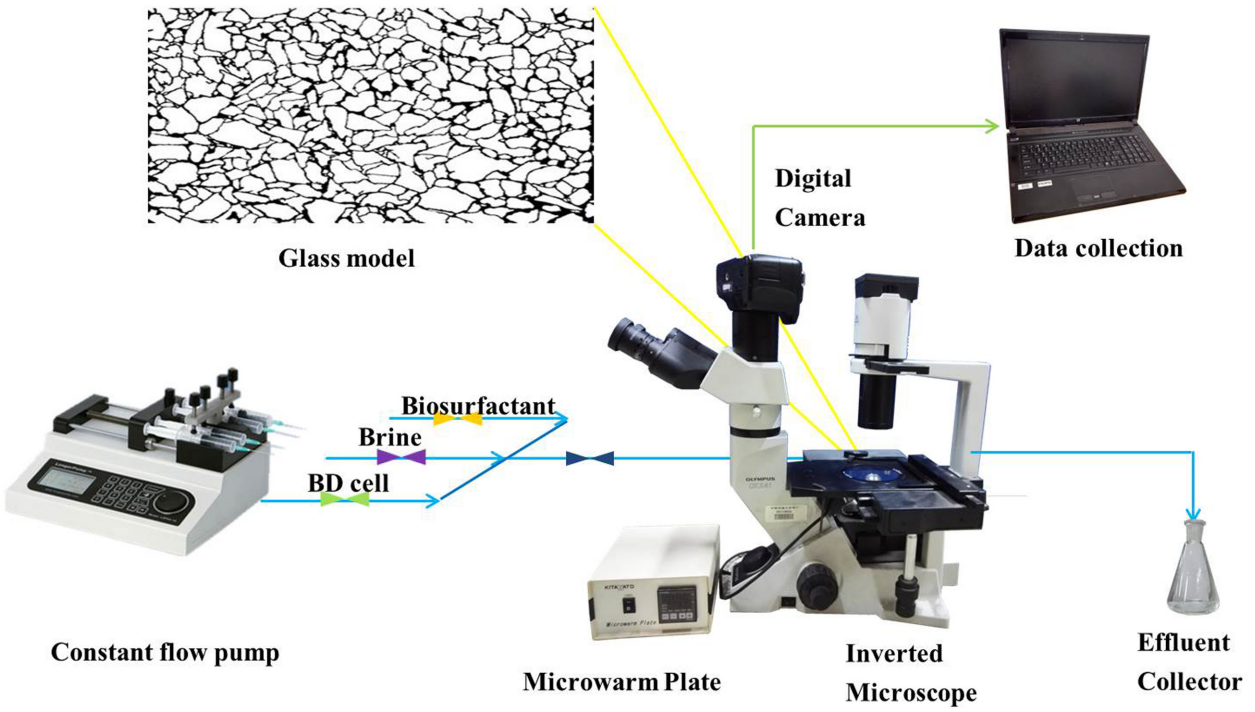

FIGURE 1 | Schematic of the glass micromodel experimental set-up.

the efficiency of strain BD in enhancing oil recovery. The manufacturing process of the micromodels has been described by Dunsmore et al. (2004). Briefly, a micromodel consisted of two glass plates, one with an interconnected, acid-etched network being annealed to the other, un-etched plate. The inner diameter of the core channels was approximately 20 μm (Supplementary Figure S1).

Micromodels were first saturated with crude oil at 90°C for 7 days. Then, fresh water was injected into the micromodels for 30 min to simulate the first water flooding. The flow rate of the flooding water was controlled at 8 μL/min with a LongerPump TS-1B constant flow pump (LongerPump, China). The pump and micromodels were connected with Masterflex 06409-13 tygon tubing (Saint-Gobain, France). The temperature of the flow system and micromodel was maintained at 35°C via a micro warm plate (Kitazato, Japan). Subsequently, cell-free fermentation broth containing biosurfactants was injected into model A for 10 min, followed by 20 min second water flooding. Into model B, bacterial colonies cultivated in MSM were continuously injected until no further oil was produced. Model B was then sealed for 36 h, followed by the second water flooding for 20 min. Photographs were taken during the flooding period every 2 min with an EOS550D digital single lens reflex camera (Canon, Inc., Japan), and the oil recovery was determined based on the change of pixel numbers of crude oil areas as evaluated using Photoshop CS3 (Xiao et al., 2013):

$$\text{Oil recovery} = \frac{Area_{initial} - Area_{flooding}}{Area_{initial}} \quad (1)$$

RESULTS AND DISCUSSION

Screening of Biosurfactant Producer

The most promising biosurfactant producer, strain BD, was selected from 77 strains isolated from oil–water mixture samples because of its high performance in surface tension reduction and oil emulsification assays. The emulsification index E_{24} and the surface tension of the BD culture were determined to be 57% and 30.27 mN/m, respectively. The isolated BD strain is characterized as motile, gram negative, and MR negative. Moreover, strain BD can grow well in the temperature range of 20–55 °C, pH range of 4–10, and salinity range of 0–20%. The nearly complete 16S rRNA gene sequence (1382 bp) of strain BD was obtained, and the phylogenetic analysis showed that it has the highest 16S rRNA sequence similarity of 99.38% with *Acinetobacter junii* (Genbank accession number KT763370) (Supplementary Figure S2). The biosurfactant producer has been isolated from a wide diversity of environments including soil, sea water, marine sediments, oil fields, and even extreme environments (Yakimov et al., 1998). The biosurfactant producer isolated from reservoirs adapted better than others to the oil reservoir environment. Until now, numerous indigenous species have been known as biosurfactant producers and have been well-studied. Bacterial genera isolated from reservoirs, including *Pseudomonas*, *Bacillus*, *Enterobacter*, *Rhodococcus*, *Paenibacillus*, *Dietzia*, *Acinetobacter*, and *Brevibacterium,* were found to produce many kinds of surfactants (Najafi et al., 2011; Zheng et al., 2012; Khajepour et al., 2014). Some of these genera were found to produce different types of surfactants

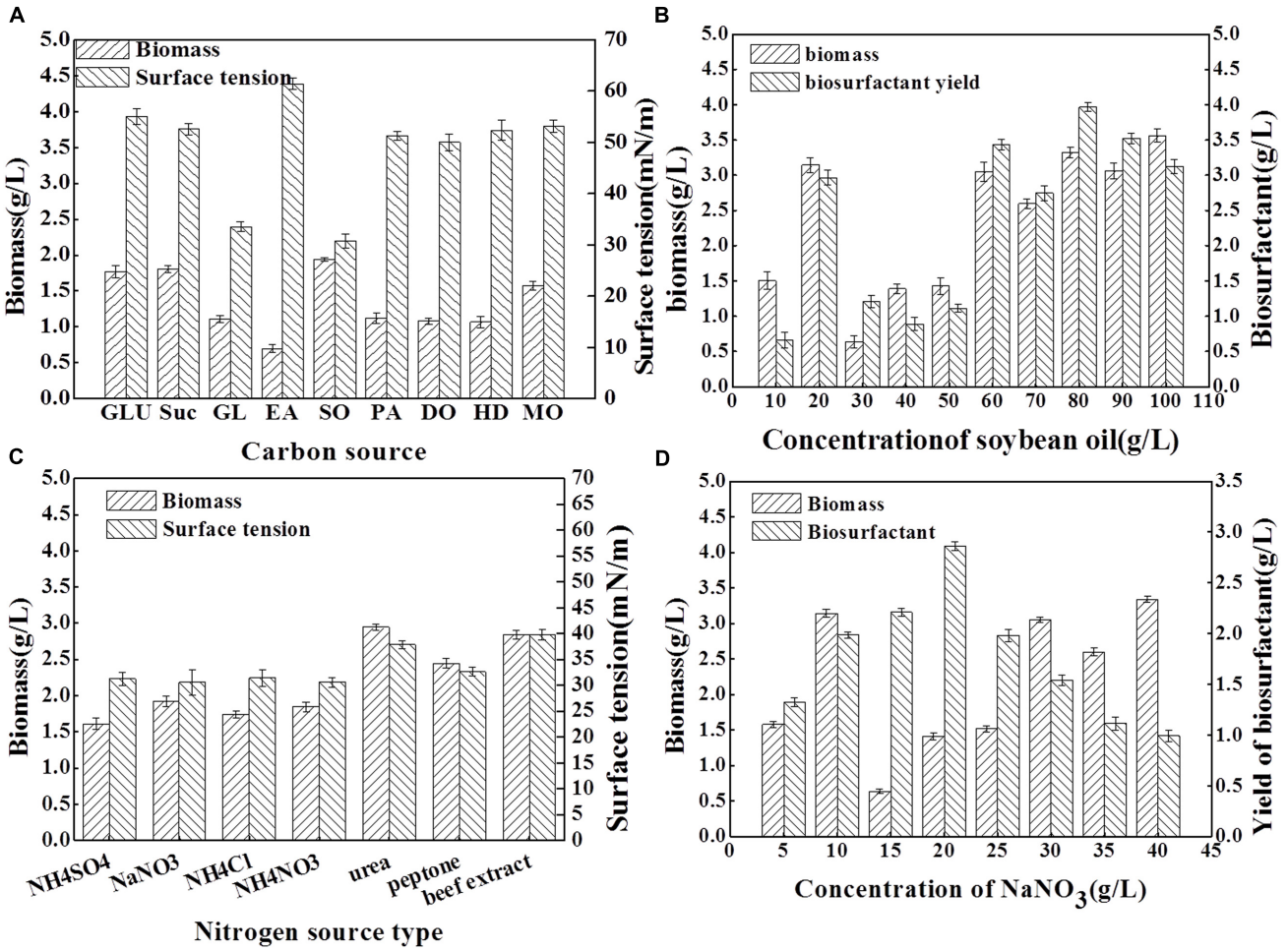

FIGURE 2 | Surface tension (mN/m), biomass yield (g dry weight L^{-1}) and biosurfactant yield (g/L) obtained for the BD strain after 96 h cultivation in mineral salt solution with different carbon and nitrogen sources. Results represent the average of three independent experiment ± standard deviation. **(A)** shows surface tension and biomass yield obtained for the BD strain cultivation in mineral salt solution with different kinds of carbon source. In **(A)**, GLU, glucose; Suc, sucrose; GL, glycerol; EA, ethanol; SO, soybean oil; PA, paraffin; DO, diesel oil; HD, hexadecane; and MO, molasses. **(B)** shows biomass yield and biosurfactant yield obtained for BD strain cultivation in mineral salt solution with different concentration of soybean oil as carbon sources. **(C)** shows surface tension and biomass yield obtained for the BD strain cultivation in mineral salt solution with different kinds of nitrogen source. **(D)** shows biomass yield and biosurfactant yield obtained for BD strain cultivation in mineral salt solution with different concentration of $NaNO_3$ as nitrogen sources.

and have multiple species. For example, *Pseudomonas* was found to produce rhamnolipids, and in several cases produced lipopeptides (Xia et al., 2011, 2014). It is well-documented that *Acinetobacter* species generally produce exocellular polymeric bioemulsifiers. The well-known bioemulsans, for example, is a kind of a bioemulsifier produced by different species of *Acinetobacter*, such as *Acinetobacter calcoaceticus* RAG-1, *Acinetobacter radioresistens* KA53, *Acinetobacter junii* BB1A, *Acinetobacter calcoaceticus* BD4, etc. (Navon-Venezia et al., 1995; Toren et al., 2001; Sen et al., 2014). These microorganisms have shown to be very efficient in bioemulsifier producing. However, few researches are there about *Acinetobacter* isolated from reservoirs as a biosurfactant (small-molecule compound) producing microbe, although it is a dominant species in many reservoirs.

Effects of Carbon and Nitrogen Sources on Biosurfactant Production

To determine the optimal medium that yields the highest biomass and biosurfactant production by *Acinetobacter junii* BD, the effects of various carbon and nitrogen sources were examined. The results are presented in **Figure 2**. As shown in **Figure 2A**, using the same concentration of carbon sources, and keeping other conditions constant, soybean oil as the sole carbon source resulted in the highest biomass yield; the surface tension of the medium was reduced from 70.22 to 30.75 mN/m which is the most significant among all of the carbon sources. Although, a high biomass yield was obtained when glucose and sucrose were used as sole carbon sources, the surface tension reduction of the medium was poor. Previous studies also reported that hydrocarbons and carbohydrates are the best

carbon sources for biosurfactant production in other isolates (Gudiña et al., 2015). Many *Acinetobacter* isolates are regarded as bioemulsifier producers; Su et al. (2009) considered C_2H_5OH as the best carbon source for bioemulsifier growth and production. In the current study, soybean oil was determined to be the appropriate carbon source for the biosurfactant production with *Acinetobacter junii* BD. **Figure 2B** shows that higher concentrations of the carbon source enhanced biomass yield. Moreover, the maxima were observed when 10% soybean oil was added. However, the biosurfactant yield attained its highest value when 8% soybean oil was added, with a concomitant high biomass yield.

Comparing the effect of nitrogen source (8 g/L) on the biomass yield and surface tension (**Figure 2C**), the maximum biomass production was obtained when the strain was cultivated on urea. The maximum emulsification capacity and lowest surface tension were observed in the culture with $NaNO_3$ as nitrogen source. Based on these results, it can be suggested that the optimum nitrogen source for BD growth differ from that for biosurfactant production. The presence of $NaNO_3$ as the sole source of nitrogen with a concentration of 1.0% (w/v) in the soybean oil-containing MSM resulted in a high level of biomass and biosurfactant production (**Figure 2D**).

Thin-Layer Chromatography (TLC) Analysis of the Purified Biosurfactants

Considering the significant reduction of surface tension in the current study, it seems that the strain BD synthesized low-molecular-weight biosurfactants in the MSM with soybean oil as the sole carbon source. As previously described, low-molecular-weight biosurfactants are generally glycolipids, phospholipids and lipopeptides (Abdel-Mawgoud et al., 2010; Gudiña et al., 2013). Two types of biosurfactants exhibited positive reactions with two brown reagents of a phenol sulfate solution (**Figure 3**). This observation indicated that different homologous series might belong to glycolipids that consisted of sugar and lipid moieties (Juliana et al., 2013).

Diversity of the BD Biosurfactants

The diversity of the biosurfactants produced by *Acinetobacter junii* BD is illustrated in **Table 1**. A series of singly charged negative ions were observed in the BD biosurfactant mix (**Figure 4A**). The mass under the negative mode exhibited deprotonated molecular ions at m/z 503.32139, which was considered to be the most abundant ion in the TLC fraction of the biosurfactant eluted with 1:1 methanol: chloroform. This ion peak was analyzed with the FT-ICR-MS data analysis software and was shown to correspond to $C_{26}H_{48}O_9$, which generated three hits in the ChemSpider database. By reviewing the TLC analysis and searching against the ChemSpider database, it was determined that the $C_{26}H_{48}O_9$ molecule was 3-({3-[(6-deoxy-α-L-mannopyranosyl) oxy]decanoyl}oxy)decanoic acid, a type of mono-rhamno-di-lipidic rhamnolipid (Abdel-Mawgoud et al., 2010). Similarly, the ion at m/z 531.35377 was assigned to $C_{28}H_{52}O_9$, which may belong to homologs of this mono-rhamno-di-lipidic rhamnolipid.

TABLE 1 | The diversity of biosurfactants produced from strain BD.

m/z	Formula	DBE	Metabolite class
503.32139	$C_{26}H_{48}O_9$	3	Mono-rhamno-di-lipidic congeners
531.35377	$C_{28}H_{52}O_9$	3	Mono-rhamno-di-lipidic congeners
557.36973	$C_{30}H_{54}O_9$	4	Mono-rhamno-di-lipidic congeners
529.33773	$C_{28}H_{50}O_9$	4	Mono-rhamno-di-lipidic congeners
475.2911	$C_{24}H_{44}O_9$	3	Mono-rhamno-di-lipidic congeners
473.27579	$C_{24}H_{42}O_9$	4	Mono-rhamno-di-lipidic congeners
649.37936	$C_{32}H_{58}O_{13}$	4	Di-rhamno-di-lipidic congeners
677.41079	$C_{34}H_{62}O_{13}$	4	Di-rhamno-di-lipidic congeners
621.34781	$C_{30}H_{54}O_{13}$	4	Di-rhamno-di-lipidic congeners
419.22967	$C_{20}H_{36}O_9$	3	Mono-rhamno-di-lipidic congeners
479.24932	$C_{22}H_{40}O_{11}$	3	Di-rhamno-mono-lipidic congeners

In the TLC fraction of the biosurfactant sample eluted with 1:2 methanol: chloroform (**Figure 4B**), the molecular ions at m/z 649.37936 corresponding to $C_{32}H_{58}O_{13}$ garnered three hits in the ChemSpider database. Based on TLC analysis and a search against the ChemSpider database, $C_{32}H_{58}O_{13}$ was assigned to 3-[(3-{[6-deoxy-2-*O*-(6-deoxyhexopyranosyl) hexopyranosyl] oxy} decanoyl) oxy] decanoic acid, a type of di-rhamno-di-lipidic rhamnolipid. $C_{32}H_{58}O_{13}$ was the most abundant di-rhamnolipid in the BD biosurfactant mix. Molecular ions at m/z 503.32139 were also detected. This result implied that $C_{26}H_{48}O_9$ was the main biosurfactant produced by BD. The unique ion at m/z 531.35355 was assigned to $C_{28}H_{52}O_9$, which was a homolog of the mono-rhamno-di-lipidic rhamnolipids.

Acinetobacter strains produce groups of compounds including high-molecular-weight bioemulsifiers, such as emulsan, alasan, and exopoly saccharide, which are effective in stabilizing oil-in-water emulsions (Navon-Venezia et al., 1995; Toren et al., 2001; Sen et al., 2014). The *Acinetobacter* strain in this study exhibited high levels of biosurfactant production. However, the molecular mass of the purified biosurfactants indicated the presence of more than 10 major and minor congeners of rhamnolipids. The largest portion of the biosurfactants was composed by the mono-rhamnolipids. Microorganisms can produce rhamnolipid homologs with various fatty acid chain

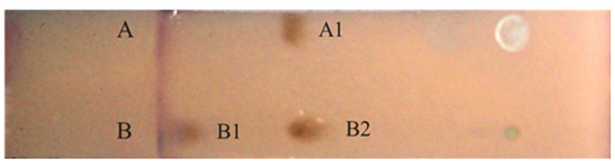

FIGURE 3 | The thin-layer chromatogram of crude biosurfactant. Lanes **(A,B)** were developed with phenol-sulfuric acid method to detect glycolipid. **(A)** The thin-layer chromatogram of rhamnose; **(B)** the thin-layer chromatogram of biosurfactant produced by BD when utilizing soybean oil as carbon source. Brown color is positive.

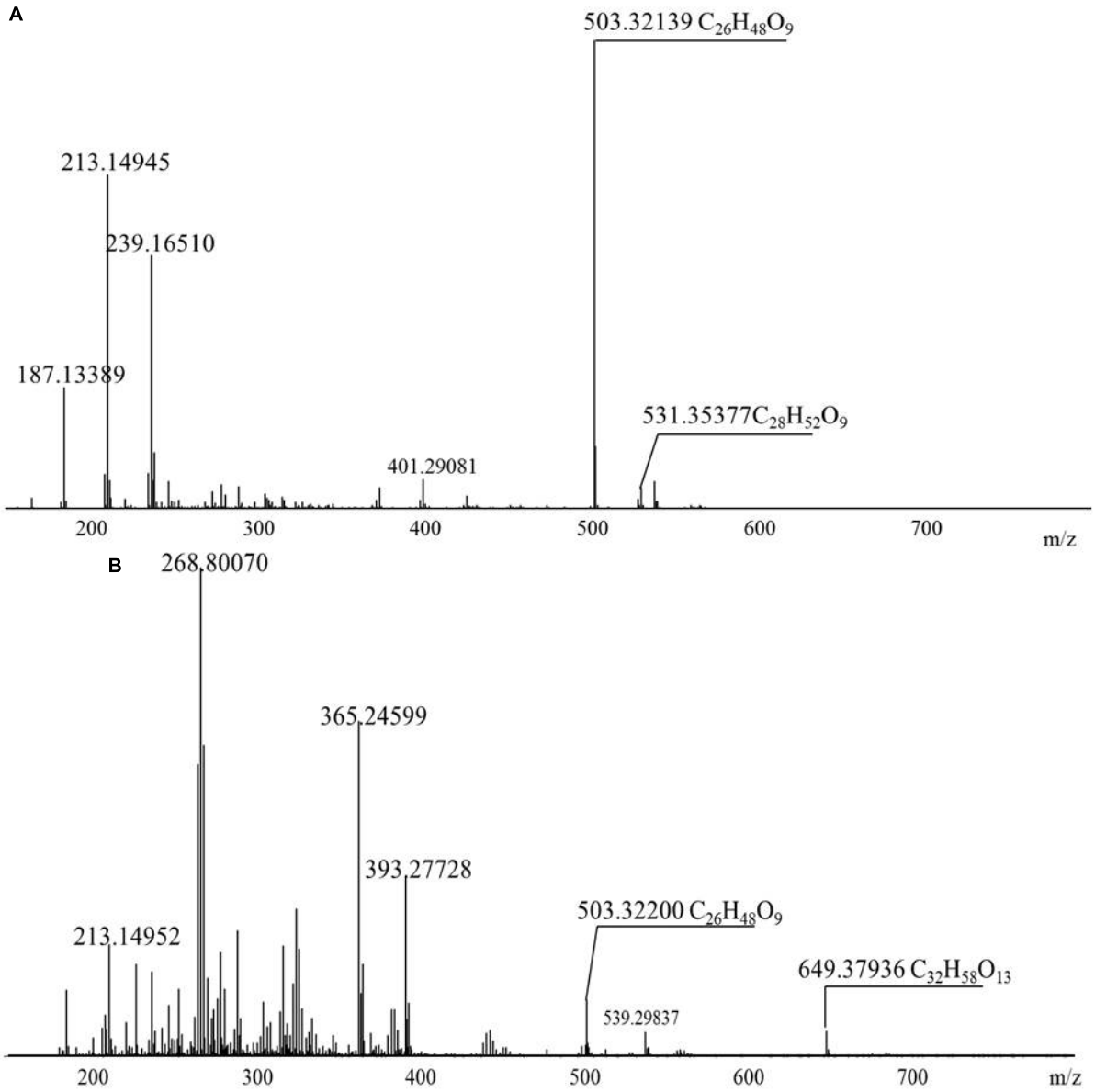

FIGURE 4 | Negative-ion electrospray FT-ICR mass spectra of the purified biosurfactant. (A) Biosurfactant eluted with 1:1 methanol:chloroform; **(B)** 1:2 methanol:chloroform.

length and number of rhamnose units. According to Nitschke et al. (2005), rhamnolipids with a longer fatty acid chain possess stronger hydrophobicity.

Analysis of Polar Components during the Biodegradation of Crude Oil

The mass spectra of the negative ions obtained from the ESI FT-ICR-MS analysis of the crude oil showed that the contour of the molecular weight spectrum of the residual crude oil shifted toward lower molecular weights during degradation by strain BD. This finding indicates that biodegradation converts larger molecules into smaller ones (Supplementary Figure S3).

Compound Class Distribution

The importance of oxygenated polar compounds as interfacial active components in crude oil is widely acknowledged as one of the primary causes of emulsion stability in crude oil mixtures (Acevedo et al., 1999; Pauchard et al., 2008). Oxygenated polar compounds also play an important role in wettability alteration. The distribution of heteroatom-containing species in the crude oil subject to various biodegradation is presented in **Figure 5**. The O2 class was predominant, followed by O1, O5, and O3 heteroatom-containing classes. Similarly, the O2 class was the most abundant species in the crude oil after biodegradation, then the abundance decreased in the sequence of O3, O4, O1, and O5.

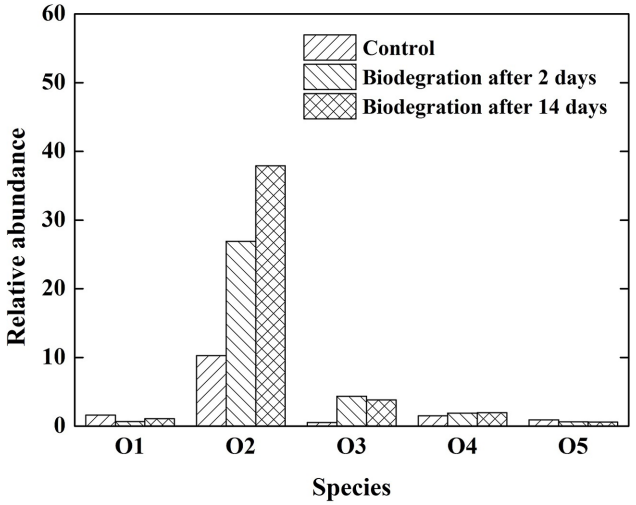

FIGURE 5 | Relative abundance of different heteroatom classes (number of heteroatoms) derived from negative-ion ESI FT-ICR mass spectrum of the crude oil.

The abundance of O2 species increased with increasing the extent of biodegradation, which is consistent with previous report (Kim et al., 2005). In this study, the increase in O2 species was the most predominant, and its abundance increased from 12.3 to 33.2% after 14 days of biodegradation. The O2 species in the crude oil were mainly carboxylic and naphthenic acids. An important mechanism by which emulsions are stabilized in crude oil systems is through the adsorption of carboxylic acids and their anions. The acids in crude oil can be adsorbed to the oil–water interface and dramatically lower the IFT (Hoeiland et al., 2001).

Distribution of *O*-Containing Compounds

The iso-abundance plots of double bonds equivalent (DBE) versus the carbon number of the O2, O3, O4 class species in crude oil before and after biodegradation are shown in **Figure 6**. The distribution of the O2 species with DBE of and carbon number provides information on the effects of biodegradation on oil displacement. The O2 species with DBE of 1 are fatty acids, whereas those with DBE of 2–7 correspond to 1- to 6-ringed naphthenic acids (Pan et al., 2013). In contrast, O2 species with a high DBE value and low carbon number are likely to be multi-ring naphthenic acids, aromatic acids, or phenols with multiple hydroxyl groups (Shi et al., 2010b). In this study, before biodegradation, the DBE of the O2 species in the crude oil was primarily in the range from 1 to 4, and their carbon numbers was mainly in the range of 10–30. However, after biodegradation by strain BD, the DBE of the O2 species narrowed to 1 and 2, and their carbon number mainly ranged from 14 to 22. It has long been known that acids in crude oil can adsorb to a crude oil–water interface and dramatically lower the IFT (McCaffery, 1976). Emulsion is stabilized by naphthenates through the formation of alkaline earth soaps, particularly calcium naphthenate, by polyvalent acids (Baugh et al., 2004; Lutnaes et al., 2006).

The relative abundance of O3 species increased during biodegradation. The relative abundance of O3 species with DBE of 1 and 2 significantly increased. These constituents are likely hydroxyl fatty acids. The O4 species in the crude oil before biodegradation was sparsely distributed in the DBE range of 1–18 and carbon number between 7 and 53. After biodegradation, the species was in the DBE range from 1 to 12 and carbon numbers from 7 to 35. The O4 species with a DBE of 2 had the highest relative abundance and were likely saturated fatty dibasic acids. The species with a DBE of 5 also became the dominant compounds after biodegradation. These species were likely dicarboxylic acids with three naphthenic rings (Mapolelo et al., 2011).

The acid components in crude oil are mainly alkyl carboxylic, alkyl benzene carboxylic, naphthenic, and fused aromatic ring acids. Some of these components may be large molecular species with a combination of fused aromatic rings, alkyl side chains, and other chemical moieties with carboxylic acid pendant groups. The acid components in crude oil can be ionized at the oil–water interface to form the anion of the acid and to dramatically lower the IFT (Gao et al., 2010). This occurrence leads to the stabilization of interfaces and emulsion. During biodegradation, the content of fatty acids, naphthenic acids, multi-ring naphthenic acids, aromatic acids, and phenols with multiple hydroxyl groups rapidly increases. The microorganisms attached to the solid surface and those that can degrade crude oil on the solid-oil surface can sweep oil from the rock and improve the mobility of petroleum.

Analysis of the Glass Micromodel Test

Glass micromodels were used to evaluate the effect of microbial enhancement of the oil recovery. **Table 2** compares the oil recovery with different injection methods in glass micromodel tests using bacteria and biosurfactants. The final oil recovery when only formation water flooding was applied was 54.6% after 60 min. A 2.8% enhancement of oil recovery was achieved by flooding with bacterial broth for 10 min. This value was further increased with a second period of formation water flooding after a 36 h shut-in period, resulting in a 9.6% improvement in total oil recovery. In another experiment, the oil recovery was increased by 13.4% after biosurfactant flooding. The result shows that strain BD and its metabolite have a great potential for enhancing oil recovery.

Biosurfactants have a great potential for enhancing oil recovery, which is also demonstrated in the literature. In the water flooding experiments investigating the MEOR potential of *Rhodococcus ruber* Z25, Zheng et al. (2012) achieved improvement of oil recovery by 8.9–25.8%. Application of biosurfactant producing strain *Enterobacter cloacae* increased the oil recovery up to 24.5% (Khajepour et al., 2014). Xia et al. (2011) reported 14.3% enhancement compared with the control condition using the biosurfactants of *Pseudomonas aeruginosa* in their oil recovery experiment. The results of oil recovery by

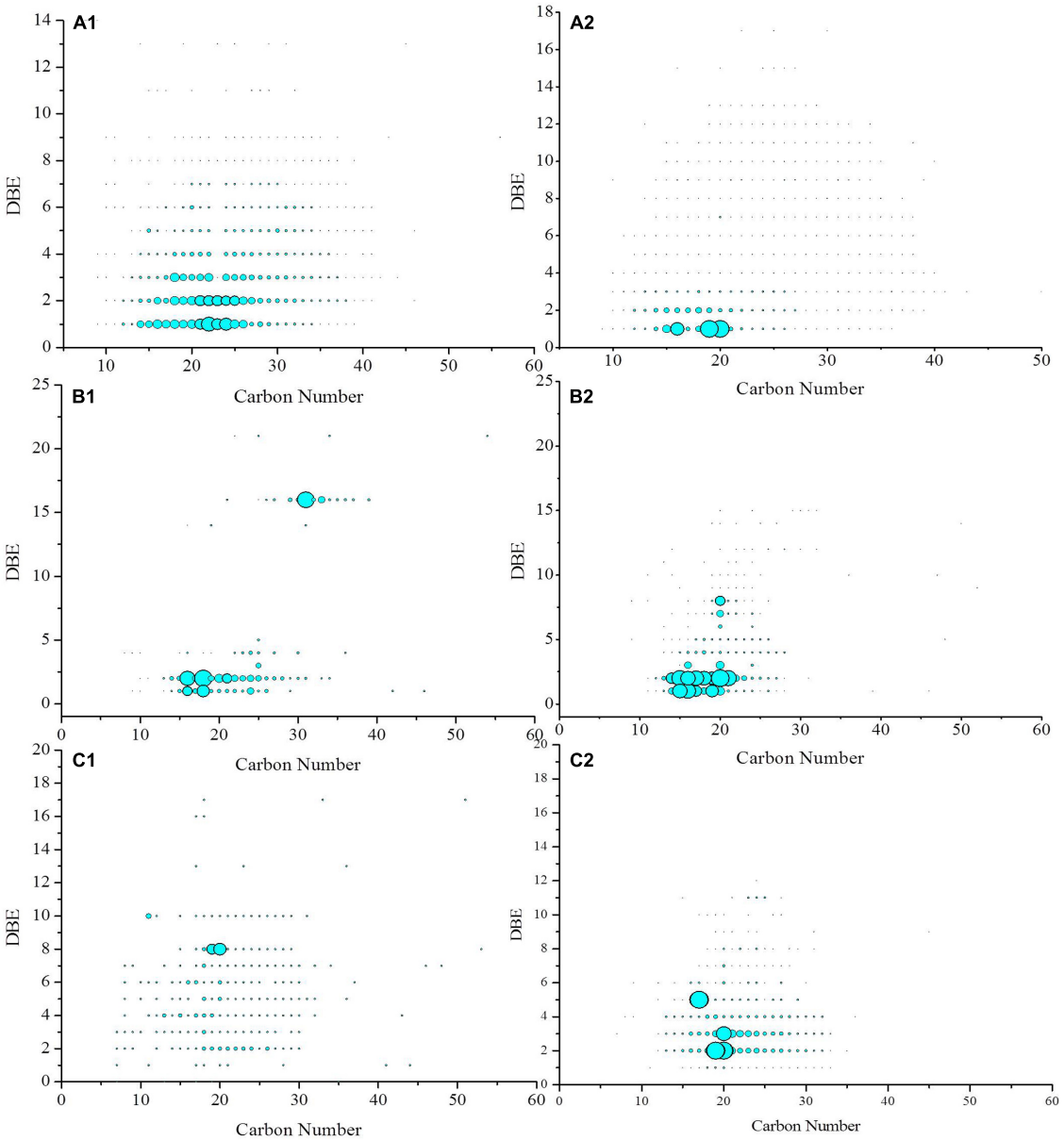

FIGURE 6 | Iso-abundance plots of DBE versus carbon number of O2 (A1,A2), O3 (B1,B2), and O4 (C1,C2) class species in crude oil before (A1,B1,C1) and after biodegradation (A2,B2,C2). The size of circles corresponds to the relative abundance of O2, O3, and O4 species in the spectra.

this study show that both the BD strain and its metabolites are effective in increasing oil recovery.

Rabiei et al. (2013) compared the results of oil recovery from *in situ* and *ex situ* tests and showed that in the quick-flooding tests, the incremental oil recovery in the *ex situ* process was higher than in the *in situ* process due to the partially purified biosurfactant that was used in the *ex situ* test. The results of this work are consistent with those of Rabiei et al. (2013). Moreover, by means of IFT and wettability measurements, Rabiei et al. (2013) concluded that IFT reduction was predominant in the quick-flooding test, whereas in the shut-in test, both IFT reduction and wettability contributed to improved oil recovery.

In this work, many photos were gathered to directly assess the MEOR mechanisms in the micromodel system. **Figure 7** compares the fluid distribution in the glass micromodels after flooding with bacterial and biosurfactant solutions for different stages. The microphotogram on the left hand side of model A shows that after water flooding, the interface between water and the residual oil was rigid, which in turn made the residual oil difficult to move further. Oil emulsification and deformation were commonly observed during both bacterial and biosurfactant flooding. The biosurfactants produced by the bacteria decreased the IFT between petroleum and formation water. The lowered IFT resulted in emulsification and deformation of the oil confined

TABLE 2 | Oil recovery of different injection methods in the micromodel.

Test project	Flooding rate	Oil displacement efficiency (%)		
		First water flooding	Second water flooding	MEOR
Model A with biosurfactant flooding	8 μL/min	49.7	63.1	13.4
Model B with bacteria flooding	8 μL/min	54.6	64.2	9.6

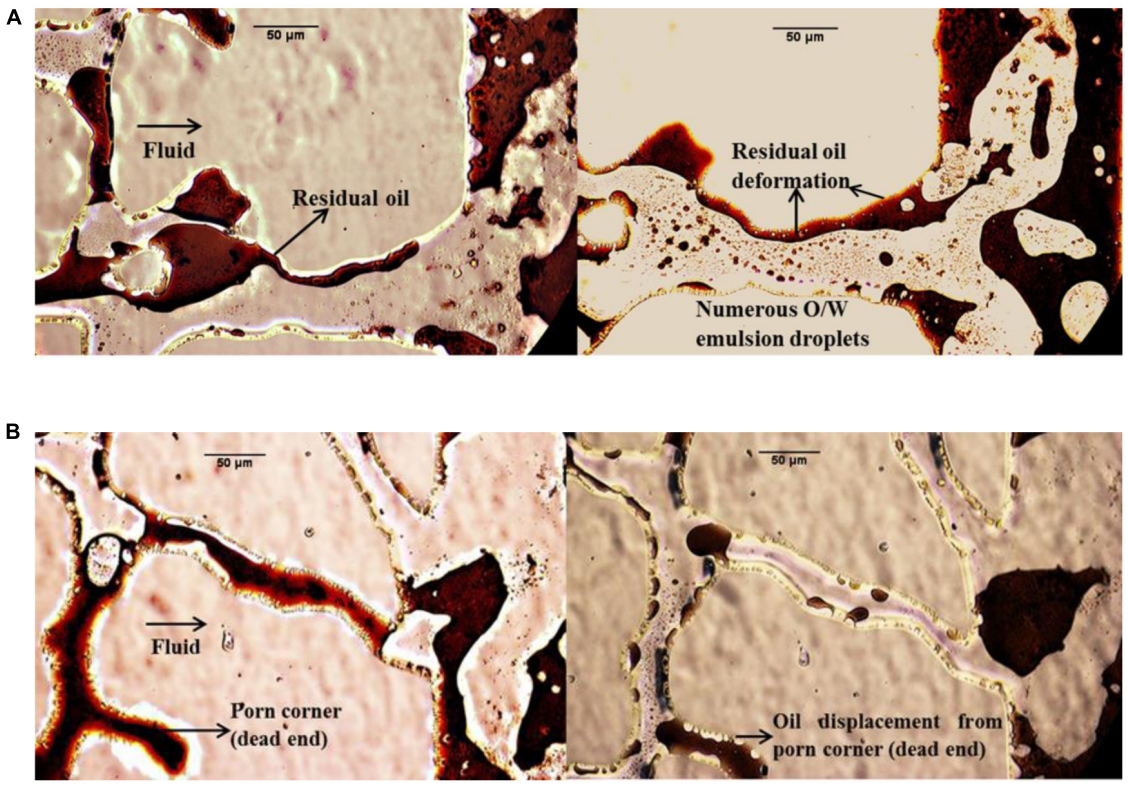

FIGURE 7 | Microphotograms showing the fluid distribution after treatment with supernatant (cell free) solution in micromodel (A) and bacterial solution in micromodel (B).

in the pores, and the oil became significantly easier to transport. This means that IFT reduction and resultant oil emulsification was the main mechanism by which oil recovery was enhanced by bacterial and biosurfactant flooding.

Wettability reversal either from oil-wet to water-wet state or from water-wet to oil-wet state increases oil recovery (Mehranfar and Ghazanfari, 2014). Sarafzadeh et al. (2013) reported that bacterial adhesion and biofilm formation can convert wettability from the neutral- or oil-wet state to the water-wet state. After a proper shut-in time period, more tertiary oil was recovered due to the wettability alteration mechanism. In this study, wettability alteration was observed after the shut-in period in bacterial flooding (**Figure 7B**). Comparing the oil area within the same dead pore in the two microphotograms of model B, it can be seen that oil displacement took place to some extent. **Figure 7B** shows that microorganisms moved into the dead pore and swept the oil, which was almost immoveable during the secondary water flooding. The wettability was changed by the attachment of the bacteria to the solid surface and the adsorption of biosurfactants and polar compounds on the solid surface.

In general, strain BD and its biosurfactants enhanced the oil recovery process after the first water flooding and intensified the second water flooding in the glass micromodels. The micromodel test showed that IFT reduction, wettability alteration, and the mobility of microorganisms were the dominant mechanisms enhancing the oil recovery.

CONCLUSION

The novel rhamnolipid-producing strain *Acinetobacter* sp. BD was isolated from the formation water of an oil reservoir. This strain showed significant rhamnolipid-producing and oil-degrading capability. The biosurfactants produced with the optimal carbon and nitrogen sources were identified through

FT-ICR-MS as different isoforms and homologs of rhamnolipids. Strain BD and its biosurfactants have great potential to enhance oil recovery, as demonstrated by flooding tests with a glass micromodel.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments, wrote the manuscript: HD. Performed the experiments: HD, HhD, PZ, KL. Analyzed the data: HD, WX. Contributed reagents and materials: ZZ. Contributed analysis tools: SS, CL. Helped perform the analysis with constructive discussions: YS, GZ. Approved the final version: ZS.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01710/full#supplementary-material

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Sophorolipids Production by *Candida bombicola* ATCC 22214 and its Potential Application in Microbial Enhanced Oil Recovery

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Biosurfactant production using *Candida bombicola* ATCC 22214, its characterization and potential applications in enhancing oil recovery were studied at laboratory scale. The seed media and the production media were standardized for optimal growth and biosurfactant production. The production media were tested with different carbon sources: glucose (2%w/v) and corn oil (10%v/v) added separately or concurrently. The samples were collected at 24 h interval up to 120 h and checked for growth (OD_{660}), and biosurfactant production [surface tension (ST) and interfacial tension (IFT)]. The medium with both glucose and corn oil gave better biosurfactant production and reduced both ST and IFT to 28.56 $\pm$ 0.42mN/m and 2.13 $\pm$ 0.09mN/m, respectively within 72 h. The produced biosurfactant was quite stable at 13–15% salinity, pH range of 2–12, and at temperature up to 100°C. It also produced stable emulsions ($\%E_{24}$) with different hydrocarbons (pentane, hexane, heptane, tridecane, tetradecane, hexadecane, 1-methylnaphthalene, 2,2,4,4,6,8-heptamethylnonane, light and heavy crude oil). The produced biosurfactant was extracted using ethyl acetate and characterized as a mixture of sophorolipids (SPLs). The potential of SPLs in enhancing oil recovery was tested using core-flooding experiments under reservoir conditions, where additional 27.27% of residual oil (S_{or}) was recovered. This confirmed the potential of SPLs for applications in microbial enhanced oil recovery.

Keywords: biosurfactant, sophorolipids, *Candida bombicola*, microbial enhanced oil recovery, core-flood experiment

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INTRODUCTION

Surfactants are amphiphilic molecules consisting of both hydrophilic and hydrophobic domains that partition preferentially at the interface between fluid phase with different degree of polarity and hydrogen bonding (such as oil and water or air and water). Surfactants intercede in nearly every product and aspect of day to day life, with a total world production exceeding 13 million tons per year (Levison, 2009). Nearly half is used in household and laundry detergents and inevitably ends up in the environment after use, which results in a negative impact on aquatic system since

traditional surfactants have low rate of biodegradation, high-ecotoxication and bio-accumulation (Mann and Boddy, 2000; Mann and Bidwell, 2001). Biosurfactants in comparison are a heterogeneous group of surface active molecules synthesized by microorganisms which reduces surface tension (ST) and interfacial tension (IFT) and plays a vital role in microbiological physiology (Al-Sulaimani et al., 2011a). Biosurfactants can be produced from renewable feedstock or even agro-industrial waste products (Joshi et al., 2008; Van Bogaert et al., 2011; Al-Bahry et al., 2013). Biosurfactants have advantages over chemical surfactants, because of their lower toxicity, higher biodegradability, better environmental compatibility and higher selectivity and specific activity at extreme temperature, pH and salinity (Marchant and Banat, 2012a). Biosurfactants have been applied in many fields including the oil industry, both for petroleum production and for incorporation into oil formulations which are considered as the largest possible market (Marchant and Banat, 2012b). Other application related to the oil industries includes oil spill bioremediation/dispersion, both inland and at sea, bioremediation of non-aqueous phase liquids (NAPL), removal/mobilization of oil sludge from storage tanks and to enhance oil recovery (Banat et al., 1991; Sen, 2008; Joshi and Desai, 2010). The second largest market for biosurfactants are emulsion polymerization for paints, paper coating and industrial coatings.

Major classes of biosurfactants are glycolipids, lipopeptides, lipoproteins, phospholipids, fatty acids, polymeric surfactants and particulate surfactants (Desai and Banat, 1997). Glycolipidic biosurfactants are extracellular surface active molecules produced by many microorganisms, e.g., sophorolipids (SPLs) produced by yeast – *Candida bombicola* (formerly called *Torulopsis bombicola*). *C. bombicola* was isolated from bumblebee honey (Kachholz and Schlingmann, 1987). SPLs are amongst one of the most promising biosurfactants. They are produced by non-pathogenic yeast strains; in contrast to rhamnolipids which represent another commercially available glycolipid surfactant that is available industrially, but produced by the opportunistic pathogenic bacteria *Pseudomonas aeruginosa* (Van Bogaert et al., 2007). Generally SPLs are produced as a mixture of slightly different molecules with variations in acetylation and lactonisation. In general, lactonic SPLs are reported to be having better ST lowering and antimicrobial activity, whereas the acidic SPLs are generally better foaming and solubility agents. Furthermore, the acetyl groups render the molecule less water soluble, but enhance their anti-viral and cytokine stimulating effects (Shah et al., 2005). Van Bogaert et al. (2007) reported the production and applications of SPLs produced by various yeast strains.

In this paper we investigated the effect of carbohydrate and oil based media on biosurfactant production by *C. bombicola* ATCC 22214. The biosurfactant was extracted and characterized using high-performance thin layer chromatography – mass spectrometry (HPTLC-MS), matrix-assisted laser desorption ionization – time of flight-mass spectroscopy (MALDI-TOF-MS) and nuclear magnetic resonance (NMR) spectroscopy (^{1}H and ^{13}C NMR). The stability of the biosurfactant under extreme conditions of pH, temperature and salinity was also investigated and finally we tested its efficacy in enhancing oil recovery using core-flood experiments.

MATERIALS AND METHODS

All of the experimental data were expressed in terms of arithmetic averages obtained from at least three independent replicates, with standard deviation (±).

Chemical and Reagents

All chemicals and reagents used were of analytical grade, media constituents for microbial studies were of microbiology grade, and the hydrocarbons had minimum of 99% purity. All chemicals, reagents and hydrocarbons were purchased from Sigma–Aldrich Co. LLC. Corn oil was purchased from local market. Light and heavy crude oil were kindly provided by local oil company, Petroleum Development Oman (PDO).

Microorganism and its Maintenance

The yeast *C. bombicola* ATCC22214 was sub- cultured from the stock culture on freshly prepared potato dextrose agar slants (PDA), composition (g/l): potato extract, 4.0; dextrose, 20.0; agar, 15.0) and glucose yeast peptone agar slants (GYPA), composition (g/l): glucose, 100; yeast extract, 30; peptone, 50; agar, 20; and incubated at (25°C) for 48 h. The agar slants were preserved at 5°C.

Seed Culture Preparation

Two types of seed media were used for developing the seed culture: potato dextrose broth (PDB) and glucose-yeast extract-urea broth (GYUB), composition (g/l): glucose, 50; yeast extract, 5; urea, 0.5. Seed media were prepared and distributed 50 ml each in 250 ml Erlenmeyer flasks and autoclaved at 121°C and 15 psi for 15 min. Seed media flasks were inoculated with a loop full of the microorganism freshly grown on GYPA or PDA agar slant, and incubated for 48 h at 25°C; 160 rpm in a rotary incubator shaker.

Preparation of Production Media

Three different production media were tested containing (g/l): yeast extract, 10; urea, 1; and different carbon sources. The pH was adjusted to 4.0 using 1M HCl, before autoclaving. Glucose and/or corn oil was used as carbon sources, as: only glucose (2% w/v), only corn oil (10% v/v) or both glucose (2% w/v) and corn oil (10% v/v). Glucose and corn oil were autoclaved separately and added to pre-sterilized production media. The flasks were inoculated with 2% (v/v) pre-grown seed media (GYUB) and incubated in a rotary shaker at 25°C, 160 rpm for 120 h. Samples were withdrawn every 24 h for different analysis: growth (OD_{660}), pH, ST, and IFT.

Surface Tension and Interfacial Tension Measurements

A pendent drop tensiometer (DSA100, KRUSS, Germany) was used to measure ST and IFT. The IFT was measured against

hexadecane as the embedding phase as reported by Al-Sulaimani et al. (2011b). All measurements were done in triplicate at ambient temperature (25 ± 2°C) and pressure (1 atm) and the average of three readings were reported for each of the three independent experiments.

Stability Studies

The stability of biosurfactant produced by *C. bombicola* was studied under various environmental parameters including wide range of temperature, pH and different salt (NaCl) concentrations. The study was carried out by changing the levels of one parameter while keeping other parameters constant using cell-free biosurfactant broth centrifuged at 10,000 × *g* for 15 min. ST and IFT were measured after each treatment to test their effect on biosurfactant activity.

Temperature Stability Tests

For the temperature stability test, 25 ml of the cell-free broth were added in 50 ml glass tubes and tightly closed with stopper to prevent evaporation. The tubes were incubated at different temperatures (40, 60, 80, and 100°C) each for 30 min, cooled down to room temperature and the biosurfactant activity was measured.

Salinity Stability Tests

Different concentrations of salt (NaCl) were added (1, 3, 5, 7, 10, 13, 15, 20, and 25% w/v) into 25 ml cell-free broth, dissolved completely and the effect on biosurfactant activity was measured.

pH Stability Tests

For the pH stability study, 25 ml of cell-free broth was adjusted using either 1N HCl or 1N NaOH to desired values (2, 4, 6, 8, 10, and 12) and the effect on biosurfactant activity was measured.

Emulsification Index (%E_{24})

The emulsification index (%E_{24}) was analyzed using previously reported procedures (Al-Wahaibi et al., 2014; Ismail and Dadrasnia, 2015): two ml of the cell-free broth was added to an equal amount of different hydrocarbons (*n*-hexadecane, heptane, hexane, *n*-tetradecane, 1-methylnaphthalene, *n*-pentane, *n*-tridecane, 2,2,4,4,6,8-heptamethylnonane, heavy crude oil and light crude oil). The solution was mixed by vortexing for 2–3 min at high speed and was left to stand for 24 h. After 24 h the height of the emulsion was measured and the emulsification index was given as the percentage of the height of the emulsified layer divided by the total height of the liquid column multiplied by 100.

Extraction of Biosurfactant

Biosurfactant was extracted and partially purified by solvent-extraction. The cells were removed by centrifugation (10,000 × *g*, 15 min at 20°C) to obtain cell-free broth. The cell-free broth (1L) was extracted twice with an equal volume of ethyl acetate, shaken vigorously in a separation funnel. The bottom aqueous layer and the top ethyl acetate layers were collected separately. The aqueous portion was re-extracted further twice with ethyl acetate. Ethyl acetate extracts were combined and the solvent was evaporated under vacuum using Rotavapor (Buchi, Switzerland) to give crude biosurfactant along with residual oil. The residual oil was removed by washing thrice with *n*-hexane. Crude biosurfactant was recovered by evaporating the *n*-hexane (Wadekar et al., 2012).

Biosurfactant Characterization

High-Performance Thin Layer Chromatography-Mass Spectroscopy

Biosurfactant was separated and analyzed using an automated HPTLC system (CAMAG, Switzerland) in the Central Analytical and Applied Research Unit (CAARU), Sultan Qaboos University, Oman. Twenty five microliter samples were spotted onto a 10 cm × 10 cm pre-coated silica gel HPTLC plate (Merck, Germany) containing green fluorescent F_{254}. These samples were spotted under a flow of nitrogen gas using automatic TLC sampler 4 (ATS 4) spotting device (CAMAG, Switzerland). The plates were developed using an automatic developing chamber ADC 2 (CAMAG, Switzerland) with remote operation from winCATS software, containing solvent systems – MP1: Chloroform: Methanol: Acetic acid (95:5:5) and MP2: Chloroform: Methanol: Water (65:15:2). The documentation and evaluation of the TLC plate was done using TLC visualizer (CAMAG) under direct UV 254 and UV 366 nm light, capturing the images. The separated bands were extracted and eluted by TLC-MS interface (CAMAG, Switzerland), based on the coordinates determined by the TLC scanner 4. The TLC-MS interface head (oval, 4 × 2 mm) was connected to the pump (11 PLUS, HARVARD APPARATUS, Holliston, MA, USA) and the extraction was performed at a flow rate of 10 µl/min, with methanol: acetonitrile (50% diluted with water) – 1:10. The interface outlet was directly connected with the ESI – MS (Qaattro UltimaTM Pt, Micromass®, UK), using Mass Lynx V4.0 software. The experimental conditions were: capillary voltage, 3.0 kV; cone voltage, 35 V; lens voltage, 0.0 V; source block temperature, 100°C; desolvation temperature, 120°C; analyzed under both positive and negative modes.

Matrix Assisted Laser Desorption Ionization –Time of Flight

All MALDI-TOF experiments were performed at CAARU, Sultan Qaboos University, on UltraFlextreme (Bruker Daltonics, Bremen, Germany) operating in positive reflectron mode in the m/z range of 50–2000 Da. Stainless steel MTP 384 target plate was used for all the molecular weight analysis. Dihydroxy benzoic acid (DHB) dried droplet protocol described in Bruker manual was employed for sample preparation and spotting. Two micro liter of 2, 5-DHB matrix (20 mg/ml) in TA 30 (30:70 v/v ACN:TFA 0.1%TFA) was premixed with 2 µl of the sample solution. One micro liter of the mixture was applied to the ground steel target plate, dried at room temperature. The spectra were acquired using FlexControl software v3.3 (Bruker Daltonics, Bremen, Germany). A SmartBeam-II laser, set at a frequency of 1000 Hz, was used for ionization. The laser strength was optimized at 25–35%. A summed spectrum was obtained for each MALDI-spot. Peaks were detected using

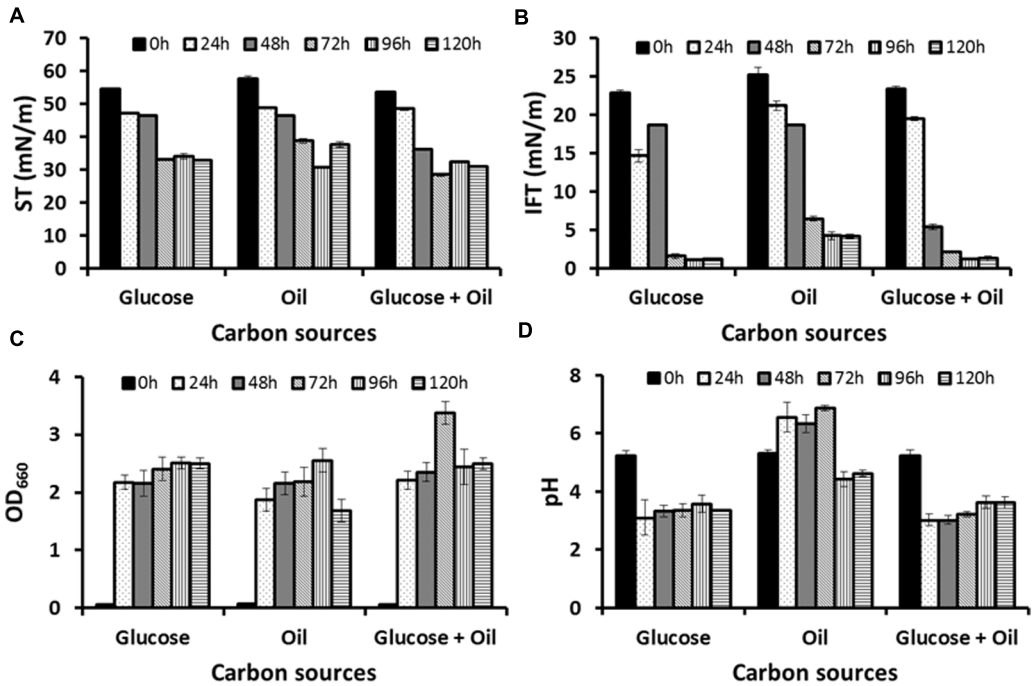

FIGURE 1 | Production profile for *Candida bombicola* ATCC 22214, when grown in medium with glucose, corn oil or both glucose and corn oil as a carbon source: (A) surface tension (ST), (B) interfacial tension (IFT), (C) Growth – OD_{660}, and (D) pH profile.

the SNAP peak detection algorithm and a baseline subtraction was carried out using "TopHat" algorithm. The MALDI-TOF spectra were externally calibrated using a commercially available peptide mix (peptide calibration standard II, Part-No #222570, Bruker Daltonics, Germany). FlexAnalysis Software v3.3 (Bruker Daltonics) was used for visualization and initial data processing.

Nuclear Magnetic Resonance spectroscopy

The NMR experiments were carried out in Bruker Avance III HD 700 MHz spectrometer equipped with 5 mm TCI H/C/N cryoprobe. The proton (^{1}H) NMR experiment was run using zg30 pulse program operating at 176.08 MHz. Acquisition parameters were as follows: 90° proton pulse width of 8.00 μs, relaxation delay of 1 s, 16 scans. The proton decoupled ^{13}C NMR experiments were carried out using composite pulse decoupling scheme operating at 176.08 MHz. Acquisition parameters were as follows: 90° proton pulse width of 8.00 μs, relaxation delay of 2 s, 512 scans. The Spectra were recorded in $CDCl_3$ at 298K and processed using TOPSPIN 3.2 software.

Core-flooding Experiments

Berea sandstone cores (1.5 inch diameter × 3 inch long) were used for core-flooding experiments. The average porosity and permeability of Berea core-plugs were 20% and 250–350 mD respectively. The formation water and crude oil used in these experiments were obtained from an Omani oil field of interest which has an average reservoir temperature of 60°C. The salinity of the formation water was between 7 and 9% its chemical composition was (kg/m^3): Sodium, 25.083; Calcium, 3.762; Magnesium, 0.878; Iron, 0.045; Chloride, 47.722; Sulfate, 0.247; Bicarbonate, 0.079. Formation water was filtered prior to use, by Millipore Membrane Filtration Unit (0.45 μm). The crude oil used for core-flood experiments was of API 36.51°. For all core-flooding experiments, cleaned Berea cores were saturated with filtered formation brine using vacuum desiccators for 24 h and pore volume (PV) was determined using the dry and wet weights of the cores. The cores were then flooded with crude oil at 24 cm^3/h until no more water was produced. The oil initially in place (OIIP) was determined, which was indicated by the volume of water displaced. The cores were subjected to water-flooding at 24 cm^3/h until no further oil was produced. The residual oil was calculated by measuring the amount of oil produced from the water-flood. Then, 5 PV of the cell-free supernatant (biosurfactant broth) was injected as a tertiary recovery stage and extra oil recovery was determined (Al-Sulaimani et al., 2011b). All core-flood experiments were carried out at 60°C to mimic the average reservoir temperature of the field of interest.

RESULTS AND DISCUSSION

Biosurfactant Production Studies

Two different media GYUB and PDB were tested as seed media of which GYUB supported better growth (GYUB: OD_{660} = 2.441 ±0.02, and PDB: OD_{660} = 2.085 ±0.03) and hence selected as a seed medium for inoculum preparation.

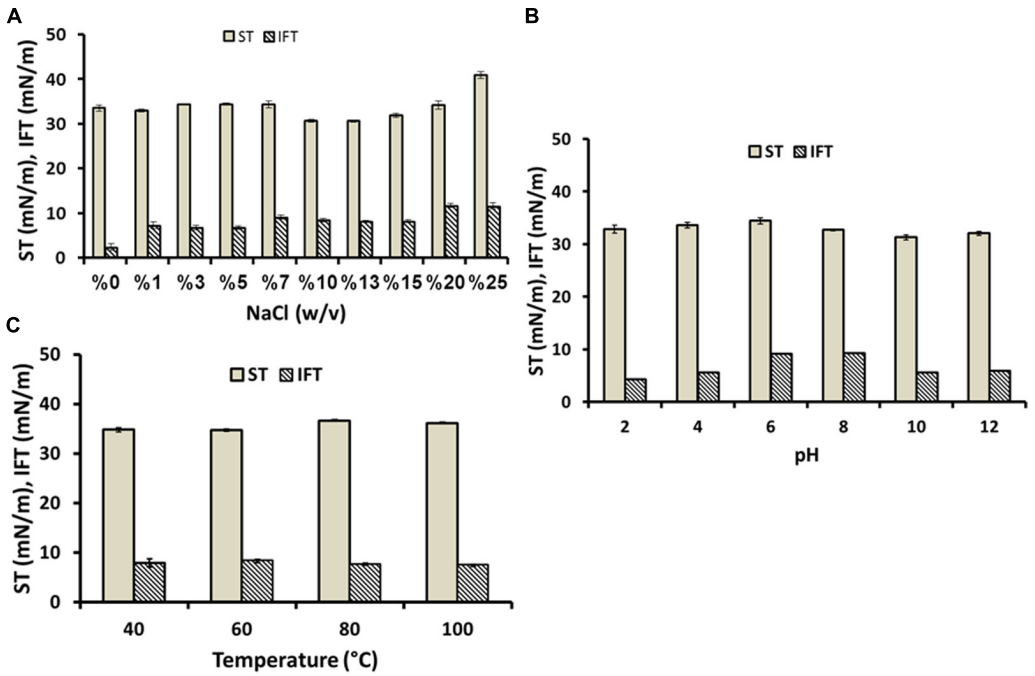

FIGURE 2 | Effect of salinity (A), pH (B), and temperatures (C) on the activity of biosurfactant produced by *C. bombicola* ATCC 22214.

To study the biosurfactant production, two different carbon sources (glucose and corn oil) were tested either alone or as a mixture of both. Growth (OD_{660}) and biosurfactant production (ST and IFT) were studied from all three different production media up to 120 h. In glucose-based production medium both ST and IFT decreased after 72 h, and reached to 33.08 ±0.05 mN/m and 1.63 ±0.29 mN/m from 54.66 ±0.26 mN/m and 22.84 ±0.36 mN/m respectively, and remained almost stable up to 120 h (**Figures 1A,B**). Maximum growth was observed at 96 h (OD_{660} = 2.508) and didn't increase further till 120 h (**Figure 1C**). However no change in pH was observed until 120 h (**Figure 1D**). In corn oil-based production medium maximum reduction in ST and IFT was observed after 96 h, and reached to 30.80 ±0.19 mN/m and 4.26 ±0.54 mN/m from 57.81 ±0.79 mN/m and 25.26 ±0.94 mN/m respectively, and remained almost stable up to 120 h (**Figures 1A,B**). Maximum growth was observed at 96 h (OD_{660} = 2.556) and decreased thereafter (**Figure 1C**). The pH decreased after 72 h and the medium became acidic (**Figure 1D**). In production medium containing 'both glucose and corn oil' as carbon sources, maximum reductions in ST and IFT were observed at 48 h and after 72 h reached to 28.56 ± 0.42 mN/m and 2.13 ± 0.09 mN/m from 53.69 ± 0.36 mN/m and 23.37 ± 0.41 mN/m respectively. The IFT was further reduced to 1.23 ± 0.02 mN/m after 96 h (**Figures 1A,B**). Maximum growth was observed at 72 h (OD_{660} = 3.372) and decreased thereafter (**Figure 1C**). The pH remained acidic throughout the experiments (**Figure 1D**).

In the production media containing both hydrophilic and lipophilic carbon sources (glucose and corn oil), reduction in ST and IFT were observed earlier as compared to production media containing either glucose or corn oil alone. At 48 h ST and IFT were reduced to 36.25 ±0.43 and 5.43 ±0.36 mN/m, respectively in medium containing 'both glucose and corn oil.' Whereas, in medium containing 'either glucose' or 'only corn oil,' STs and IFTs were reduced to ∼46.51 ±0.33 and ∼18.66 ±0.15 mN/m, respectively. Other researchers also reported that the highest production of SPLs was achieved when glucose was combined with a lipophilic carbon source (Zhou et al., 1992; Davila et al., 1994; Rau et al., 1996; Pekin et al., 2005). SPLs fermentation is a two-step process in which production occurs after growth when nitrogen source has been utilized (Cooper and Paddock, 1984; Davila et al., 1992). SPLs production has been described for cultures with only one carbon source, such as glucose (Hommel et al., 1994) or *n*-alkanes (Davila et al., 1994). However, production was significantly higher when two carbon sources (a carbohydrate based and lipidic one) were simultaneously provided. The various reported carbohydrate based substrates were glucose, sucrose (Klekner et al., 1991), lactose (Zhou and Kosaric, 1993), fructose or mannose (Göbbert et al., 1984). The reported lipidic substrates were *n*-alkanes (Davila et al., 1994), vegetable oils or waste frying oil (Zhou et al., 1992; Shah et al., 2007) or animal fats (Deshpande and Daniels, 1995). Continuous feeding of the lipid substrate, such as fatty acids (Rau et al., 1996) or fatty acid methyl or ethyl esters (Davila et al., 1992), has been shown to improve fermentation performances. The medium should also contain a source of nitrogen such as yeast extracts or corn steep liquor or additional nitrogen sources such as urea or ammonia, citrate buffering compounds and small amount of minerals such as Mg^{2+}, Fe^{3+}, Ca^{2+}, Zn^{2+}, and Na^{+} (Davila et al.,

1992, 1997). We also observed quicker biosurfactant production in medium containing yeast extract and urea with both glucose and corn oil and therefore it was used for further studies.

Stability Studies

Temperature, pH and salinity are known to be one of the most important environmental factors influencing the performance of any component (including biosurfactants) to be used for enhanced oil recovery (EOR) purpose. For *ex situ* microbial enhanced oil recovery (MEOR) applications, biosurfactant must be stable at range of high temperature (≥50°C), effective at wide range of pH and salt concentration between 7 and 9% to ensure its applicability to induce oil recovery (Al-Sulaimani et al., 2011b). As shown in **Figure 2A**, the biosurfactant showed no changes in ST till 10–13% salt concentration, and at 15–25% concentration it increased from 31.93 to 40.90 mN/m. Whereas IFT increased to 7.28 mN/m after adding 1% salt and thereafter it remained stable till 15% salt concentration. Which showed clearly that even at high salt concentrations, biosurfactant can perform well and still retain some of its surface activity. As shown in **Figure 2B**, ST was quite stable at all pH range with values ranging between 31.0 and 34.0 mN/m. Whereas IFT was stable between pH < 6 and > 8, with corresponding value of 4.31–5.98 mN/m respectively, and at pH values between 6 and 8 it showed an increase in IFT value (**Figure 2B**). Thermal stability profile (**Figure 2C**) reveals that at different temperatures (40–100°C) the biosurfactant showed stability at all temperatures tested. These results indicated that the produced biosurfactant is suitable for MEOR applications as it was stable under extreme condition such as salinity, pH and temperature. This study showed that biosurfactants was quite stable at most salt concentration tested (13–15%), over a wide range of pH values tested (2–12), and at different temperatures (40–100°C). It is comparable with other types of biosurfactants reported by several researchers (Joshi et al., 2008, 2015; Ghojavand et al., 2008; Gudiña et al., 2010; Al-Sulaimani et al., 2011b).

Emulsification Index (%E_{24})

Emulsification is one of the features of biosurfactant which aids in enhancing the trapped oil from the oil wells. As shown in **Figure 3**, biosurfactant emulsified a variety of hydrocarbons. It gave high %E_{24} with heavy crude oil (68.75%), and with *n*-hexadecane, light crude oil, *n*-tetradecane, *n*-pentane, *n*-tridecane and 2,2,4,4,6,8-heptamethylnonane also formed stable emulsions, with %E_{24} between 34 and 35%. Whereas it showed lower %E_{24} (23.86–29.55%) against hexane, 1-methylnaphthalene and heptane. Higher %E_{24} with 'heavy-crude oil' can potentially help in heavy-oil recovery. Al-Wahaibi et al. (2014) reported lipopeptide type of biosurfactant produced by isolate *Bacillus subtilis* B30 in minimal medium or molasses medium, which showed around 15–55% emulsification of various hydrocarbons including light or heavy oils. Ismail and Dadrasnia (2015) also reported %E_{24} of novel *Bacillus* strain 139SI as 69% with crude oil, thus aiding in bioremediation of oil polluted water. We also observed similar results with biosurfactant produced by *C. bombicola*.

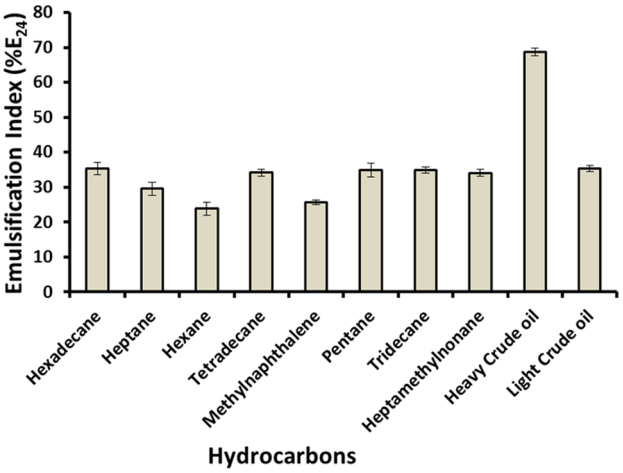

FIGURE 3 | Emulsification index of different hydrocarbons with biosurfactant produced by *C. bombicola* ATCC 22214.

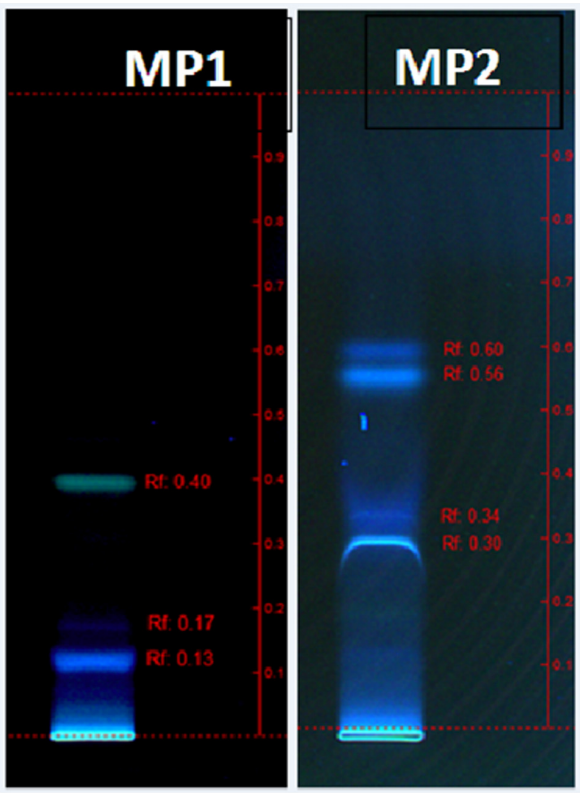

FIGURE 4 | Separation of biosurfactant by HPTLC in two different mobile phases MP1 and MP2.

Extraction and Characterization of Biosurfactant

The biosurfactant was extracted and purified by solvent extraction with ethyl acetate followed by washing with hexane

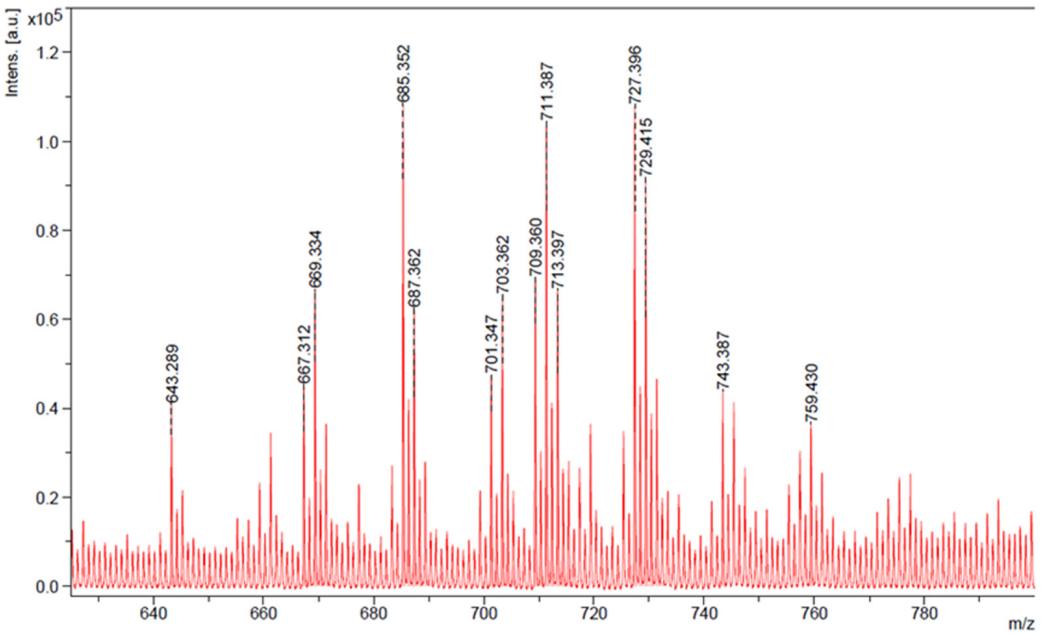

FIGURE 5 | Matrix-assisted laser desorption ionization – time of flight-mass spectroscopy (MALDI-TOF-MS) mass spectrum of produced biosurfactant.

which resulted in 2.42 g/l of crude product as yellowish brown powder (Supplementary Figure S1), after evaporation of hexane. In order to confirm the activity of the crude biosurfactant, ST and IFT were analyzed. The ST and IFT were found to be 30.32 ± 0.31 mN/m and 2.66 ± 0.38 mN/m respectively, which were quite similar to the values of ST and IFT of biosurfactant in broth, before extraction.

The characterization of the surface active components from the crude biosurfactant mixture is important to determine its molecular structure since their structural features may provide very useful information for future research purposes. The biosurfactant obtained using glucose-corn oil based production medium was identified and characterized by different analytical techniques. TLC or HPTLC has been reported as a useful technique for initial qualitative or quantitative analysis of biological active compounds (Al-Wahaibi et al., 2014). Thus we analyzed biosurfactant by HPTLC using two mobile phases, and the separated bands were directly extracted and analyzed for MS detection. Both the MP systems gave good separation of biosurfactant components and the visualization was better under UV 366 nm (**Figure 4**), and the extracted bands showed mass in the range of 300–795 (Supplementary Figure S2). The MALDI-TOF further confirmed the mass of the produced SPLs, of which major peaks were in the range of 643–759 Da (**Figure 5**). It is reported that typical structure of biosurfactant produced by *C. bombicola* – SPLs consists of a sophorose sugar β-glycosidically linked to terminal (or subterminally) hydroxylated fatty acid with chain lengths of 16–18, as a mixture of compounds. Where precursor fatty acids added to the production medium leads to formation of different forms of SPLs, which contains both acid and lactone SPLs, but lactones frequently represent the largest fraction of the product (Asmer et al., 1988; Davila et al., 1994; Joshi-Navare et al., 2013). We have used corn oil as a precursor, which is a mixture of fatty acids and thus multiple forms of SPLs with diverse hydrophobic moieties were expected. This was confirmed with the MALDI-TOF-MS analysis of the purified biosurfactant. The major peaks from the mass spectrum were correlated to sodium adducts $[M^+ + Na^+]$ of different forms of SPLs, as derived from oleic acid (*m/z* 669, 687, 711, 729) were detected (**Figure 5**). Where major ions at *m/z* 711 and *m/z* 729 can be attributed to the $[M^+ + Na^+]$ adduct ions for the lactone and free acid forms of the major diacetylated SPLs respectively (Asmer et al., 1988; Kurtzman et al., 2010). Other ions at *m/z* 669 and *m/z* 687 correspond to the monoacetylated forms of the major SPLs, whereas the non-acetylated forms were not observed in current biosurfactant. The observed difference between these two sets of ions (729–711 and 687–669) can be attributed to the mass difference between the free acid form and the ester-linked lactone form of SPLs respectively. Thus the mass of produced SPLs can be calculated as from ∼646 to 706. Kurtzman et al. (2010) reported production and characterization of SPLs by multiple species of the *Starmerella* (*Candida*) *bombicola* yeast clade, using high-throughput MALDI-TOF-MS analysis. They reported SPLs production using oleic acid as fatty acid precursor along with other components in the production medium, and thus observed only oleic acid containing SPLs. Whereas we have used corn oil as fatty acid precursor and observed other minor SPLs derived from other types of acids present in corn oil, as sodium adducts $[M^+ + Na^+]$ derived from palmitic (*m/z* 685, 703), linoleic (*m/z* 709, 727), and trace amounts of stearic (*m/z* 713) and arachidic (*m/z* 759) acid (**Figure 5**). The mass difference (18 Da) between the two sets of ions derived from palmitic and

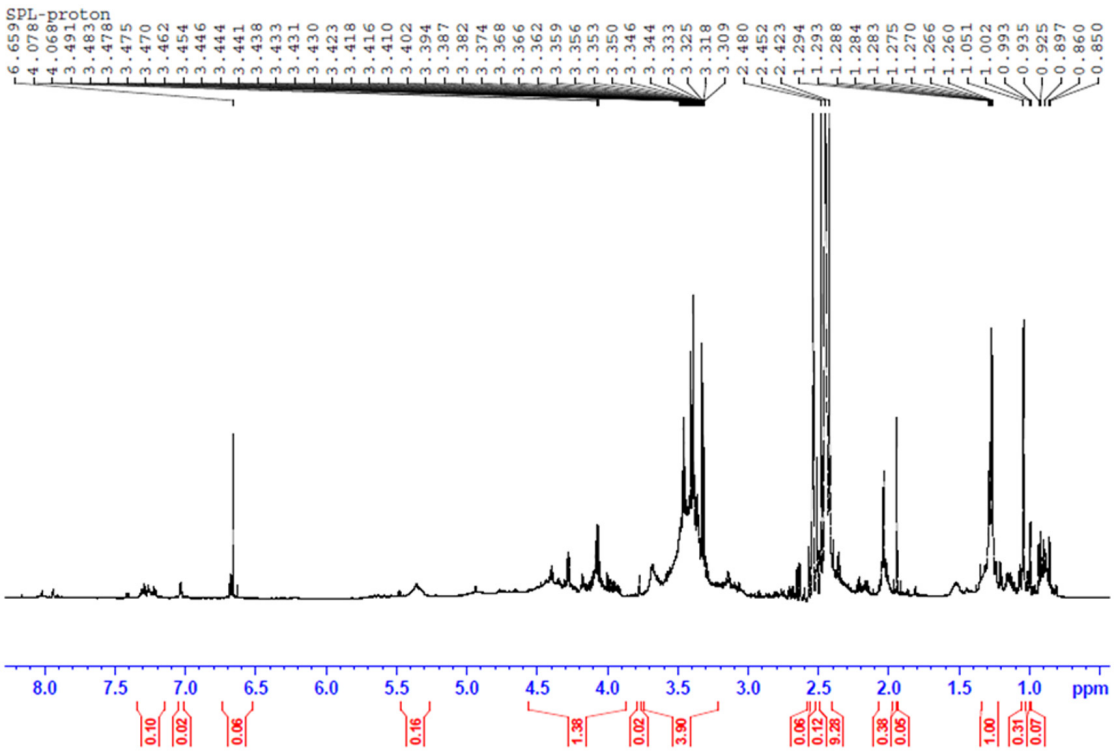

FIGURE 6 | The proton (^{1}H) NMR spectrum of produced biosurfactant.

linoleic acids are again indicative of the free acid and lactone forms of the minor SPLs respectively. Similar type of SPLs ions were identified and reported previously from *C. bombicola* and other yeast species using fast atom bombardment MS or MALDI-TOF MS (Asmer et al., 1988; Kurtzman et al., 2010; Joshi-Navare et al., 2013).

The structures of biosurfactants were further confirmed by ^{1}H, and proton decoupled ^{13}C – NMR and the results are shown in **Figures 6** and **7**. The ^{1}H NMR spectrum of the purified biosurfactant in $CDCl_3$ was assigned to a typical glycolipid-type structure and characteristic proton chemical shift peaks could be observed (**Figure 6**). Multiple signals of protons of ($-CH_3$) below 1.0 ppm and of ($-CH_2$) between 1.0 and 1.4 ppm revealed the existence of a linear alkane, and signals at 5.3–5.4 ppm revealed presence of ($-CH = CH-$) group in the fatty acid chain. The signal at 5.3–5.4 ppm is attributable to an unsaturated hydrocarbon moiety, also consistent with the oleic acid derived SPLs signatures in MALDI-TOF-MS analysis. Protons of ($-CH_2$) bonded to carboxylic group of fatty acid resonated at ∼1.99 ppm, and ∼2.09 ppm revealed the presence of ($-COCH_3$) group in biosurfactant. Resonance of protons belonging to sophorose moiety resulted in peaks within the region 4–4.5 ppm, and the other protons of sugar were resonated at 3.18–3.8 ppm.

The ^{13}C NMR spectrum of the biosurfactant showed the presence of two (=CH−) groups in the fatty acid chain moiety corresponding to signals at 130.41 ppm and 128.99 ppm; and few more (=CH−) groups in the fatty chain moiety were resonated between 128 and 130 ppm, which may be probably due to contribution from other interfering group of biosurfactant in the sample (**Figure 7**). In addition, several ($-CH_2-$) groups in the fatty chain moiety were also resonated at 20∼35 ppm. The spectrum also revealed signals of glucose- C-1″ at 101.24 ppm, glucose-C-6′, and glucose-C-6″ at 64.08, 62.29 ppm; the other carbon atoms of glucose were resonated between 70∼78 ppm. The peaks derived from the carbonyl groups (−CO−) were shown at 170∼177 ppm. The chemicals shifts were comparable to previous reports (Daverey and Pakshirajan, 2009, 2010; Wadekar et al., 2012; Joshi-Navare et al., 2013). The MALDI-TOF-MS and NMR results further confirmed the biosurfactant as a mixture of SPLs with mono and diacetylated SL as mixture of different fatty acids in acidic or lactonic form.

Core-flood Experiments

An area of considerable potential for biosurfactant application is in the field of MEOR. Since oil is a critical energy source that drives industrialization and sustained economic development of the world (Youssef et al., 2006). McInerney et al. (2005) documented that current oil production technologies recover only about one-third to one-half of the oil originally present in an oil reservoir. So, EOR methods were developed to recover oil remaining in reservoirs after primary and secondary recovery procedures. MEOR is an important tertiary recovery technology which utilizes microorganisms and/or their metabolites for residual oil recovery (Banat, 1995) which was attempted more than 60 years ago (Hitzman, 1991). We checked the potential of

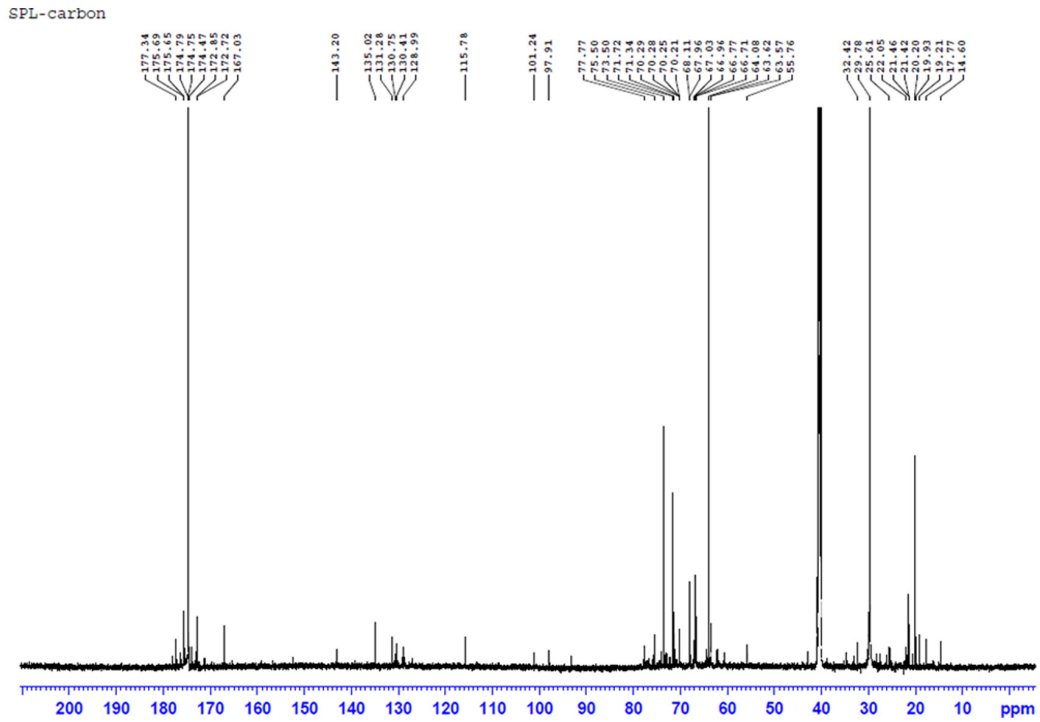

FIGURE 7 | The proton decoupled-carbon (^{13}C) NMR spectrum of produced biosurfactant.

produced SPLs in MEOR using core-flooding experiments. The PV of the core-sample was 18 cm^3. Initial oil saturation (So_i) was calculated to be 55–60% after oil flooding. It was found that after injecting 5–8 PV of formation water, no more oil was produced and residual oil saturation (So_r) was about 20%. Extra oil recovery was observed after injecting 4–5 PV SPLs, where 27.27% of So_r was produced (**Figure 8**). An increase in oil recovery in *ex situ* MEOR experiments was detected following SPLs injection. This is similar to reports shown by other researchers for different types of biosurfactants (Al-Sulaimani et al., 2011b; Castorena-Cortés et al., 2012; Joshi et al., 2015). No study was reported before to test the ability of SPLs in enhancing oil recovery using core flood experiments. To the best of our knowledge, this study was the first to explore the efficiency of SPLs to recover oil and it can be used in biosurfactant based MEOR applications. There are various experiment at laboratory scale that have been used to prove the effectiveness of using biosurfactants for microbial enhancing oil recovery, such experiments include sand-pack columns or core-flooding and field trials (Yakimov et al., 1997; Youssef et al., 2006; Joshi and Desai, 2013; Al-Wahaibi et al., 2014). This research is the first report to investigate the ability of SPLs produced by *C. bombicola* ATCC22214 to enhance oil recovery using core-flooding. Biosurfactants like SPLs are considered as green alternatives for chemical counterparts, as it can be produced from renewable sustainable resources. Production economy is still considered as bottleneck for widespread biosurfactants applications, which hinders the field-scale applications. The cost of production can be drastically reduced when agro-industrial waste products (like molasses) or used waste frying oil (as fatty acid supplement) be used as raw material for SPLs production at large scale fermentation (Makkar et al., 2011; Banat et al., 2014). Once scaled-up, these SPLs can be used for biosurfactant based MEOR or environmental bioremediation, without much purification so that the cost of application can be further reduced. Thus large scale production of SPLs using cheaper raw materials and field-scale MEOR application are further proposed.

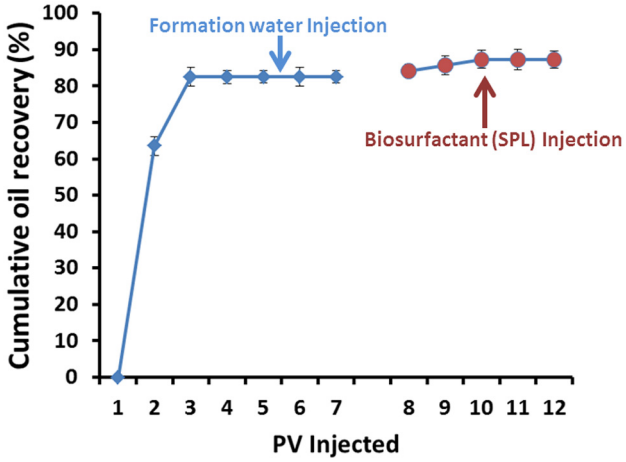

FIGURE 8 | Cumulative oil recovery as a function of pore volumes (PV) injected of formation water and biosurfactant-SPLs.

CONCLUSION

This study shows that corn oil could be used as a carbon source along with glucose to produce SPLs by the yeast *C. bombicola* ATCC22214. The SPLs showed reduction of ST and IFT; high $\%E_{24}$ against various hydrocarbons including light and heavy crude oils, and also showed high stability under extreme conditions of salinity, pH and temperature. It was characterized as a mixture of SPLs, using different analytical techniques. To the best of our knowledge this is the first report on using SPLs for testing MEOR applications. Core-flooding studies revealed that it can recover 27.27% of residual oil (S_{or}) trapped between the pores of Berea sandstone cores, which highlight the potential applications of SPLs in MEOR.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01324

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Biocatalytic Desulfurization Capabilities of a Mixed Culture during Non-Destructive Utilization of Recalcitrant Organosulfur Compounds

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We investigated the biodesulfurization potential of a mixed culture AK6 enriched from petroleum hydrocarbons-polluted soil with dibenzothiophene (DBT) as a sulfur source. In addition to DBT, AK6 utilized the following compounds as sulfur sources: 4-methyldibenzothiophene (4-MDBT), benzothiophene (BT), and 4,6- dimethyldibenzothiophene (4,6-DM-DBT). None of these compounds supported the growth of AK6 as the sole carbon and sulfur source. AK6 could not grow on dibenzylsulfide (DBS) as a sulfur source. The AK6 community structure changed according to the provided sulfur source. The major DGGE bands represented members of the genera *Sphingobacterium*, *Klebsiella*, *Pseudomonas*, *Stenotrophomonas*, *Arthrobacter, Mycobacterium,* and *Rhodococcus*. *Sphingobacterium* sp. and *Pseudomonas* sp. were abundant across all cultures utilizing any of the tested thiophenic S-compounds. *Mycobacterium/Rhodococcus* spp. were restricted to the 4-MDBT culture. The 4-MDBT culture had the highest species richness and diversity. Biodesulfurization of DBT by resting cells of AK6 produced 2-hydroxybiphenyl (2-HBP) in addition to trace amounts of phenylacetate. AK6 transformed DBT to 2-hydroxybiphenyl with a specific activity of 9 ± 0.6 μM 2-HBP g dry cell weight^{-1} h^{-1}. PCR confirmed the presence in the AK6 community of the sulfur-specific (4S) pathway genes *dszB* and *dszC*. Mixed cultures hold a better potential than axenic ones for the development of a biodesulfurization technology.

Keywords: mixed cultures, biodesulfurization, dibenzothiophene, *stenotrophomonas,* 4S pathway

INTRODUCTION

The increasing global human population accompanied by extensive fossil energy consumption has posed serious threats to the environment and human health (Maass et al., 2015). Organosulfur (thiophenes) compounds found in crude oil and diesel are of particular concern due to their hazardous impact on human health and the ecosystem (Kilbane, 2006; Morales et al., 2010). Moreover, the sulfur oxide gases resulting from fuel combustion are a major cause of acid rain.

Governments and environmental organizations worldwide have recognized the problem and implemented strict regulations and legislations that limit the amount of sulfur in transportation fuels.

Hydrodesulfurization (HDS) is commonly applied by oil refineries to reduce sulfur content in refined products (Konishi et al., 2000). Nonetheless, HDS has many disadvantages. It is cost-intensive, environmentally polluting, and not sufficiently efficient. Consequently, there has been an increasing interest in the development of alternative desulfurization technologies that circumvent the drawbacks associated with the conventional HDS (Ohshiro and Izumi, 1999). The petroleum refineries are facing the problem that crude oil feeds are becoming heavier with high sulfur content, which means high sulfur levels in both straight-run and secondary processed diesel oil (Bhatia and Sharma, 2006). This will make the HDS process more economically and technically challenging, particularly with the stringent environmental legislations that tend to limit sulfur content of transportation fuels to less than 10 ppm (Monot and Warzywoda, 2008).

Non-destructive (sulfur-specific) microbial desulfurization or biodesulfurization (BDS) has been proposed as an alternative or complementary technology. BDS exploits the ability of dedicated microorganisms to remove sulfur from many organosulfur compounds that are commonly found in crude oil and diesel. As compared to physicochemical techniques like HDS, biocatalytic processes are environmentally friendly, cost-effective, specific, and more efficient (Kilbane, 2006). During the past two decades, many microorganisms have been isolated and characterized based on their unique ability to specifically remove the sulfur atom from organosulfur substrates without breaking the carbon skeleton. This desulfurization mechanism preserves the calorific value of the treated fuel.

The most common biodesulfurization pathway reported to date is the 4S pathway discovered initially in *Rhodococcus erythropolis* IGTS8 (Gallagher et al., 1993; **Figure 1**). The 4S pathway is well-characterized at the biochemical and molecular levels. It proceeds via two cytoplasmic monooxygenases (DszC, DszA) supported by a flavin reductase (DszD) and a desulfinase (DszB). DBT monooxygenase (DszC) catalyzes the sequential conversion of DBT to DBT sulfoxide (DBTO) and DBT-sulfone ($DBTO_2$). $DBTO_2$ monooxygenase (DszA) catalyzes the oxidative C-S bond cleavage producing 2-(2′-hydroxybiphenyl) benzene sulfinate (HBPS). DszB, an aromatic sulfinic acid hydrolase, affects a nucleophilic attack of a base-activated water molecule on the sulfinate sulfur to produce 2-hydroxybiphenyl (2-HBP) as a dead-end product and sulfite as a bioavailable sulfur for microbial growth. DszD delivers the reducing equivalents ($FMNH_2$) needed for the functionality of DszC and DszA. The oxygen atom incorporated at each step of the pathway is derived from atmospheric oxygen.

The genes involved in DBT desulfurization (*dszA, dszB, dszC*) are organized as one operon (*dsz* operon) and transcribed in the same direction under the control of a single promoter. The three genes are clustered on a 120-kb linear plasmid of the *R. erythropolis* IGTS8 strain. A fourth gene, *dszD*, that encodes a flavine reductase is located on the chromosome.

Despite the progress that has been achieved during the last two decades, biodesulfurization has not been applied on a commercial scale yet. This is attributed to many issues related to the stability and catalytic efficiency of the microbial biocatalyst in addition to other technical problems (Monot and Warzywoda, 2008). The majority of the research conducted on microbial desulfurization has adopted axenic cultures of selected microorganisms. However, it is worth investigating biodesulfurization capabilities of microbial consortia to benefit from the cooperative or synergistic microbe-microbe interactions (McGenity et al., 2012; Mikesková et al., 2012). Recently, engineered synthetic bacterial consortia have shown enhanced desulfurization and revalorization of oil sulfur compounds (Martínez et al., 2016).

The aim of this study was to enrich a mixed culture from soil polluted with petroleum hydrocarbons and to study its biocatalytic desulfurization potential using various organosulfur compounds as a sulfur source. Furthermore, we investigated the dynamics of the microbial consortium when challenged with different sulfur sources.

MATERIALS AND METHODS

Chemicals

Organosulfur compounds were purchased from Fluka (Switzerland), Acros (USA), and Sigma-Aldrich (USA). Other chemicals and culture media were from Difco (France), Fluka (Switzerland), Sigma (USA), Qiagen (Germany), and Promega (USA). Deionized water was used to prepare all media and solutions.

Soil Samples and Bacteria

Soil samples contaminated with used motor lubricating oil, diesel, benzene, and grease were collected (top surface layer, 10 cm in depth) from the neighborhood of mechanic workshops in Fahaheel district, Kuwait. The AK6 bacterial

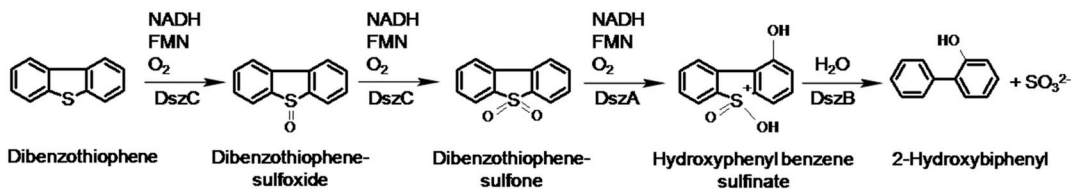

FIGURE 1 | The 4S pathway of non-destructive biodesulfurization of dibenzothiophene (Gallagher et al., 1993).

consortium was enriched from the contaminated soil. A reference biodesulfurization strain, *R. erythropolis* IGTS8, was obtained from The American Type Culture Collection (ATTC 53968, USA).

Culture Media and Growth Conditions

Commercially available Lauria-Bertani (LB) agar and broth media were prepared according to the instructions of the supplier. Sulfur-free chemically defined medium (CDM) had the following composition (per litter): KH_2PO_4 1.08 g; K_2HPO_4, 5.6 g; NH_4Cl, 0.54 g; $MgCl_2.6H_2O$, 0.2 g; $CaCl_2.2H_2O$, 0.044 g; $FeCl_2.4H_2O$, 1.5 mg, vitamins (cyanocobalamin 0.2 mg, pyridoxamine-HCl 0.6 mg, thiamin-HCl 0.4 mg, nicotinic acid 0.4 mg, p-aminobenzoate 0.32 mg, biotin 0.04 mg, Ca-pantothenate 0.4 mg), and trace elements ($ZnCL_2.7H_2O$ 70 μg, $MnCl_2.4H_2O$ 100 μg, $CuCl_2$ 20 μg, $CoCl_2.6H_2O$ 200 μg, $Na_2MoO_4.2H_2O$ 40 μg, $NiCl_2.6H_2O$ 20 μg, H_3BO_3 20 μg). Routinely, the carbon source was glucose (10 mM) and the sulfur source was either $MgSO_4.7H_2O$ (1 mM) or an organosulfur compound (0.1 mM). The tested organosulfur substrates were dibenzothiophene (DBT), benzothiophene (BT), 4-methyldibenzothiophene (4-MDBT), 4,6-dimethyldibenzothiophene (4,6-DM-DBT), and dibenzylsulfide (DBS). All organosulfur compounds were added to the CDM from 100 mM ethanol stocks except 4,6-DM-DBT which was prepared in acetone. The final concentration of either ethanol or acetone in the culture media was 0.1% (vol/vol). $MgSO_4$ was replaced by $MgCl_2.6H_2O$ when organosulfur compounds were used either as the sole sulfur source or as the sole sulfur and carbon source (in this case glucose was omitted). All liquid cultures were incubated in an orbital shaker (180 rpm) at 30°C. All cultures on solid media were incubated at 30°C for 48 h. Liquid cultures were routinely grown in duplicate in 250-mL Erlenmeyer flasks containing 100 mL of the growth medium. The uninoculated medium was routinely included as a negative control.

Enrichment of the AK6 Mixed Culture

Soil samples (2 g) were inoculated into 100 mL of sterilized CDM supplemented with 0.1 mM DBT as a sulfur source and 10 mM of glucose as a carbon source. The enrichment flasks were incubated on a rotary shaker for 4–7 days until turbidity appeared. Subsequently, 1 mL from those original enrichments was transferred to a fresh medium with the same composition and further incubated under the same conditions for the same time. This sub-culturing was repeated 4 times. To check whether AK6 is a pure or mixed culture, samples from enrichment cultures were serially diluted in sterile saline solution (0.9% NaCl) and aliquots from those culture dilutions (100 μL) were spread over LB-agar plates and incubated for 72 h.

Growth of AK6 on Different Sulfur Sources

The AK6 mixed culture was grown in CDM containing glucose as a carbon source and one of the organosulfur compounds as a sole sulfur source. Another set of cultures was prepared in which the organosulfur compounds served as carbon and sulfur source (no glucose was added). Inocula were prepared from cultures containing the respective sulfur source. AK6 was also grown in CDM containing DBT added as solid without solvent (ethanol) and glucose. To test the ability of AK6 to utilize ethanol as a carbons source, it was cultured in CDM containing ethanol (0.1%) as a sole carbon source and $MgSO_4$ as a sole sulfur source. In another experiment, AK6 was grown in CDM containing 2-HBP (0.1 mM dissolved in ethanol) as a carbon source in the presence of $MgSO_4$ (1 mM) as a sulfur source. The inoculum for this culture originated from a starter culture grown in the same medium. Growth was monitored by measuring culture turbidity (Optical Density at 600 nm, OD_{600}) after time intervals until the culture entered the stationary phase. The biomass yield was measured as dry cell weight (dcw) by drying cell pellets at 105°C for 15 h.

Biodesulfurization of DBT by Cell Suspension of AK6

AK6 was grown as described earlier in 1 L Erlenmeyer flasks (duplicates) containing 400 mL of CDM-DBT-glucose and the cells were harvested at the mid-exponential phase ($OD_{600} = 0.7$) by centrifugation (10,000 rpm for 10 min). The cell pellet was washed twice with phosphate buffer (50 mL, 100 mM, pH 7) and resuspended in 25 mL of the same buffer (2.6 g dcw L^{-1}). Both cell suspensions were incubated with 1.0 mM DBT in 100 mL Erlenmeyer flasks at 30°C with shaking (180 rpm) for 4 h. A flask containing phosphate buffer and DBT only without cells was included as a negative control. Samples (1 mL) were retrieved at intervals, and the cells were removed by centrifugation (14,000 rpm for 5 min). Benzotiophene was then added as an internal standard (1 mM). The biodesulfurization intermediates were extracted twice from the cell-free supernatants with one volume of ethylacetate. The organic phase evaporated by centrifugation under vacuum and the residue was resuspended in 200 μL ethanol for HPLC analysis. The produced 2-HBP was quantified from a standard curve by estimating the peak area. The biodesulfurization activity of the AK6 cell suspension was calculated as the amount of 2-HBP produced per g dcw per hour (μM 2-HBP g dcw^{-1} h^{-1}).

Biodesulfurization Pathway Intermediates

AK6 was grown as described earlier in 2 L Erlenmeyer flasks containing 1000 mL of CDM-DBT-glucose and the cells were harvested in the mid-exponential phase by centrifugation (10,000 rpm for 10 min). The cell pellet was washed with 50 mL phosphate buffer (100 mM, pH 7) and resuspended in 25 mL of the same buffer (6.4 g dcw L^{-1}). *R. erythropolis* IGTS8 was grown in CDM-DBT-glucose and treated under the same conditions. The cells of IGTS8 were harvested in the exponential phase after 22 h of incubation, washed and resuspended in 25 mL phosphate buffer (14.8 g dcw L^{-1}). Both cell suspensions were incubated with 1.0 mM DBT in 100 mL Erlenmeyer flasks at 30°C with shaking (200 rpm) for 18 h. A flask containing phosphate buffer and DBT only without cells was included as a negative control. All assays were then centrifuged (10,000 rpm for 5 min) to remove the cells. The pH of the supernatants was adjusted to 2 with 25% HCl. Extraction of the intermediates was performed twice by adding one volume (25 mL) of ethylacetate to the

acidified supernatants in a separating funnel. After vigorous shaking for 5 min, the mixtures were kept undisturbed to allow phase separation. The aqueous phase was drained, and the organic phase from both extractions was pooled and collected in clean glass flasks. The volume of ethylacetate was reduced by centrifugation under vacuum to 0.5 mL from which samples were analyzed by HPLC and GC/MS

High-Performance Liquid Chromatography (HPLC)

HPLC was performed on Thermo-Dionex UHPLC 3000 equipped with a photodiode array (Thermo, USA), using an Acclaim^{TM} 120 C18 column (5 μm column, 120 A). Cell-free culture supernatants (500 μL) were extracted once with one volume of ethylacetate that was then evaporated and replaced by 50 μL ethanol. Also, samples from the cell suspension assays were treated in the same way. The mobile phase was an acetonitrile-water mixture (60 and 80%) pumped at a flow rate of 1 mL/min and the injected volume was 10 μL. Detection of the organosulfur compounds and 2-HBP was performed at various wavelengths (233, 235, and 248 nm). Authentic organosulfur compounds and 2-HBP were used for comparison. The concentrations of DBT and 2-HBP were estimated from the peak area using standard curves prepared for both compounds.

Gas Chromatography-Mass Spectrometry (GC-MS)

The intermediates of DBT biodesulfurization were analyzed with a Gas Chromatograph hyphenated to a mass spectrometer detector (GC-MS) operated in the scanning mode. The system was fitted with a non-polar chromatographic column (DB-1 ht, 30 m long, 0.1 mm ID, and 0.1 μm film thickness). The column was operated under a constant flow of helium as a carrier gas (1.0 mL/min). The GC oven was ramped from 70 to 350°C at 10°C/min. The samples were injected manually into the GC/MS. The collected mass spectra were matched with the NIST mass spectral database.

Detection of the Biodesulfurization Genes

Promega Wizard Genomic DNA Purification Kit was used to isolate genomic DNA from cells of the AK6 consortium harvested from CDM cultures with glucose and DBT after 96 h (OD_{600} = 1.2). Primers listed in **Table 1** were adopted for the amplification of the *dsz* genes. Genomic DNA from *R. erythropolis* IGTS8 was included as a positive control. The 20-μL PCR assays contained 1 μL (5 ng) of template DNA, 2 μL of each primer (2 pmol/μL), 10 μL Qiagen *Taq* PCR master mix, and 5 μL nuclease-free water. Initial denaturation at 95°C for 2 min was followed by 1 min at 94°C, 1.5 min at 47 or 55°C (annealing), 1 min at 72°C, and eventually a final extension step for 10 min at 72°C.

PCR-DGGE Analysis of the Community Structure

Extraction of Total Community DNA

Cell pellets of AK6 were harvested (toward the end of the exponential phase) from CDM cultures containing glucose as a carbon source and different organosulfur compounds. The cell pellets were washed once with 100 mM K-phosphate buffer (pH 7.0) and used as a source for genomic DNA. Total community DNA was extracted with the Ultra Clean Soil DNA purification kit (MoBio Laboratories, USA) according to the manufacturer's instructions. After air drying, DNA pellets were resuspended in 10 μL TE buffer and kept at −20°C until use.

Amplification of the 16S rRNA Genes by PCR

Amplification of the 16S rRNA genes for DGGE analysis was performed using GC-clamp primers (EUB341F-GC: 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGG GGGGCCTACGGGAGGCAGCAGCAG-3′ and EUB517R: 5′-ATTACCGCGGCTGCTGG-3′) which correspond to positions 341 and 517 in *Escherichia coli* genome (Muyzer et al., 1993). Amplifications were performed in 25-μL reactions containing: 2.5 μL of 10x *Taq* buffer (100 mM Tris-HCl, pH 8), 1.25 mM $MgCl_2$, 100 μM dNTPs (Invitrogen, USA), 1.2 μM forward and reverse primers (Invitrogen, USA), 0.5 U *Taq* DNA polymerase (Invitrogen, USA), and about 5 ng of template DNA. PCR was performed in Thermal Cycler (Applied Biosystem 2720, USA). A touchdown PCR program was implemented as follows: initial denaturation at 95°C for 5 min; 5 cycles of 94°C for 40 s, annealing at 65°C for 40 s, and extension at 72°C for 40 s; 5 cycles of 94°C for 40 s, annealing at 60°C for 40 s, and extension at 72°C for 40 s; 10 cycles of 94°C for 40 s, annealing at 55°C for 40 s, and extension at 72°C for 40 s; 10 cycles of 94°C for 40 s, annealing at 50°C for 40 s, and extension at 72°C for 40 s, followed by a final hold at 72°C for 7 min. Amplicons were analyzed by electrophoresis in 1% agarose gels with the size markers (1 kb DNA ladder, Invitrogen, USA) and visualized using ethidium bromide.

TABLE 1 | Primers used in this study.

Primer name	Primer sequence (5′–3′)	Target gene	Product size (bp)	References
bdsAf	tcgatcagttgtcagggg	*dszA*	547	Davoodi-Dehaghani et al. (2010)
bdsAr	ggatggaccagactgttgac			
bdsBf	atcgaactcgacgtcctcag	*dszB*	422	Davoodi-Dehaghani et al. (2010)
bdsBr	ggaacatcgacaccaggact			
dszCf	acacaccatatgacactgtcacctgaaaaggagc	*dszC*	1250	Kayser et al. (2002)
dszCr	acacacagatcttcaggaggtgaagccgggaatcggg			

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed using Dcode Mutation Detection System (Bio-Rad Laboratories Ltd., Hertfordshire, UK). PCR products were electrophoresed in 0.5 × TAE buffer (1 × TAE buffer is 0.04 M Tris base, 0.02 M sodium acetate, and 10 mM EDTA, pH 7.4) on 8% acrylamide gel containing 25–50% denaturating gradient of formamide and urea. DGGE was conducted at 60°C for 5 h at voltage of 200 V. The gel was stained with SYBR Green I Nucleic acid gel stain (Cambrex Bio Science Rockland, USA), photographed and analyzed for DGGE band profile with a UV gel documentation system (Bio-Rad Laboratories Inc., CA, USA).

Sequencing and Analysis of the DGGE Bands

Dominant DGGE bands were cut off with a sterile scalpel and eluted by incubation in 100 μL TE buffer at 100°C for 5 min. The supernatant was used as a template for PCR amplification. Reamplification of 16S rRNA genes from excised DNA fragments was performed using bacterial primers EUB314F without GC clamp and EUB517R. Amplification was verified by electrophoresis on 1% agarose gel. PCR products were purified using PCR-Clean kit (Promega, USA) according to instructions manual. PCR products were directly sequenced using a BigDye terminator cycle sequencing (Sanger et al., 1977) at GenoScreen sequencing facility (Genoscreen, Lille, France). Sequences of the 16S rRNA genes were analyzed using Blast search facility on NCBI (National Center for Biotechnology Information, National Library of Medicine, USA) database (www.ncbi.nlm.nih.gov/BLAST/). Sequences were compared with their closest matches in the GenBank with nucleotide-nucleotide BLAST to obtain the nearest phylogenetic neighbors. Numerical analysis of the DGGE fingerprints was performed using Quantity One 1D software (BioRad). The total number of DGGE bands was used to reflect the richness of AK6 operational taxonomic units (OTUs; Duarte et al., 2009). Bacterial diversity was estimated based on densitometric measurements and Shannon diversity index (H') (Duarte et al., 2009) according to the following equation:

$$H' = -\sum P_i \quad (\ln P_i)$$
$$P_i = n_i/N_i \qquad (1)$$

P_i is the relative intensity of DNA band in the fingerprint, n_i is densitometrically measured intensity of individual DNA band, and N_i is the total amount of DNA in the fingerprint. The relative intensity of each band (Pi) was used to express the relative frequency of each phylotype (Moreirinha et al., 2011).

The 16S rRNA gene sequences obtained from the AK6 mixed culture were deposited in GenBank under accession numbers LC011106–LC011116.

RESULTS

Enrichment of the DBT-Desulfurizing Mixed Culture AK6

The adopted enrichment procedure produced a microbial culture (AK6) that grew in mineral salts medium containing glucose as a carbon source and DBT as a sole sulfur source after repeated subculturing. The AK6 culture appeared yellow to orange in color and was also able to grow (moderate growth) in mineral salts medium containing DBT (dissolved in ethanol) as both a carbon and sulfur source (no glucose). Spread plates of AK6 culture (on glucose and DBT) dilutions revealed several morphologically distinct colonies indicating that AK6 is a mixed culture.

Biodesulfurization Spectrum of AK6

The AK6 culture grew on the organosulfur substrates DBT, BT, 4-MDBT, and 4,6-DM-DBT (all dissolved in ethanol or acetone) as a sole sulfur source in the presence of glucose as a carbon source (**Figure 2**). DBS (dissolved in ethanol) as a sulfur source supported only residual growth of AK6. HPLC analysis of culture samples revealed the transformation of DBT to a product that co-migrated with authentic 2-HBP. HPLC analysis also clearly showed a decrease in the peaks of BT, 4-MDBT, 4,6-DM-DBT (data not shown). In contrast, no remarkable change occurred in the peak corresponding to DBS. AK6 also gave good growth on 2-HBP (dissolved in ethanol) as a carbon source in the presence of $MgSO_4$ as a sole sulfur source (**Figure 2**). Utilization of 2-HBP by AK6 was confirmed by HPLC (**Figure S1**).

The AK6 culture gave a moderate growth (maximum OD_{600} = 0.4) in cultures containing DBT as carbon and sulfur source (no glucose). In this case, DBT was added from an ethanol stock solution. In contrast, AK6 did not grow on DBT when it was added to the medium as a solid, without ethanol or any other carbon source. Similarly, AK6 showed reduced growth (maximum OD_{600} = 0.25) when 4-MDBT (from ethanol stock solution) was added to the culture in the absence of any other carbon or sulfur sources. AK6 did not grow on either BT, 4,6-DM-DBT, or DBS as the carbon and sulfur source. Moreover, AK6 grew well in CDM containing ethanol as a sole carbon source and $MgSO_4$ as a sulfur source (OD_{600} of 0.8 after 72 h of incubation).

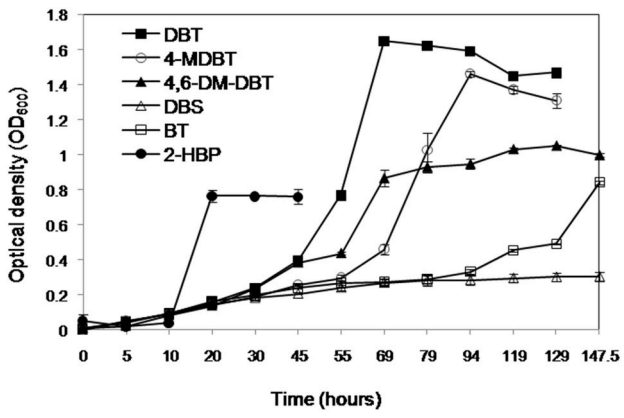

FIGURE 2 | Growth of the AK6 mixed culture on different organosulfur compounds as sulfur sources in the presence of glucose as a carbon source. Growth on 2-HBP as a carbon source in the presence of inorganic sulfate as a sulfur source is also shown.

DBT Biodesulfurization Activity of the AK6 Cell Suspension

The biodesulfurization activity of the AK6 mixed culture was estimated as the amount of 2-HBP produced at different time intervals in resting cell assays with DBT as a substrate. After 1 h of incubation, the AK6 cells produced 6.05 ± 0.13 μM 2-HBP g dcw^{-1} h^{-1}. This amount increased after 2 h to 9 ± 0.6 μM 2-HBP g dcw^{-1} h^{-1}. However, after 4 h of incubation the concentration of 2-HBP declined to 1.9 ± 0.3 μM g dcw^{-1} h^{-1}. This indicates that 2-HBP might be consumed with time or, alternatively, transformed into other products.

DBT Biodesulfurization Intermediates

HPLC confirmed the biotransformation of DBT by both cell suspensions of AK6 and IGTS8 (positive control). HPLC also revealed a peak comigrating with authentic 2-HBP in both assays (**Figure 3**). GC-MS analysis revealed several peaks (**Figures 4A,B**). In all analyzed samples (control, AK6 and IGTS8), two major peaks which were assigned to DBT (at around 15 min, m/z 184) and dibutyl phthalate plasticizer (at 16.5 min, m/z 278) and two minor peaks which were assigned to biphenyl (at 10 min, m/z 154) and benzoic acid (at 8.5 min, m/z 122) were observed. One new major peak (at 12 min, m/z 170) which was assigned to 2-HBP was observed only in AK6 and IGTS8 treatments in addition to the above- mentioned compounds but to less extent. A minor peak (cannot be seen in total ion chromatogram, at around 9 min, m/z 136) which was assigned to phenylacetate was detected only in AK6 assay. Integration of the peak areas for both DBT and 2-HBP revealed that 90% of the added DBT substrate was consumed and nearly 11% of the utilized DBT substrate were recovered as 2-HBP in assays of both AK6 and IGTS8.

Biodesulfurization Genes

Using Genomic DNA isolated from the AK6 community as a template, it was possible to amplify two of the genes commonly found in bacteria possessing the 4S desulfurization pathway, namely, *dszB* and *dszC*. No PCR product corresponding to the *dszA* gene could be obtained (**Figure 5**).

Changes in the AK6 Community Structure (PCR-DGGE)

To gain insight into the dynamics of the microbial diversity present in AK6 in response to different sulfur sources, bacterial communities in AK6 cultures amended with various organosulfur compounds were monitored using

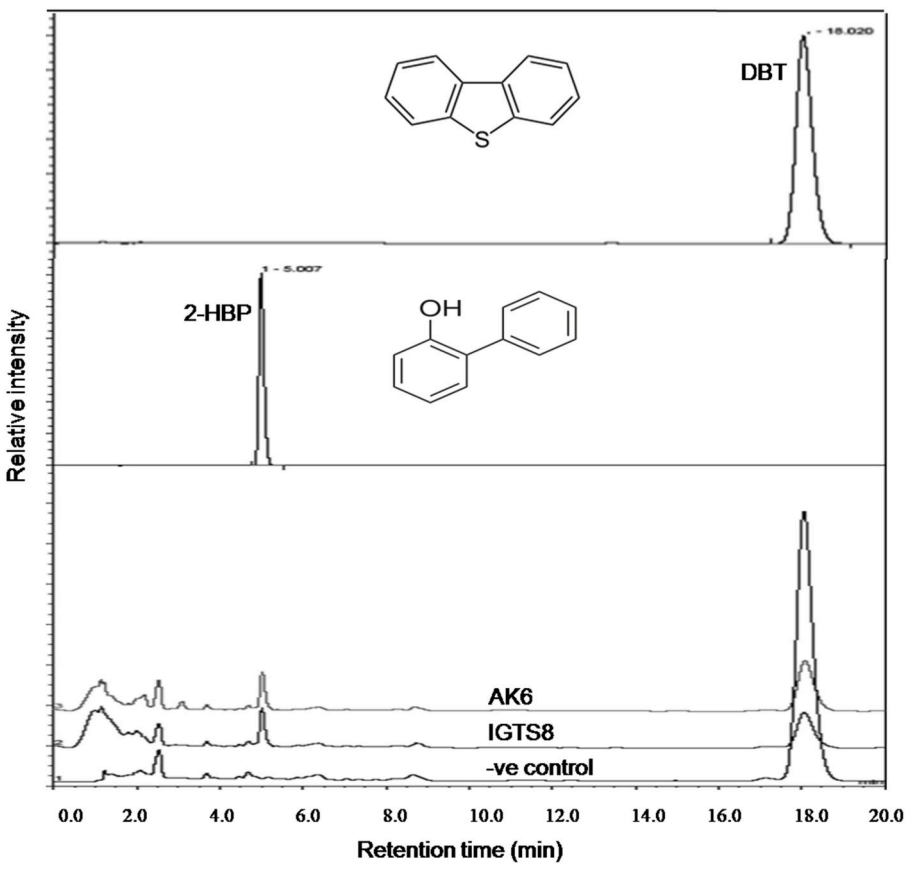

FIGURE 3 | HPLC chromatogram showing the biotransformation of DBT in resting cell assays for the AK6 mixed culture and the reference strain *R. erythropolis* IGTS8. The −ve control assay contained buffer and substrate only.

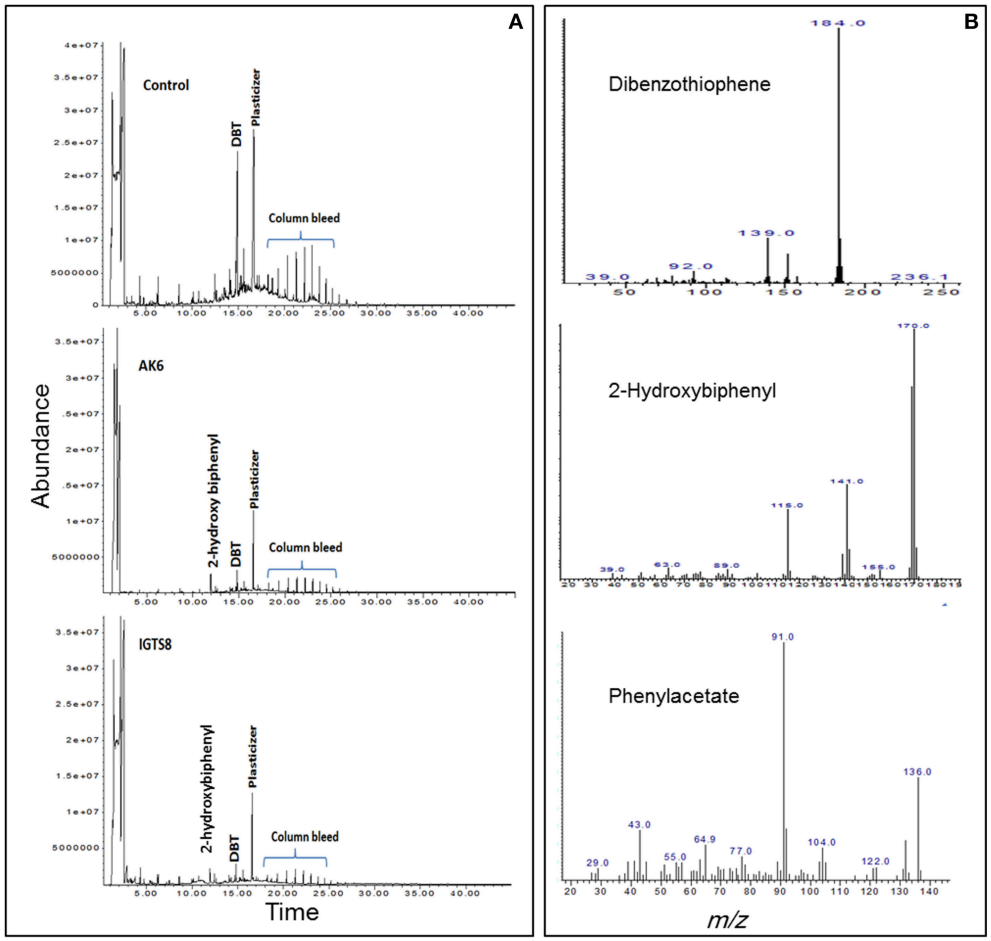

FIGURE 4 | (A) Gas chromatogram showing the biotransformation of DBT in resting cell assays for the AK6 mixed culture and the reference strain *R. erythropolis* IGTS8. **(B)** Mass spectra for compounds detected in ethylacetate extracts of the resting cell assays.

culture-independent PCR-DGGE analysis of 16S rRNA genes. For identification purposes, 16S rRNA genes recovered from dominant DGGE bands were sequenced. The results of homology search and closest matches for the sequences obtained are shown in **Table 2**. DGGE banding pattern for different cultures reveals a community structure change depending on the provided sulfur source (**Figure 6**). DGGE profile of cultures supplemented with BT showed the predominance of three major OTUs (operational taxonomic units) identified as members of the genera *Sphingobacterium*, *Stenotrophomonas*, and *Pseudomonas*. Cultures amended with 4,6-DM-DBT showed more diverse population with at least ten OTUs comprised of *Sphingobacterium*, *Klebsiella*, *Pseudomonas*, and *Stenotrophomonas* species. In contrast, the population in cultures grown on DBT in the absence of glucose was restricted to fewer genera assigned to *Sphingobacterium*, *Stenotrophomonas*, *Pseudomonas*, and *Cellulosimicrobium*/*Arthrobacter* (**Figure 6**, Band 7, **Table 2**), with the latter being more abundant and characteristic to the DBT/no glucose culture. Banding patterns for cultures supplemented with either 4,6-DM-DBT, DBS, or DBT/glucose were similar, consistent and showed almost no change in community structure compared to each other. Dominant DGGE bands from these cultures were affiliated to the genera *Sphingobacterium*, *Klebsiella*, *Pseudomonas*, *Stenotrophomonas*, and uncultured members of *Stenotrophomonas*. The 4-MDBT culture had the largest number of DGGE bands. However, *Klebsiella* spp.-related sequences were lacking. One characteristic feature of the 4-MDBT culture is the presence of *Mycobacterium*/*Rhodococcus* spp. (**Figure 6**, Band 11, **Table 2**) along with other dominant bacteria.

Estimation of bacterial diversity was possible using numerical analysis of the DGGE fingerprints. Overall bacterial richness exhibited different trends with different sulfur sources. The bacterial community enriched on 4-MDBT had the highest operational taxonomic OTU richness. The lowest richness was observed in the DBT/no glucose cultures. OTU richness correlated positively with bacterial diversity. Diversity as measured by H′ index, was variable depending on the sulfur source (**Figure 7A**). Cultures

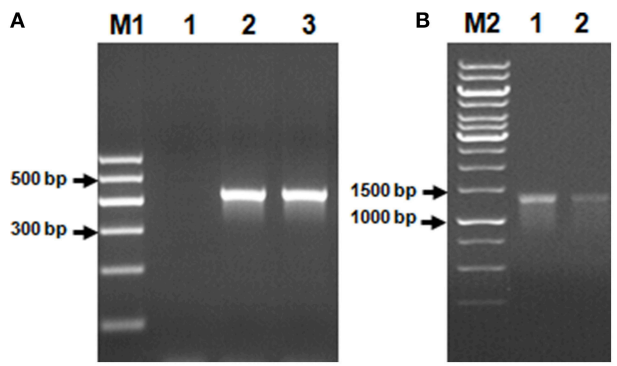

FIGURE 5 | Genes of the 4S biodesulfurization pathway detected in the AK6 mixed culture. Genomic DNA from the reference strain *R. erythropolis* was included as a positive control. Lane **A1**: negative (no-template) control, lane **A2**: *dszB* gene in the reference strain, lane **A3**: *dszB* gene in AK6, lane **B1**: *dszC* gene in the reference strain, lane **B2**: *dszC* gene in AK6. M1 and M2 are DNA markers.

grown on 4-MDBT had relatively high species diversity followed by those grown on BT. On the other hand, cultures grown on DBT without glucose had relatively low species diversity. Besides diversity, species abundance also varied depending on the utilized sulfur source (**Figure 7B**). Species abundance provided complementary insights regarding the effect of the provided sulfur source on bacterial community structure. Specifically, the most abundant OTUs across all cultures were members of the genera *Sphingobacterium* and *Pseudomonas*. Some OTUs appeared to be sulfur source-specific. In this regard, *Mycobacterium/Rhodococcus* sp. and *Cellulosimicrobium/Arthrobacter* sp. were restricted to the 4-MDBT and DBT/no glucose cultures, respectively.

DISCUSSION

We selected some thiophene compounds to investigate the biodesulfurization potential of the microbial mixed culture AK6. These compounds were selected because they constitute the major fraction of organosulfur in crude oil and diesel. Thiophenic compounds account for about 70% of the sulfur contained in crude oil (Borgne and Quintero, 2003). Furthermore, they are resistant to the conventional hydrodesulfurization, and it is important to remove them to drastically reduce the sulfur content of diesel as mandated by environmental regulations (Ohshiro and Izumi, 1999). The adopted enrichment procedure produced a microbial culture that utilized DBT as a sole sulfur source in the presence of glucose as a carbon source. In agreement with these results, many authors reported the isolation of biodesulfurization-competent microorganisms from soil contaminated with hydrocarbons or crude oil (Monot and Warzywoda, 2008; Mohamed et al., 2015). The observation of several morphologically distinct colonies on spread plates confirmed that AK6 is a mixed culture. Deployment of mixed cultures and engineered consortia in biodesulfurization research, though very rare, has been reported by some authors. Li and Jiang (2013) and Jiang et al. (2014) studied biodesulfurization of model thiophenic compounds and heavy oil by mixed cultures enriched from oil sludge. Most recently, Martínez et al. (2016) reported enhanced desulfurization of oil sulfur compounds by using engineered synthetic bacterial consortia.

Growth of the AK6 culture on several organosulfur compounds in the presence of glucose and HPLC analysis confirmed the ability of AK6 to utilize those substrates as sulfur sources, except DBS. Moreover, lack of growth on the tested organosulfur compounds in the absence of glucose suggests that AK6 can't utilize them as a carbon source. The moderate growth observed on DBT (as a carbon and sulfur source) is most likely due to the utilization of ethanol in which DBT was dissolved. The capability of different bacteria to utilize ethanol as an efficient carbon source for enhanced desulfurization of DBT has been reported (Aggarwal et al., 2013). In line with this, AK6 grew on ethanol as a sole carbon source in the presence of $MgSO_4$ as a sulfur source. Furthermore, AK6 could not grow when DBT was added as solid to the growth medium (no glucose, no ethanol). Altogether, the growth experiments and HPLC analysis confirmed the joint capability of the bacterial assortment present in AK6 to utilize a broad spectrum of organosulfur substrates (DBT, BT, 4-MDBT, 4,6-DM-DBT) only as a sulfur source.

The genetic determinants of the 4S pathway have been the target for enormous genetic boosting strategies. Nevertheless, none of the genetically improved axenic cultures has shown biodesulfurization rate meeting the industrial requirements for the development of a commercial biodesulfurization process (Kilbane, 2006; Boniek et al., 2015; Mohamed et al., 2015). Interestingly, other host factors which are not linked with the genetics of the 4S pathway have shown synergetic effect enhancing the biodesulfurization rate (Kilbane, 2006). Accordingly, a consortium of biodesulfurization-competent strains such as AK6 would be a rich pool of these host factors for more efficient and robust biodesulfurization process. The AK6 mixed culture seems to contain both desulfurizing as well as non-desulfurizing bacteria which are adapted to co-metabolize the 4S pathway intermediates. This mosaic nature of AK6 is expected to fit better than axenic monocultures for the development of biotechnological processes targeting desulfurization of a broad range of sulfur compounds present in crude oil and diesel. Furthermore, deployment of mixed cultures may avoid the decay of the biodesulfurization activity resulting from the accumulation of the inhibitory intermediates of the 4S pathway (Abin-Fuentes et al., 2013; Martínez et al., 2016). It remains, however, to identify which members of the AK6 community are essential for the utilization of each of the tested organosulfur compounds. In other words, it is interesting to investigate if the type of the sulfur source has an impact on the structure of the AK6 community. One approach to address these issues would be the time-dependent isolation of the bacterial strains from each AK6 culture. Then, each isolate should be investigated individually and in different qualitative and quantitative combinations with others regarding substrate spectrum, biodesulfurization efficiency, and mechanism. The latter should cover the pathway intermediates and the genetic background. Obviously, this culture-dependent approach is laborious and time-consuming (van Hamme et al., 2003).

TABLE 2 | Bacterial species identified in AK6 cultures and their phylogenetic affiliations.

DGGE Bands	Accession No.	Closest Matches	Similarity (%)	Accession No.	Phylogenetic Affiliation
A1	LC011106	*Sphingobacterium* sp. S2842	100	KJ939323	Bacteroidetes/Sphingobacteriaceae
		Uncultured *Sphingobacterium* sp. clone OTU0876	100	KM059710	Bacteroidetes/Sphingobacteriaceae
		Sphingobacterium siyangense ALS-4	100	KJ638991	Bacteroidetes/Sphingobacteriaceae
		Sphingobacterium multivorum M-A-02/11-10-1	100	KF777399	Bacteroidetes/Sphingobacteriaceae
B2	LC011109	*Klebsiella pneumoniae* R18	100	KM017982	Proteobacteria/Enterobacteriaceae
		Uncultured *Klebsiella* sp. clone TSK	100	KF649832	Proteobacteria/Enterobacteriaceae
		Erwinia chrysanthemi DSM 4610T	100	HG515379	Proteobacteria/Enterobacteriaceae
		Klebsiella sp. XMR21	100	KM241871	Proteobacteria/Enterobacteriaceae
B3	LC011110	*Klebsiella pneumoniae* QLR-8	99	KM096437	Proteobacteria/Enterobacteriaceae
		Klebsiella variicola R39	99	KM019912	Proteobacteria/Enterobacteriaceae
		Klebsiella sp. M.pstv.26.2	99	KM108517	Proteobacteria/Enterobacteriaceae
		Klebsiella sp. F018	99	KJ846494	Proteobacteria/Enterobacteriaceae
E4	LC011115	*Pseudomonas* sp. ESBL485B15-13-4E	100	KJ831548	Proteobacteria/Pseudomonadaceae
		Pseudomonas plecoglossicida RS 1	100	KJ508408	Proteobacteria/Pseudomonadaceae
		Pseudomonas sp. FSBRY17	100	KJ200400	Proteobacteria/Pseudomonadaceae
		Pseudomonas putida FUM1A3	100	KC195910	Proteobacteria/Pseudomonadaceae
A5	LC011107	*Stenotrophomonas maltophilia* B8R	99	DQ466570	Proteobacteria/Xanthomonadaceae
		Stenotrophomonas sp. SO5.1	99	KC859435	Proteobacteria/Xanthomonadaceae
		Stenotrophomonas sp. CDRIG20	99	JN574752	Proteobacteria/Xanthomonadaceae
		Xanthomonas sp. JAPE1	99	KF952249	Proteobacteria/Xanthomonadaceae
A6	LC011108	*Pseudomonas* sp. NTN153	99	LK936599	Proteobacteria/Pseudomonadaceae
		Pseudomonas sp. SCU-B128	99	KJ000799	Proteobacteria/Pseudomonadaceae
		Pseudomonas sp. SF90	99	JX134078	Proteobacteria/Pseudomonadaceae
		Pseudomonas sp. X10	99	EU285592	Proteobacteria/Pseudomonadaceae
C7	LC011114	*Cellulosimicrobium* sp. L414	100	KJ944168	Actinobacteria/Promicromonosporaceae
		Uncultured *Arthrobacter* sp. clone R48	100	KC922044	Actinobacteria/Micrococcaceae
		Cellulomonas hominis PuiC5.18	100	LM994741	Actinobacteria/Cellulomonadaceae
		Cellulosimicrobium cellulans S17	100	KJ947163	Actinobacteria/Promicromonosporaceae
B8	LC011111	*Pseudomonas putida* NBFPASM-RAS176	99	KJ917221	Proteobacteria/Pseudomonadaceae
		Uncultured *Pseudomonas* sp. clone SDn2-35	99	JX493326	Proteobacteria/Pseudomonadaceae
		Pseudomonas azotifigens 6H33b	99	NR041247	Proteobacteria/Pseudomonadaceae
		Uncultured *Proteobacterium* clone Upland-16-5526	99	JF986228	Proteobacteria/Environmental sample
B9	LC011112	Uncultured *Stenotrophomonas* sp. clone DVASW-J329	99	KF722572	Proteobacteria/Xanthomonadaceae
		Uncultured *Stenotrophomonas* sp. clone WCD37	99	KJ123780	Proteobacteria/Xanthomonadaceae
		Uncultured *Stenotrophomonas* sp. clone DVBSD-J259	99	KF463873	Proteobacteria/Xanthomonadaceae
		Uncultured bacterium clone MW75	99	JN868813	Bacteria/Environmental sample
B10	LC011113	*Pseudomonas hibiscicola* R8-737	99	JQ659977	Proteobacteria/Pseudomonadaceae
		Stenotrophomonas maltophilia TCCC11385	99	FJ393320	Proteobacteria/Xanthomonadaceae
		Stenotrophomonas sp. F802	99	AY371433	Proteobacteria/Xanthomonadaceae
		Pseudomonas sp. NTN153	99	LK936599	Proteobacteria/Pseudomonadaceae
F11	LC011116	*Mycobacterium* sp. SR34	100	KF896115	Actinobacteria/Mycobacteriaceae
		Mycobacterium sp. RDB-148	100	AB730319	Actinobacteria/Mycobacteriaceae
		Rhodococcus sp. ZWL3NT	100	JX512559	Actinobacteria/Nocardiaceae
		Rhodococcus sp. NCCP-309	100	AB734810	Actinobacteria/Nocardiaceae

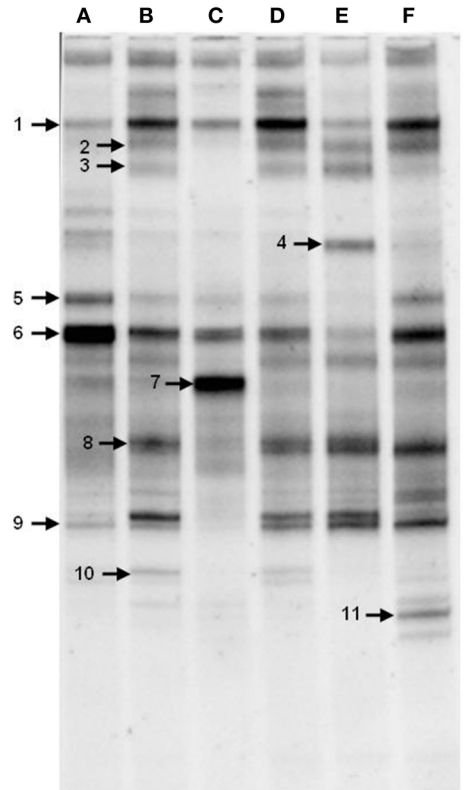

FIGURE 6 | DGGE profiles of 16S rRNA gene fragments amplified from total community DNA recovered from AK6 cultures grown on different sulfur sources. Glucose was provided as carbon source for all cultures and dibenzothiophene was also tested without glucose addition. lane **A**, benzothiophene; lane **B** 4,6-dimethyldibenzothiophene; lane **C**, dibenzothiophene (without glucose); lane **D**, dibenzothiophene; lane **E**, dibenzylsulfide; lane **F**, 4-methyldibenzothiophene. Faint DGGE bands representing PCR artifacts were neglected.

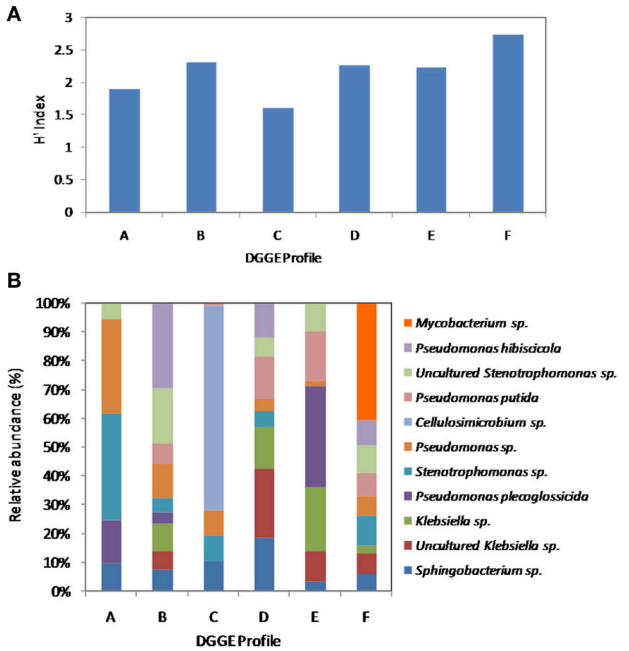

FIGURE 7 | Numerical analysis of the DGGE fingerprints revealing bacterial diversity among AK6 cultures grown on different sulfur sources. (A) Sulfur source-dependent change in the diversity index (H′). **(B)** Species richness and abundance of various bacterial genera across the different AK6 bacterial communities. Glucose was provided as carbon source for all cultures and dibenzothiophene was also tested without glucose addition. A, benzothiophene; B 4,6-dimethyldibenzothiophene; C, dibenzothiophene (without glucose); D, dibenzothiophene; E, dibenzylsulfide; F, 4-methyldibenzothiophene.

Due to the inherent bias and limited recovery efficiency of the culture-dependent approach, it is probable that we miss one or more of the community members essential for the utilization of a particular sulfur source. Furthermore, isolation of a microorganism in a pure culture does not necessarily reveal its role in the community.

Culture-independent molecular characterization techniques have been shown to be more useful for analyzing microbial components of consortia responding actively in desulfurization of thiophene substrates (Duarte et al., 2001). Direct amplification of the *dsz* genes, which code for the biodesulfurization enzymes via the 4S pathway, or testing their expression rates by RT-qPCR would not be discriminative for the identification of the active bacterial groups in AK6 because of the high sequence similarity of the *dsz* genes among different bacterial groups. Alternatively, 16S rRNA gene-based qPCR for the identification and quantification of biodesulfurization-active bacterial components in the AK6 culture would be applicable. However, this could be misleading because other non-biodesulfurizing bacteria in AK6 which can utilize and grow on the intermediates of the 4S pathway will also be detected. Therefore, we chose to perform the investigations using the PCR-based DGGE analysis of 16S rRNA gene fragments amplified directly from total community DNA recovered from the mixed culture. This culture-independent technique excludes the bias associated with the culture media, provides identification of microorganisms by sequencing the gel bands, and gives an overview of bulk changes in community structure (van Hamme et al., 2003). Analysis of AK6 community dynamics revealed qualitative as well as quantitative changes depending on the utilized sulfur source. This variation reflects the metabolic specialization of the bacterial components of AK6. As one might expect, the dominant strains in each culture are those having the ability to desulfurize or (co)metabolize the respective organosulfur compound. In fact, all detected 16S rRNA gene sequences are related to those of bacterial genera that are known to be biodesulfurization-competent, hydrocarbon degraders, or inhabitants of hydrocarbons-polluted environments (Duarte et al., 2001; Mohebali and Ball, 2008; Bhatia and Sharma, 2012; Ismail et al., 2014). The presence, in the majority of the cultures, of *Pseudomonas*, *Sphingobacterium*, *Klebsiella,* and *Stenotrophomonas* spp.-related sequences indicates the significant role of these bacteria in the biodesulfurization of different organosulfur substrates. This can be reconciled for *Pseudomonas*, *Klebsiella*, and *Stenotrophomonas* spp., which have been reported in several biodesulfurization studies. In

contrast, there are no reports in the literature concerning biodesulfurization by *Sphingobacterium* spp. However, these bacteria have been implicated in the biodegradation of polycyclic aromatic hydrocarbons (PAH) and lubricating oil in addition to biosurfactants production (Kanaly et al., 2000; Noparat et al., 2014). Accordingly, *Sphingobacterium* spp. might play an indirect role in the utilization of the organosulfur substrates by providing the community with essential nutrients and surfactants or detoxification of toxic degradation intermediates (Sathishkumar et al., 2008; McGenity et al., 2012; Mikesková et al., 2012; Todorova et al., 2014). Nonetheless, a direct role in the biodesulfurization process can't be ruled out

The 16S rRNA gene sequences retrieved from DGGE band number 7, which existed exclusively in AK6 cultures supplemented with DBT only (dissolved in ethanol) as the sole carbon and sulfur source, were identical to Actinobacterial genera like *Cellulosimicrobium* and *Arthrobacter*. It can be proposed that these bacteria play a significant role in DBT metabolism. The observed moderate growth in this culture was supported most probably by the solvent ethanol as a carbon source and DBT as a sulfur source. Some *Arthrobacter* spp. can utilize DBT and its alkylated derivatives as a sulfur source (Lee et al., 1995; Duarte et al., 2001). Accordingly, *Arthrobacter* spp. are better candidates for the DGGE band 7 than *Cellulosimicrobium* spp. which have not been reported as biodesulfurization-competent. However, it can't be excluded that *Cellulosimicrobium* spp. identified in band 7 are not involved in the biodesulfurization process. Instead, they might have grown on the biodesulfurization intermediates or fed on the debris of dead cells, particularly sugar components (Vogt et al., 2005).

The DGGE band 11 was detected only in the 4-MDBT culture. Therefore, the underlying organisms appear to be involved in the utilization of 4-MDBT as a sulfur source. This is consistent with many studies that reported the biodesulfurization capabilities of some *Mycobacterium* and *Rhodococcus* spp. (Mohebali and Ball, 2008). The DGGE bands detected in the DBS/glucose cultures represent bacterial components of AK6 that gave the residual growth in those cultures. This residual growth was probably supported by glucose and sulfur traces in the growth medium.

The DGGE data provided interesting insights into the effect of the sulfur source on the structure of the AK6 community and the bacterial members that are probably the major contributors. It is worth noting that for the DGGE analysis we focused only on the major bands in the DGGE gels. Therefore, we can't exclude the presence of other bands, minor or not detectable on the gels; that represent other contributors to the biodesulfurization process, which might be directly or indirectly involved. In this context, it has been also reported that a single DGGE band might represent multiple strains (Sekiguchi et al., 2001; Al-Awadhi et al., 2013). Furthermore, the PCR-associated limitations due to primer specificity and differential/preferential amplification of 16S rRNA genes might lead to bias in the structure of the microbial community (Polz and Cavanaugh, 1998; Sipos et al., 2007). This obviates the need for deeper and more conclusive analysis through a combination of approaches to circumvent the limitations inherent to each approach. Our current study on the AK6 mixed culture opened several interesting questions that represent the basis for further in-depth investigations such as: How many bacterial strains exist in each AK6 culture? Which components are indispensable for the biodesulfurization process? What is the role of each AK6 member in the biodesulfurization process? How the AK6 community behaves in the presence of a mixture of organosulfur sources? Can the AK6 community survive in biphasic media? Can the AK6 mixed culture remove sulfur from diesel and gasoline? How active and efficient is AK6 in biodesulfurization as compared to axenic cultures? How reproducible is the AK6 community? All these questions need to be addressed by applying metagenomics and deeper sequencing via next generation techniques. Metagenomic investigations by sequencing and analysis of the 16S rRNA gene pool should enable comprehensive fingerprinting of the AK6 community under different culturing conditions. Alternatively, direct shotgun sequencing on the metagenome shall allow *de novo* assembly of the microbial community as well as compositional analysis in terms of the functional genes. This, in addition to systems biology approaches like metatranscriptomics and metaproteomics coupled to the use of isotope-labeled substrates and biochemical analysis, should provide essential information that will allow the development and engineering of microbial consortia for efficient and economically viable biorefining processes for the fossil fuel industry. In this context, Martínez et al. (2016) have reported a novel approach utilizing engineered synthetic bacterial consortia for enhanced desulfurization and revalorization of oil sulfur compounds. This new approach was developed to overcome inhibition of the Dsz enzymes by the 4S pathway intermediates, and to enable efficient production of value-added intermediates, e.g., 2-(2′-hydroxyphenyl) benzene sulfinate (HBPS), that are difficult to obtain with monocultures.

The detection of 2-HBP, a characteristic end product of the 4S pathway, in the AK6 resting cell assays confirms that DBT biodesulfurization follows the 4S pathway. This is consistent with the detection of *dszB* and *dszC* genes. The lack of a *dszA* PCR product may be due to an insufficient specificity of the used primers. Alternatively, DszA might be lacking in the AK6 community, and another enzyme compensates its catalytic role in the 4S pathway. These findings together with the detection of sequences related to *Rhodococcus*, *Mycobacterium*, *Arthrobacter*, *Stenotrophomonas*, and *Klebsiella* spp., known to harbor the 4S pathway, provide a solid evidence that these bacteria are key players in the biodesulfurizing cultures. It can't be, however, excluded that other hydrocarbons biodegradation/biotransformation pathways are involved. The actual involvement of the 4S and other pathways in the AK6-mediated biodesulfurization process should be further investigated by monitoring temporal changes in gene expression via RT-qPCR.

Previous studies on biodesulfurization of DBT reported specific activities higher and lower than those reported here for AK6 resting cells. These differences could not be attributed solely to the microbial cultures tested in the different studies.

The culture conditions, assay design, and analytical approach should also be considered (Kilbane, 2006; Mohamed et al., 2015). The amount of 2-HBP recovered in the cell suspension assays was tiny and decreased with time. Some authors reported that 2-HBP production was not stoichiometric with the amount of transformed DBT (Davoodi-Dehaghani et al., 2010; Mohamed et al., 2015). The AK6 resting cells originate from DBT-glucose cultures, which include the majority of the bacterial groups detected in AK6 consortium. Accordingly, further transformation of DBT or 2-HBP into other aromatic products can't be excluded. In line with this, the ability of AK6 to grow on 2-HBP as a carbon source might lead to biodegradation/biotransformation of 2-HBP to more polar products. The samples were further analyzed by full-scan mode GC/MS to test this possibility. Dibutylphthalate, a well-known plasticizer, was detected in control samples lacking AK6 and to a lesser extent in AK6 and IGTS8. Therefore, the biotic formation of this compound as a product of DBT or 2-HBP transformations by AK6 was excluded.

Benzoate, a confirmed intermediate in the degradation of either DBT via the angular dioxygenase pathway (Nojiri et al., 2001) or 2-HBP which is encoded by the *hbp* genes (García et al., 2014) was detected to a lower extent in AK6 compared to the control. None of the preceding intermediates that lead to the formation of benzoate in these pathways could be detected. Besides, benzoate was also detected in the cell-free control assays. Accordingly, the biotic generation of benzoate from DBT or 2-HBP by AK6 was also excluded. Similarly, the formation of biphenyl by AK6, a possible intermediate in an extended pathway for desulfurization of DBT (Akhtar et al., 2009), was excluded since it was detected mainly in control samples and to lower extent in AK6 assay. Although the transformation profiles of DBT by AK6 consortium and the reference IGTS8 strain were similar (**Figure 4**), a minor peak assigned to phenylacetate was detected only in AK6 assay. Pure cultures have not been reported to produce phenylacetate as an intermediate in the degradation pathways of DBT or 2-HBP. However, since AK6 is a consortium containing a variety of hydrocarbon-degrading bacteria, the biotic formation of phenylacetate can't be ruled out. Since AK6 is a mixed culture and 2-HBP is a toxic phenolic biocide that can damage cell membranes, we can speculate that phenylacetate originates from 2-HBP via an unknown detoxification mechanism by one or more of the AK6 bacterial components. However, the likelihood of 2-HBP conversion to phenylacetate by AK6 bacteria lacking the biodesulfurization activity can't be ruled out. In this case, phenylacetate is not considered as a new intermediate in an extended 4S pathway, rather it is a product of cometabolism of a sulfur-free substrate (2-HBP).

The proposed transformation of 2-HBP to phenylacetate could probably enhance the biodesulfurization activity of AK6 by eliminating the toxic and inhibitory effect of 2-HBP. This is further corroborated by the ability of AK6 to grow on 2-HBP as a carbon source.

Application of mixed microbial cultures for biodegradation and biotransformation of hydrocarbons is perceived as advantageous. This is due to interspecific and intraspecific interactions that enable microbial consortia to out-perform pure cultures (McGenity et al., 2012; Mikesková et al., 2012; Seth and Taga, 2014). Kim et al. (2009) reported a higher efficiency of phenanthrene degradation by a microbial consortium as compared to the degradation efficiency of the individual component strains. Although the microbial interactions in mixed populations or consortia are not well understood, some types of interactions have been reported in the literature such as collaborative degradation or transformation of the substrate, removal, or sequestration of toxic intermediates, provision of essential metabolites and coenzymes (Konopka et al., 2015).

To summarize, it seems that the major peak of dibutylphthalate and the minor peaks of biphenyl and benzoate detected in the assays most likely are contaminants, and the possibility of the biotic formation of phenylacetate needs further investigation.

CONCLUSIONS

A mixed bacterial culture AK6 was enriched from hydrocarbons-polluted soil based on its biodesulfurization competency. The type of the utilized organosulfur source had an impact on the structure of the AK6 community. The AK6 culture showed a good biodesulfurization substrate spectrum and higher DBT biodesulfurization efficiency. Biodesulfurization of DBT proceeds via the non-destructive 4S pathway and, probably other pathways. Mixed cultures hold a promising potential for the development of biocatalytic desulfurization technology and deserve further in-depth investigations.

AUTHOR CONTRIBUTIONS

WI designed research, analyzed and interpreted data, and wrote the manuscript. WE and MM conducted experiments and wrote the manuscript. AA and AE conducted experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00266

Figure S1 | HPLC analysis of 2-HBP utilization by AK6 growing in mineral salts medium with 2-HBP as a carbon source and $MgSO_4$ as a sole sulfur source.

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High Frequency of *Thermodesulfovibrio* spp. and *Anaerolineaceae* in Association with *Methanoculleus* spp. in a Long-Term Incubation of *n*-Alkanes-Degrading Methanogenic Enrichment Culture

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In the present study, the microbial community and functional gene composition of a long-term active alkane-degrading methanogenic culture was established after two successive enrichment culture transfers and incubated for a total period of 1750 days. Molecular analysis was conducted after the second transfer (incubated for 750 days) for both the active alkanes-degrading methanogenic enrichment cultures (T2-AE) and the background control (T2-BC). A net increase of methane as the end product was detected in the headspace of the enrichment cultures amended with long-chain *n*-alkanes and intermediate metabolites, including octadecanoate, hexadecanoate, isocaprylate, butyrate, isobutyrate, propionate, acetate, and formate were measured in the liquid cultures. The composition of microbial community shifted through the successive transfers over time of incubation. Sequences of bacterial and archaeal 16S rRNA gene (16S rDNA) and *mcrA* functional gene indicated that bacterial sequences affiliated to *Thermodesulfovibrio* spp. and *Anaerolineaceae* and archaeal sequences falling within the genus *Methanoculleus* were the most frequently encountered and thus represented the dominant members performing the anaerobic degradation of long-chain *n*-alkanes and methanogenesis. In addition, the presence of *assA* functional genes encoding the alkylsuccinate synthase α subunit indicated that fumarate addition mechanism could be considered as a possible initial activation step of *n*-alkanes in the present study. The succession pattern of microbial communities indicates that *Thermodesulfovibrio* spp. could be a generalist participating in the metabolism of intermediates, while *Anaerolineaceae* plays a key role in the initial activation of long-chain *n*-alkane biodegradation.

Keywords: alkanes degradation, long-chain alkanes, microbial community, 16S rRNA gene, *Thermodesulfovibrio*, *Anaerolineaceae*, *Methanoculleus*, methanogenesis

INTRODUCTION

Quantitatively, alkanes are one of the most significant component of petroleum hydrocarbons (Head et al., 2010). They were long considered to be recalcitrant to biodegradation in the absence of molecular oxygen, nitrate, and/or sulfate (Heider et al., 1998). In recent years, alkanes biodegradation via methanogenesis has been a topic of increasing interests thanks to the first report of a successful enrichment culture converting long-chain alkanes (specifically hexadecane) to methane (Zengler et al., 1999). Many relevant researches on methanogenic alkanes-degradation studies appeared afterward (Anderson and Lovley, 2000; Jones et al., 2008; Gray et al., 2011; Wang et al., 2011; Li et al., 2012; Mbadinga et al., 2012; Zhou et al., 2012; Aitken et al., 2013; Cheng et al., 2013b; Berdugo-Clavijo and Gieg, 2014; Embree et al., 2014; Sherry et al., 2014; Liang et al., 2015). The initial activation of alkane degradation under anaerobic conditions may involve fumarate addition to the parent alkane, hydroxylation/carboxylation and, in some specific cases, intra-hydroxylation (Callaghan, 2013). Among the above mentioned activation mechanisms, addition to fumarate appear as the most prevalent and almost the best characterized mechanism in anaerobic hydrocarbon degradation (Aitken et al., 2013). The gene *assA*/*masD* coding the alkylsuccinate synthetase has eventually been considered as a valuable biomarker for detecting fumarate addition pathway in alkane degradation (Callaghan et al., 2010).

Microbial communities capable of degrading petroleum hydrocarbons under methanogenic conditions are often complex consortia, at least consisting of various fermenting bacteria, syntrophic bacteria and methanogens at least. Generally, the constitution of microbes from different hydrocarbons impacted environments like aquifers, sediments, and soils can be dramatically different. However, among them some microbial taxa appeared with relatively high frequency, a good example of this is that many researches shared the similar microorganisms members of the *Syntrophaceae* (*Smithella*/*Syntrophus*) which was inferred to have the ability of degradation of alkane (Bakermans and Madsen, 2002; Kasai et al., 2005; Allen et al., 2007; Gray et al., 2011; Ramos-Padron et al., 2011; Cheng et al., 2013a; Embree et al., 2014; Tan et al., 2014a,b). A survey that collated published 16S rRNA gene data from 26 culture-independent analyses of methanogenic hydrocarbon impacted environments showed that bacterial sequences affiliated with *Firmicutes* were detected at the highest frequency followed by γ-*proteobacteria*, δ-*proteobacteria*, ε-*proteobacteria*, β-*proteobacteria*, *Bacteroidetes*, *Actinobacter*, α-*proteobacteria*, *Chloroflexi*, *Thermotogae*, *Nitrospira*, *Spirochaetes*, *Acidobacter*, *Planctomycetes* and OP11 (Gray et al., 2010). So far, at least 19 anaerobic, alkane-oxidizing microorganisms have been isolated (Webner, 2012; Khelifi et al., 2014; Schouw et al., 2016), while 17 of the isolated strains were affiliated with the phylum of *Proteobacteria*, other two strains belongs to *Firmicutes* and *Archaeoglobales,* respectively. The large proportion of non-cultivable and metabolically inactive organisms interferes with the identification of the active responsible for the degradation. In addition, the various syntrophic associations in methanogenic consortia and the obligate anaerobic conditions make isolation of them in pure cultures difficult and impossible with the current available techniques. Consortium obtained through enrichment culturing with long-term stability for methanogenic alkane degradation can eliminate the inactive members considerably so that the essential ones can be accumulated. In the present research work, a methanogenic alkanes-degrading consortium was established after enrichment culturing and long-term of incubation amended with a mixture of *n*-alkanes (C_{15}–C_{20}) as the sole sources of carbon and energy. The succession pattern of microbial communities together with the diversity and abundance of potential functional genes were analyzed via PCR based clone libraries construction coupled with quantitative real-time PCR analysis in this study. The degradation intermediates and the final product methane were measured during degradation at the same time.

MATERIALS AND METHODS

Enrichment Cultures

Inoculum was obtained from an initial methanogenic enrichment culture from Menggulin petroleum reservoir production water (Huabei oilfield, China) amended with *n*-alkanes as described previously (Li et al., 2012). About 10 ml (20%) of inoculum were transferred into an autoclaved serum bottle (internal volume 120 ml) containing 50 ml of sterilized basal medium prepared by the Hungate technique (Bryant, 1972) and then sealed with a butyl rubber stopper (Bellco Glass, Inc., Vineland, NJ, USA) and aluminum crimp seal. The basal medium composition was described elsewhere (Wang et al., 2012). Active enrichment cultures were amended with the mixture of *n*-alkanes (C_{15}–C_{20}) as the sole carbon and energy sources (three replicates). Autoclaved controls were prepared in the same way but sterilized three times (three replicates). Background controls were prepared without addition of any organic carbon source (three replicates). The mixture of *n*-alkanes (C_{15}–C_{20}) contained *n*-pentadecane (C15; ≥99%), *n*-hexadecane (C16; ≥99%), *n*-heptadecane (C17; ≥99%), *n*-octadecane (C18; ≥99%), *n*-nonadecane (C19; ≥99%), and *n*-eicosane (C20; ≥99%) (Sigma–Aldrich, Milwaukee, WI, USA). About 30 μl of the *n*-alkanes mixture were added to each of the empty serum bottles except the background controls under a stream of N_2 gas before sterilization. All of the cultures were incubated at 37°C in the dark.

Chemical Analysis

Gas chromatography (GC) was used to measure the production of methane in the headspace gas of serum bottle during the incubation. Two hundred microliters of the headspace gas taken by gas-tight syringe were injected onto GC by a micro-syringe for analysis. Program setting of the GC analysis was: the initial column temperature at 60°C for 12 min, then increased to 200°C at a rate of 15°C/min, the final temperature at 200°C sustained for 24 min. Temperature of injector and flame ionization detector (FID) was maintained at 200°C. An external standard curve of methane was used for converting peak areas

of methane into their respective concentrations ($R^2 = 0.994$, $n = 6$).

Gas Chromatography-Mass Spectrometer (GC-MS; Agilent Technologies, Inc.) was used for the detection of residual *n*-alkanes and intermediate metabolites in the aliquot phase through the incubation. For the analysis of intermediate metabolites containing mainly long-chain fatty acids (LCFAs) and volatile fatty acids (VFAs), 5 ml of culture aliquot were taken and the pH was adjusted with ammonia water to >12 and then dried in an oven at 110°C. Esterification was performed by adding 0.5 ml of 10% butanol/sulfate solution at 90°C for 60 min. Extraction of LCFAs and VFAs was conducted with 0.5 ml of *n*-hexane and 0.5 ml *n*-dodecane, respectively, and the extracts were injected onto the GC-MS. For LCFAs analysis, program setting was oven temperature maintained at 120°C for 3 min, increased at the rate of 8°C/min to 260°C for 10 min while for VFAs, oven temperature was maintained at 60°C for 1 min and then increased at the rate of 15°C/min to 130°C. Residual *n*-alkanes in the aliquot samples were extracted with *n*-hexane as described previously (Wang et al., 2011).

DNA Extraction and PCR Amplification

At the end of incubation period, 10 ml of active culture (three replicates) and the background control (three replicates) were withdrawn from each serum bottle, then centrifuged at 1200 × *g* for 10 min. The biomass pellet after centrifugation was used for DNA extraction by using AxyPrep^{TM} Bacterial Genomic DNA Maxiprep Kit (Axygen Biosciences, USA) according to the manufacturer' instructions.

The universal primer sets of 8F/805R (Savage et al., 2010) and 340F/1000R (Gantner et al., 2011) were used for bacterial and archaeal 16S rRNA gene amplification, respectively. For bacterial 16S rRNA gene, PCR amplification reaction was performed according to the followings: 5 min for initial denaturation at 95°C, followed by 38 cycles of 95°C for 30 s, 52°C for 45 s, 72°C for 60 s, and a final elongation step at 72 C for 10 min. For archaeal 16S rRNA gene, PCR amplification conditions were as follows: 5 min for initial denaturation at 95°C, followed by 10 cycles of 95°C for 30 s, 60°C for 30 s (decreased by 0.5°C per cycle to 50°C), 72°C for 60 s. After touchdown, 30 additional cycles at annealing temperature of 50°C were performed, followed by the final elongation step at 72°C for 10 min.

Alkylsuccinate synthetase genes (*assA*) and methyl coenzyme-M reductase genes (*mcrA*) as the functional genes in the process of methanogenic degradation of alkanes were also investigated. PCR primer sets of assA2F/assA2R (Callaghan et al., 2010) and ME3MF/ME2R (Hales et al., 1996; Nunoura et al., 2008) were used for the PCR amplification, respectively. PCR thermal cycles for *assA* were carried out as follows: 5 min for initial denaturation at 95°C, followed by 10 cycles of 95°C for 30 s, 60°C for 30 s (decreased by 0.5°C per cycle to 55°C), 72°C for 60 s. After touchdown, 28 additional cycles at annealing temperature of 55°C were performed, followed by the final elongation step at 72°C for 10 min. PCR cycles for *mcrA* were as follows: 5 min for initial denaturation at 95°C, followed by 38 cycles of 95°C for 30 s, 52°C for 45 s, 72°C for 60 s, and a final elongation step at 72°C for 10 min.

Construction of 16S rRNA Gene, and *assA* and *mcrA* Functional Gene Libraries

After the PCR products were gel purified by using Gel Extraction Kit (Axygen Biosciences, USA), the purified DNA fragments were cloned into *E. coli* using pMD19®-T simple vector kit (TaKaRa Bio Inc., Japan). The white clones were picked randomly into 1 ml of Luria Broth (LB) medium amended with ampicillin and incubated for 24 h at 37°C. PCR primer set M13-47 (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and RV-M (5′-GAGCGGATAACAATTTCACACAGG-3′) was used for positive clone detection. Sequencing of the positive clones was accomplished on an ABI 377 automated sequencer. Chimeric sequences of 16S rRNA gene sequences were excluded by Bellerophon (Huber et al., 2004). Valid sequences with more than 97% similarity were classified through the BLASTclust of MPI bioinformatics toolkit (Biegert et al., 2006) to arrive operational taxonomic unit (OTU). Functional genes were translated through ExPASY translation tool[1]. Sequences were compared to the GenBank Nucleotide Sequence Database using BLAST (Altschul et al., 1990) to identify the nearest matches in the GenBank database. Phylogenetic and molecular evolutionary analyses were conducted using MEGA6.0 software (Tamura et al., 2013) with neighbor-joining method (Saitou and Nei, 1987) and 1000 bootstrap replicates.

Quantitative PCR

Quantitative PCR of target genes were performed by using SYBR Green I Real-Time PCR (BioRad CFX96 thermocycler, Bio-Rad Laboratories Inc., USA). We amplified 16S rRNA gene of *Archaea* and *Bacteria*, each target gene was amplified with specific primers ARC787F/ARC1059R (Yu et al., 2005) and BAC338F/BAC805R (Yu et al., 2005), respectively. These two genes were amplified separately in a SYBR Green I Real-Time PCR reaction with a 10-fold dilution series of plasmids containing target DNA sequences as a calibration standard for establishing the standard curve. Quantitative PCR reaction (20 μl) was composed of SYBR Green Realtime PCR Master Mix-Plus (10 μl; TaKaRa Bio Inc., Japan), Plus Solution 2 μl (TaKaRa Bio Inc., Japan), PCR primers (1 μl of each), 4 μl of sterile water, and 2 μl DNA template (27.4 ng/μl for T2-AE and 15.8 ng/μl for T2-BC). The conditions were as follows: pre-denaturation for 3 min at 95°C, followed by 38 cycles of denaturation at 94°C for 20 s, annealing temperature was 60°C and 57°C, respectively, for 30 s, elongation at 72°C for 60 s.

Nucleotide Sequences Accession Numbers

The sequences generated in this study were deposited in GenBank under accession numbers KP109826-KP109908 and KP341767-KP34176769.

[1]http://web.expasy.org/translate/

RESULTS

Headspace Methane and Intermediate Metabolites

During the incubation of the second enrichment transfer, the lag phase in the amended enrichment cultures (T2-AE) was only 92 days and 44.76 μmol of methane were generated while in the background control without amendment of any alkanes (T2-BC) approximately 0.28 μmol of methane were detected (**Figure 1**). Intermediate metabolites including LCFAs (Supplementary Figure S1) and VFAs (Supplementary Figure S2), octadecanoate, hexadecanoate, isocaprylate, butyrate, isobutyrate, propionate, acetate, and formate were detected in the enrichment cultures only. Quantity of residual *n*-alkanes were detected through the GC-MS with cetyl chloride as the surrogate standard. The net consumption of total *n*-alkanes was 53.31 μmol by subtracting the residual *n*-alkanes in the active enrichment cultures from autoclaved controls. Stoichiometry of methane production showed methane accumulated in headspace accounted for 6.23% of theoretically predicted value (Supplementary Table S1).

Phylogenetic Analysis of Bacteria

A total of 100 positive clones were picked for sequencing bacteria 16S rRNA genes from both the active enrichment (T2-AE) and background control cultures (T2-BC). After removing vector parts and checking chimeras, 81 and 82 valid sequences, we obtained for the active culture and the control, respectively. The valid sequences were classified through BLASTclust of MPI bioinformatics toolkit (Biegert et al., 2006) with 97% similarity and 10 OTUs for T2-AE and seven OTUs for T2-BC were obtained (**Figure 2**). The OTU of "T2-AE-24 (KP109862)" which contains the most clone sequences belongs to *Thermodesulfovibrio* within the phylum of *Nitrospirae* in the active enrichment cultures (T2-AE). Followed by the OTU of "T2-AE-10 (KP109861)" belongs to *Anaerolineaceae* within the phylum of *Chloroflexi* which covers 27 clone sequences. Bacteria in the genus of *Thermodesulfovibrio* having the highest abundance constituted 49% of all of the 81 clones (**Figure 2**). Family *Anaerolineaceae* as the dominant bacteria made up 33%. *Acetothermia*, *Aminicenantes*, *Actinobacteria*, and unclassified bacteria composed the remaining 18% (**Figure 2**). In T2-BC, OTU of "T2-BC-10 (KP109845)" affiliated with *Thermodesulfovibrio* within the phylum of *Nitrospirae* representing the most clone sequences of 71 in the whole 82 valid clones (**Figure 2**). Bacteria in the genus of *Thermodesulfovibrio* were the most dominant bacteria of 87%, while *Anaerolineaceae* occupied 1%. The remaining 12% comprised *Actinobacteria*, *Bacteroidetes*, *Actinobacteria*, *Synergistia*, and *Firmicutes* (**Figure 2**).

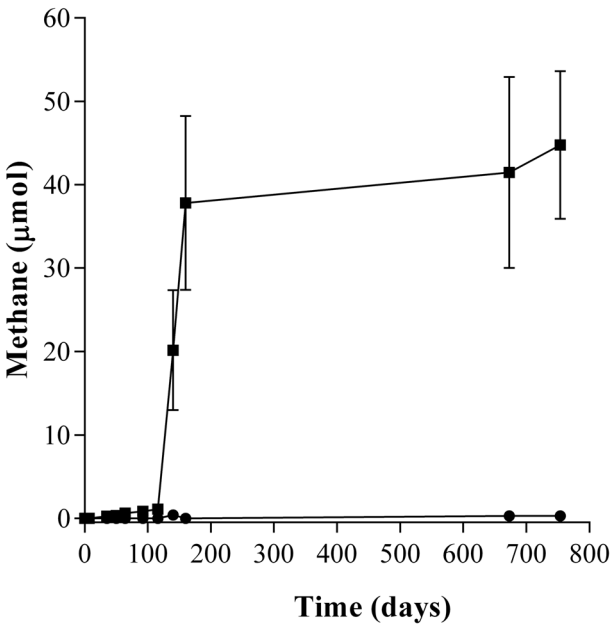

FIGURE 1 | Methane production of the *n*-alkanes degradation consortium under methanogenic conditions. (■) incubated with *n*-alkanes mixture (C_{15}–C_{20}) as the sole sources of carbon and energy (T2-AE; three replications), and (●) without any *n*-alkanes and other carbon sources as the background control cultures (T2-BC; three replicates).

Phylogenetic Analysis of Archaea

Positive clones were also picked for sequencing archaea 16S rRNA gene. A total of 61 and 51 valid sequences were obtained for the active enrichment (T2-AE) and background control cultures (T2-BC), respectively, after the removal of vector and chimeras checking. Valid sequences were analysis through BLASTclust of MPI bioinformatics toolkit (Biegert et al., 2006) with 97% similarity to generate the OTUs. There were two OTUs for active enrichment cultures (T2-AE) and four OTUs for background control cultures (T2-BC) (**Figure 3A**). For the active enrichment cultures (T2-AE), the OTU of "T2-AE-10 (KP109885)" contains the majority 61 clones of the total 62 clones. All of the 62 clone sequences belong to *Methanoculleus* within the *Methanomicrobiales* order (**Figure 3A**). For the background control (T2-BC), the OTU of "T2-BC-13 (KP109880)" had 46 of the total 51 clones. This OTU affiliated with *Methanolinea* in the order of *Methanomicrobiales* occupying 90% of all valid clone sequences. *Methanobacterium*, *Methanocella*, and *Methanomethylovorans* constituted the remaining 10% of the clone library (**Figure 3A**).

Diversity and Phylogenetic Analysis of *assA* and *mcrA* Genes

Functional gene of *assA* was only detected in the active enrichment cultures (T2-AE) by using the primer set of assA2F/assA2R (Callaghan et al., 2010). Valid sequences were used for phylogenetic analysis and resulted in three OTUs at 97% similarity (**Figure 4**). Most sequences were in the OTU of "T2-AE-*assA*-13 (AKK23578)" which contains 32 of the total 39 clone sequences. This OTU has a 100% similarity with the sequence (AIU94859) from production water of an oil reservoir of Jiangsu oil field (Bian et al., 2015). Background control cultures (T2-BC) generated no detectable products by PCR reactions.

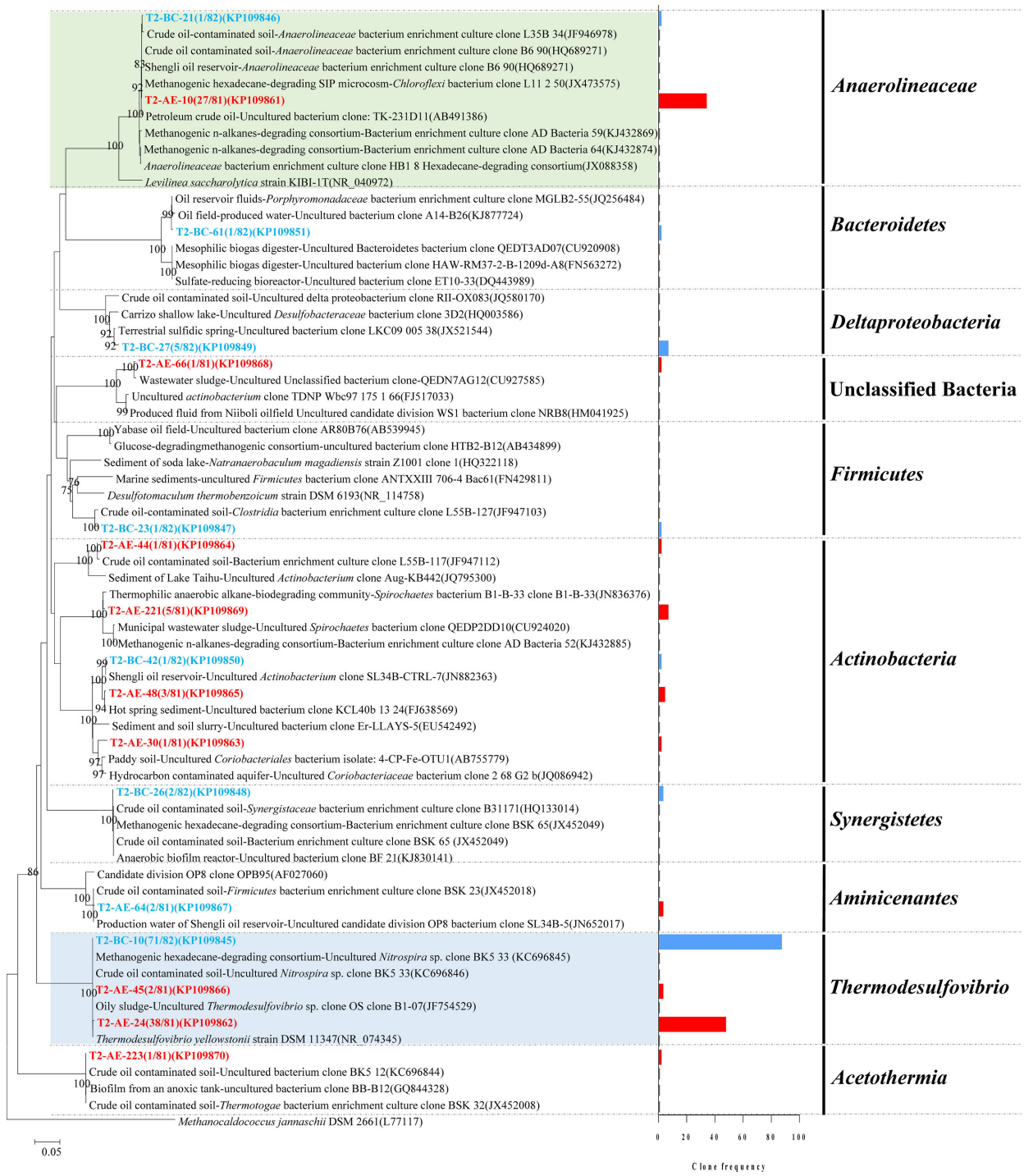

FIGURE 2 | Phylogenetic tree of bacterial 16S rRNA gene sequences from methanogenic alkanes-degrading enrichment cultures (T2-AE; in red) and background control cultures (T2-BC; in blue). The tree is rooted with *Methanocaldococcus jannaschii* DSM 2661 (L77117) as the out group. The OTUs are shown with clone names and accession numbers. One-thousand bootstraps were performed with neighbor-joining method. Bootstrap values below 75% are not shown. Frequency of clones is shown in the bar graph.

Both the T2-AE and T2-BC cultures detected the functional gene *mcrA* by using the prime set of ME3MF/ME2r (Nunoura et al., 2008) and obtained two and seven OTUs, respectively. All of the clone sequences in T2-AE cultures affiliated with *Methanoculleus* within the order of *Methanomicrobiales* (**Figure 3B**). This result was coincident with archaeal 16S rRNA

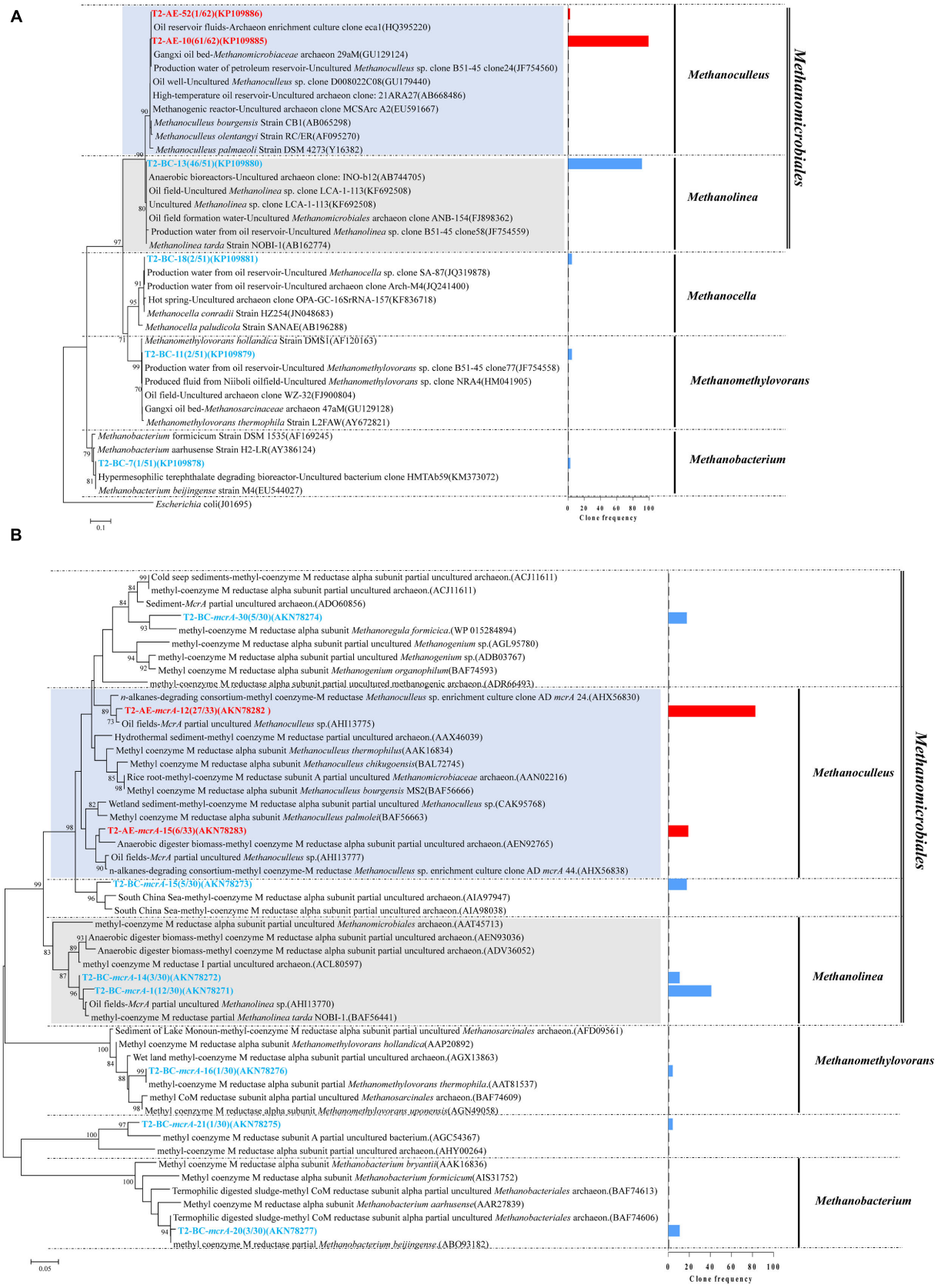

FIGURE 3 | Continued

FIGURE 3 | Phylogenetic tree of archaeal 16S rRNA gene sequences from methanogenic alkanes-degrading enrichment cultures (T2-AE; in red) and background control cultures (T2-BC; in blue). This tree is rooted with outgroup sequence from *Escherichia coli* (J01695) as an outgroup. The OTUs are shown with clone names and accession numbers. Topology of the tree was obtained by the neighbor-joining method. One-thousand time bootstraps testing were performed. Bootstrap values below 70% are not shown. Frequency of clones are shown in the bar graph **(A)**; Phylogenetic tree of deduced amino acid sequences of methyl coenzyme-M reductase genes (*mcrA*) from methanogenic alkanes-degrading enrichment culture (in red) and background control cultures (T2-BC; in blue). Topology of the tree was obtained by the neighbor-joining method. The evolutionary distances were computed using the Poisson correction method. One-thousand time bootstraps testing were performed. Bootstrap values below 70% are not shown **(B)**.

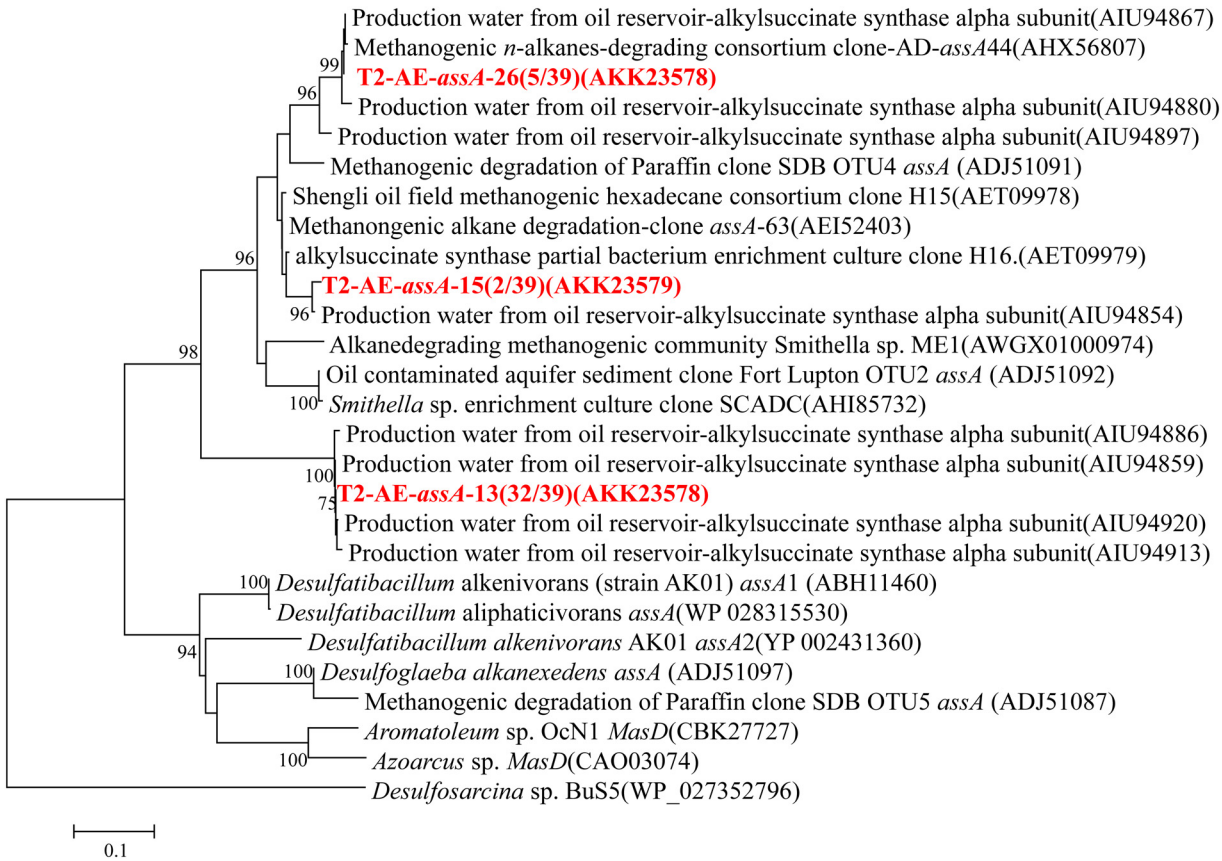

FIGURE 4 | Phylogenetic tree of deduced amino acid sequences of alkylsuccinate synthetase genes (*assA*) genes from methanogenic alkanes-degrading enrichment culture (in red). The topology of the tree was obtained by the maximum likelihood method. Bootstrap values ($n = 1000$ replicates), values below 75% are not shown.

gene clone library. The OTU of "T2-AE-*mcrA*-12 (AKN78282)" showed 97% similarity with the sequence from the *n*-alkanes-degrading consortium (AHX56830). Except the OTU of "T2-BC-*mcrA*-21 (AKN78275)," *mcrA* functional gene sequences in T2-BC cultures had three clear classifications: *Methanomicrobiales*, *Methanobacteriales,* and *Methanosarcinales* (**Figure 3B**).

Quantitative PCR

16S rRNA genes of *Archaea* and *Bacteria* were amplified for both T2-AE and T2-BC to detect the whole archaea and bacteria 16S rRNA gene copies. Gene abundance (gene copies per milliliter of culture aliquot) of *Archaea* was 4.17×10^7 (copies/ml) and 1.01×10^6 (copies/ml) in T2-AE and T2-BC, respectively. Gene abundance (gene copies per milliliter of culture aliquot) of *Bacteria* was 1.48×10^7 (copies/ml) and 1.62×10^6 (copies/ml) for T2-AE and T2-BC, respectively. For *Archaea* and *Bacteria,* 16S rRNA gene qPCR reaction, amplification efficiency was 105.6% and 102.3%, R^2-values was greater than 0.989 and 0.986, respectively.

DISCUSSION

The entire duration of nearly 5 years of enrichment culturing included the first transfer (about 950 days) and a subsequent second transfer (over 750 days), both of the two transfers and incubations received the mixture of *n*-alkanes (C_{15}–C_{20}) as the sole carbon and energy sources. During the incubation, the variations of microbial community in different enrichment transfers were investigated. Microbial community

of the Menggulin petroleum reservoir production water sample (OS) and the initial transfer of incubation after 400 days (T1-400) were analyzed by our laboratory and reported previously (Li et al., 2012). In the current research, microbial communities in the second transfer from both the active enrichment cultures (T2-AE) and the background control (T2-BC) were investigated after 750 days of incubation.

High Frequency of *Thermodesulfovibrio* and *Anaerolineaceae*

Clear variation of bacteria community was observed after the 5 years of methanogenic alkanes degrading enrichment culturing (**Figure 5**). In the original petroleum reservoir production water sample (OS), the dominant bacteria were *Gammaproteobacteria* (46.6%) and *Betaproteobacteria* (32.9%). After nearly 400 days of incubation with addition of a mixture of *n*-alkanes (T1-400) initially, *Actinobacteria* (52.2%) and *Thermodesulfovibrio* (39.1%) were detected as the dominant bacteria. It is clear that the period of *Actinobacteria* as the dominant bacteria only maintained for a short period of time at the beginning. Then the abundance of *Actinobacteria* decreased sharply to 6.2% as a minority after another 1350 days of incubation (including a second transfer of incubation for 750 days) in T2-AE, whereas *Thermodesulfovibrio* remained the most frequently encountered genus and increased gradually to 49.4%. At the same time, *Anaerolineaceae* rose

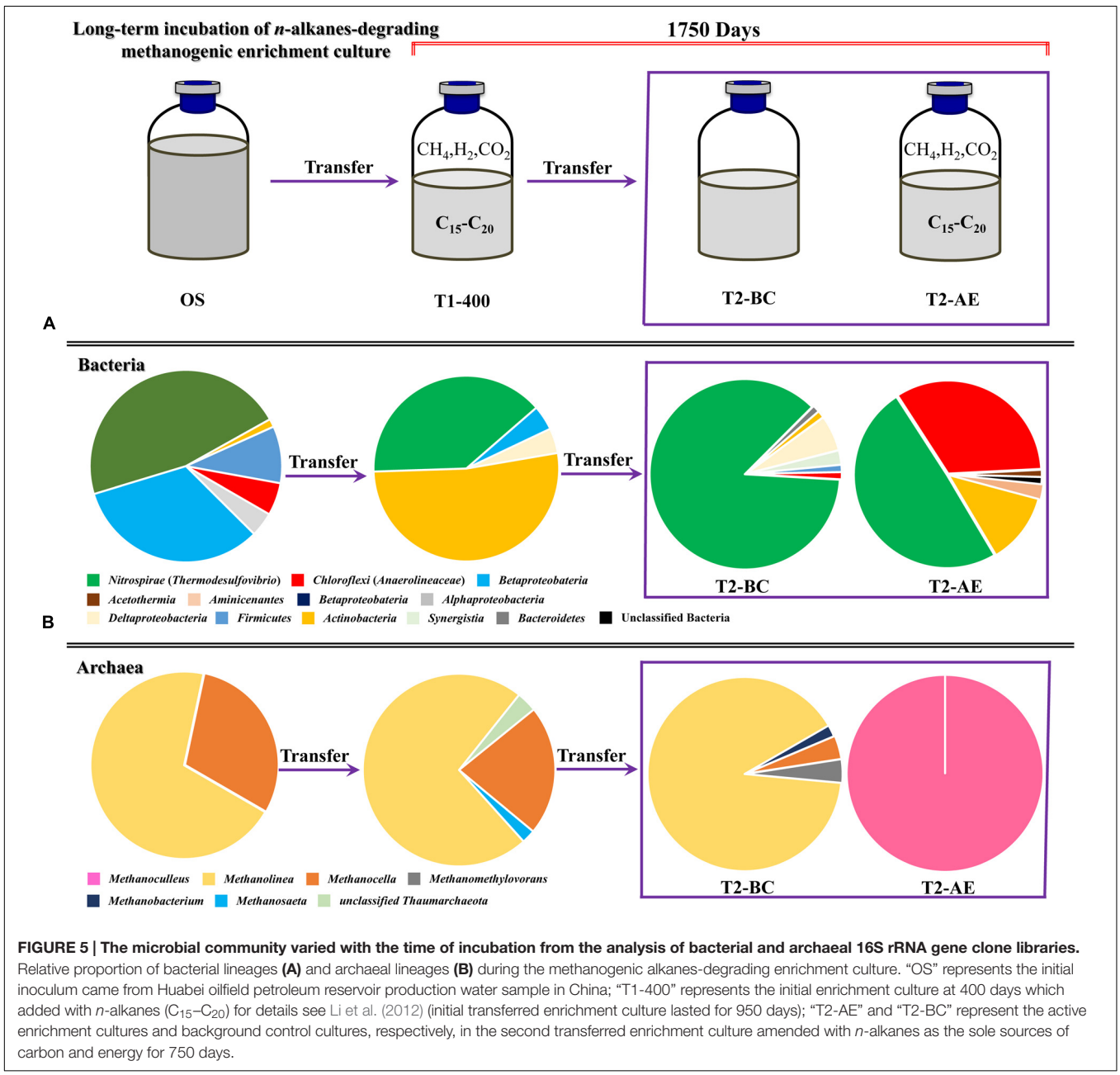

FIGURE 5 | The microbial community varied with the time of incubation from the analysis of bacterial and archaeal 16S rRNA gene clone libraries. Relative proportion of bacterial lineages **(A)** and archaeal lineages **(B)** during the methanogenic alkanes-degrading enrichment culture. "OS" represents the initial inoculum came from Huabei oilfield petroleum reservoir production water sample in China; "T1-400" represents the initial enrichment culture at 400 days which added with *n*-alkanes (C_{15}–C_{20}) for details see Li et al. (2012) (initial transferred enrichment culture lasted for 950 days); "T2-AE" and "T2-BC" represent the active enrichment cultures and background control cultures, respectively, in the second transferred enrichment culture amended with *n*-alkanes as the sole sources of carbon and energy for 750 days.

conspicuously to 33.3% as the dominant bacteria. Similar results were also observed in our earlier *n*-alkanes-degrading consortium originated from an oily sludge (Liang et al., 2015). Thus, *Thermodesulfovibrio* and *Anaerolineaceae* as the most dominant bacteria implies a major role they play in the long-term methanogenic alkanes-degrading consortium.

Thermodesulfovibrio was the only overwhelming majority (86.6%) in the control cultures (T2-BC). *Anaerolineaceae* occupied only 1.2%, a significant difference from the 33.3% in T2-AE sample. Since both T2-AE and T2-BC were the second enrichment transfers of incubation from the same inoculum initially, such major difference in consortium composition should be attributed to the amendment of organic carbons. When the microorganisms were subject to a starvation condition (T2-BC), abundance of bacteria like *Anaerolineaceae* and *Actinobacteria* dropped because of the lack of organic carbons available. Comparing to the background control T2-BC, the dominant *Anaerolineaceae* in the active enrichment transfer culture T2-AE should be closely related to the alkanes-degrading process. The total bacteria in T2-AE experienced an obvious surge comparing to T2-BC according qPCR analysis showing the alkanes-degrading activity.

Thermodesulfovibrio within the phylum *Nitrospirae*, is a sulfate-reducing microorganism (SRM) that could use sulfate as the terminal electron acceptor in their energy metabolism (Sherry et al., 2013). OTU of *Thermodesulfovibrio* in this research shared the highest identity (100%) with *Thermodesulfovibrio yellowstonii* strain DSM 11347^T (NR_074345), which is able to use lactate, pyruvate, and hydrogen as electron donors in the presence of sulfate (Henry et al., 1994). Interestingly, the medium had no sulfate available, and methane was the main end product. Theoretically *Thermodesulfovibrio*-like species could cooperate with other bacteria or methanogens syntrophically in the alkanes-degrading processes. The presence of this bacterium in similar environments is also reported by others (Sekiguchi et al., 1998; Magot et al., 2000; Pérez-Jiménez et al., 2001; Roest et al., 2005; Li et al., 2007, 2012; Mbadinga et al., 2012; Cheng et al., 2013b; Liu et al., 2014).

Since the general appearance as the majority both in T1-400, T2-AE, and T2-BC, *Thermodesulfovibrio* as a generalist may participate in the intermediate metabolism, such as the degradation of fatty acids. While *Anaerolineaceae* as a specialist should be a key player in the degradation process and may likely take part in the initial activation of alkane for degradation since the obvious increase in T2-AE in clear contrasting with OS, T1-400 and T2-BC. This seems logic because many researches have implied the potential of alkane degradation. *Chloroflexi* was firstly reported to be involved with toluene degradation in a 10-year toluene-degrading methanogenic consortium. Several researches also showed evidence that *Anaerolineaceae* was one of the frequently encountered microbial species in petroleum hydrocarbon conditions (Winderl et al., 2008; Savage et al., 2010; Gray et al., 2011; Sherry et al., 2013; Sutton et al., 2013; Liang et al., 2015). In an enrichment culture metabolizing low-molecular-weight alkanes (*n*-propane and *n*-pentane) under mesophilic sulfate-reducing conditions, *Anaerolineaceae* as one of the community members showed the potential of hydrocarbon degradation for this lineage organisms (Savage et al., 2010). In a recent study of methanogenic biodegradation of short-chain *n*-alkanes revealed that members of the *Anaerolineaceae* may either be directly involved in activation and biodegradation of *n*-octane and *n*-decane or act as scavengers of metabolic intermediates (Shahimin et al., 2016). Thus, comparative analysis of 16S rRNA genes sequenced from the cultures with and without alkanes amendment indicated that members of the family *Anaerolineaceae*, which constitutes more than 33% of the bacterial community 16S rRNA genes in *n*-alkanes amended culture, may play a key role in the anaerobic metabolism of long-chain alkanes in the long-term incubation.

The obligate anaerobic, non-photosynthetic and multicellular filamentous family *Anaerolineaceae* in *Chloroflexi* phylum contains seven isolated strains in the five genera (Sekiguchi et al., 2003; Yamada et al., 2006, 2007; Grégoire et al., 2011). When comparing *Anaerolineaceae* in T2-AE (KP109861) with the isolated strain, the highest identity is only at 91% with *Levilinea saccharolytica* strain KIBI-1^T (NR_040972), indicating that this *Anaerolineaceae* in petroleum hydrocarbon environment may be a new division different from the isolated strains. Interestingly, *Anaerolineaceae* clone (KP109861) in this research has a very high identity (99%) with the *Anaerolineaceae* clone (KJ432869) in our former *n*-alkanes-degrading consortium from oily sludge, suggesting that they should be in the same division within the family of *Anaerolineaceae*.

Methanoculleus in the Methanogenic Process from Long-Term Incubation

Methanogens in the long-term methanogenic alkanes-degrading enrichment culture experienced sharp changes especially in T2-AE sample (**Figure 5**). Both archaeal 16S rRNA gene clone library and functional gene *mcrA* clone library showed that the only methanogen in T2-BC sample was *Methanoculleus*. In contrast, methanogens in T2-BC were mostly *Methanolinea* (90.2%) and others were *Methanobacterium*, *Methanocella,* and *Methanomethylovorans* which was consistent with the archaeal community in the sample of OS and T1-400 while *Methanoculleus* was undetected. The phylum *Euryarchaeota* contains six methanogenic orders: *Methanomicrobiales*, *Methanobacteriales*, *Methanococcales*, *Methanosarcinales*, *Methanopyrales,* and *Methanocellales* (Garrity and Holt, 2001; Ferry, 2010). Both genera of *Methanolinea* and *Methanoculleus* are in *Methanomicrobiales* order. The succession of *Methanolinea* and *Methanoculleus* suggests a competitive growth relationship between these two hydrogenotrophic methanogens. Comparing to the isolated methanogens, the OTU of *Methanoculleus* in T2-AE shows a 99% identity with *Methanoculleus receptaculi*, while, OTU of *Methanolinea* in T2-BC has a 99% identity with *Methanolinea tarda*. *M. receptaculi* was reported to take acetate as the growth factor with minimal doubling time of 8.2 h (Cheng et al., 2008). *M. tarda* can use acetate, yeast extract or Coenzyme M stimulates as growth factors with minimal doubling time of 144 h (Imachi et al., 2008). Since acetate and a faster growth rate than *Methanolinea* was detected in T2-AE, it is natural that *Methanoculleus* finally turned to be the most dominant methanogens in T2-AE sample.

Possible Metabolic Pathway of Methanogenic Degradation of *n*-Alkanes

The presence of *assA* functional gene in the active enrichment cultures (T2-AE) suggests that fumarate addition should be involved in the initial activation of alkanes in this methanogenic alkanes-degrading culture (**Figure 6**). Moreover, the non-detectable *assA* functional gene in the background control (T2-BC) confirmed the activity of *assA* functional gene in T2-AE sample. By contrast, the total amount of *Bacteria* and *Archaea* gene copies in T2-AE sample were much higher than those in T2-BC by quantitative PCR analysis. All of these results demonstrate the methanogenic alkanes-degrading activities in T2-AE sample. After initial activation, alkanes converted to LCFAs and VFAs, including octadecanoate, hexadecanoate, isocaprylate, butyrate, isobutyrate, propionate, acetate, and formate. And this intermediate metabolites-degrading phase should be responsible mostly by *Chloroflexi* (more specifically, *Anaerolineaceae*) since the community genomic analyses indicated that most members in the phylum of *Chloroflexi* are anaerobic acetogenic microbes which also have the genomes of complete Wood-Ljungdahl pathway and beta-oxidation of saturated fatty acids (Hug et al., 2013). Growth of some *Methanoculleus* members requires acetate even though they do not convert it to methane (Barret et al., 2015) and they were reported to produce methane from H_2/CO_2 and from formate (Oren, 2014). Thus, in methanogenic phase, *Methanoculleus* as the methanogens generated methane through hydrogenotrophic methanogenesis or methylotrophic methanogenesis (**Figure 6**).

CONCLUSION

The obvious increase of *Thermodesulfovibrio* and *Anaerolineaceae* in alkanes-dependent methanogenic culture after a long-term of incubation showed that these two bacteria played a crucial role in the degradation process of alkanes. However, whether they act directly on the activation of alkanes is still not clear. It is quite certain that alkanes-degrading bacteria cooperated with methanogens *Methanoculleus* formed a new methanogenic *n*-alkanes-degrading microbial community in this long-term incubation consortium. Our knowledge about the key degraders in petroleum hydrocarbon system is still quite limited under

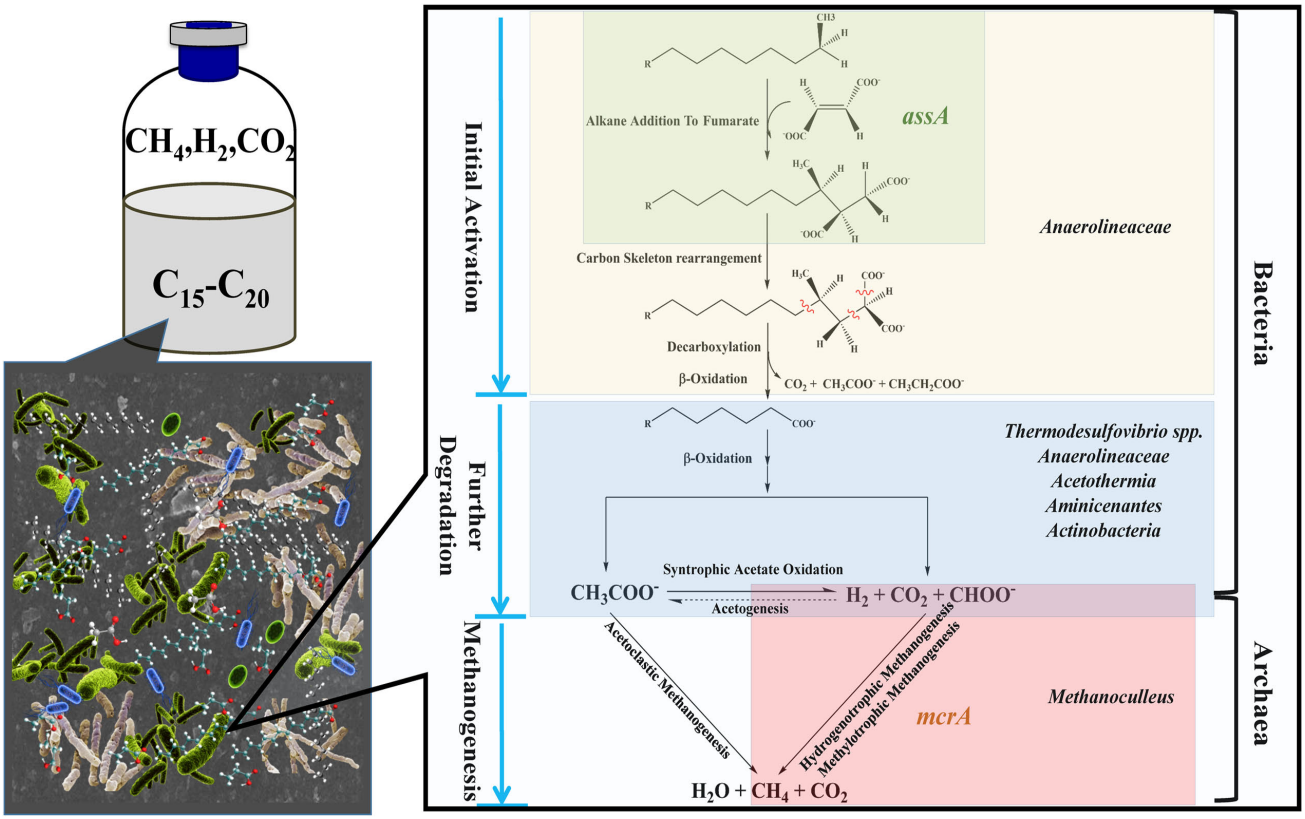

FIGURE 6 | Possible metabolic pathway of methanogenic degradation of *n*-alkanes, based on the pathway proposed by Wilkes et al. (2002) and Callaghan et al. (2012). In the methanogenic *n*-alkanes-degrading culture, metabolic pathway from *n*-alkanes to methane can be presumed. Fumarate addition could be involved in the initial activation of alkanes since the detection of *assA* functional gene. In the phase of methanogenesis, hydrogen, carbon dioxide, and formate were produced through syntrophic acetate oxidation pathway, followed by the production of methane through hydrogenotrophic or methylotrophic methanogenesis. Microorganisms are positioned according their probable role within microbial consortia.

anaerobic conditions, further researches should focus on unravel of the specific role of potential alkanes-degrading bacteria.

AUTHOR CONTRIBUTIONS

L-YW, J-DG, and B-ZM conceived the study. BL and L-YW performed all the experiments and drafted the manuscript. ZZ helped in the experimental part and data analysis. SM was involved in the discussion on the interpretation of the results. LZ helped the analysis of GC-MS data about residual *n*-alkanes and intermediate metabolites. J-FL and S-ZY were committed to all the experiments. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01431

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Community Structure in Methanogenic Enrichments Provides Insight into Syntrophic Interactions in Hydrocarbon-Impacted Environments

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The methanogenic biodegradation of crude oil involves the conversion of hydrocarbons to methanogenic substrates by syntrophic bacteria and subsequent methane production by methanogens. Assessing the metabolic roles played by various microbial species in syntrophic communities remains a challenge, but such information has important implications for bioremediation and microbial enhanced energy recovery technologies. Many factors such as changing environmental conditions or substrate variations can influence the composition and biodegradation capabilities of syntrophic microbial communities in hydrocarbon-impacted environments. In this study, a methanogenic crude oil-degrading enrichment culture was successively transferred onto the single long chain fatty acids palmitate or stearate followed by their parent alkanes, hexadecane or octadecane, respectively, in order to assess the impact of different substrates on microbial community composition and retention of hydrocarbon biodegradation genes. 16S rRNA gene sequencing showed that a reduction in substrate diversity resulted in a corresponding loss of microbial diversity, but that hydrocarbon biodegradation genes (such as *assA/masD* encoding alkylsuccinate synthase) could be retained within a community even in the absence of hydrocarbon substrates. Despite substrate-related diversity changes, all communities were dominated by hydrogenotrophic and acetotrophic methanogens along with bacteria including *Clostridium* sp., members of the Deltaproteobacteria, and a number of other phyla. Microbial co-occurrence network analysis revealed a dense network of interactions amongst syntrophic bacteria and methanogens that were maintained despite changes in the substrates for methanogenesis. Our results reveal the effect of substrate diversity loss on microbial community diversity, indicate that many syntrophic interactions are stable over time despite changes in substrate pressure, and show that syntrophic interactions amongst bacteria themselves are as important as interactions between bacteria and methanogens in complex methanogenic communities.

Keywords: methanogenesis, hydrocarbon biodegradation, syntrophy, microbial community composition, co-occurrence network analysis

INTRODUCTION

Since the dawn of the industrial age, widespread use and processing of petroleum products has led to an increase in the hydrocarbon contamination of a wide range of environments. Despite increasing environmental awareness and improved remediation technologies, contamination of the subsurface with hydrocarbon mixtures remains a problem, as the fate of hydrocarbons in the subsurface is not fully understood especially under anoxic conditions. The exposure of subsurface environments to heavy organic loads such as hydrocarbons leads to the rapid development of anoxic conditions in which the majority of hydrocarbon biodegradation is thought to proceed via methanogenesis (Jones et al., 2008). This process is also important in many fossil energy reservoirs, wherein hydrocarbon metabolism over geologic time has led to the accumulation of biogenic methane in gas caps overlying oil legs (Jones et al., 2008). Many studies have now demonstrated that diverse hydrocarbon substrates can be biodegraded under methanogenic conditions (e.g., as reviewed in Foght, 2008; Gray et al., 2010; Gieg et al., 2014).

Methanogenic hydrocarbon metabolism requires the presence of at least two groups of organisms in order to proceed in a thermodynamically favorable manner: the syntrophic bacteria that catalyze the activation and subsequent degradation of hydrocarbons to methanogenic substrates (e.g., acetate, formate, CO_2, and H_2), and methanogenic archaea that bioconvert these simpler substrates to CH_4 (plus CO_2 or H_2O). Methanogenic communities degrading hydrocarbon mixtures are typically diverse (Gray et al., 2010; An et al., 2013; Tan et al., 2015a), but how these microorganisms coordinate their metabolisms to utilize diverse hydrocarbons as carbon and energy sources and to conserve sufficient energy to support life is poorly understood (Gieg et al., 2014). Furthermore, the mechanisms involved in hydrocarbon activation are not fully understood, though fumarate addition has emerged as a key mechanism for the activation of aliphatic, substituted monoaromatic hydrocarbons, and substituted polycyclic aromatic hydrocarbons under various anaerobic electron-accepting conditions (Foght, 2008; Widdel and Musat, 2010; Callaghan, 2013). Alkylsuccinate synthase (encoded by the *assA*/*masD* gene; *assA* will be the designate name used in this study) is the key enzyme responsible for addition of alkanes to fumarate (Callaghan et al., 2008; Grundmann et al., 2008), while benzylsuccinate synthase (*bssA*) adds fumarate to substituted aromatic hydrocarbons (Heider, 2007).

In this study, we describe four new methanogenic enrichment cultures that were used to assess community changes as a result of decreased substrate diversity and that were probed for the presence of fumarate addition genes. Two cultures degrading the long-chain fatty acids (LCFA) palmitate and stearate were established from a whole crude oil-degrading methanogenic culture (Gieg et al., 2008) as the inoculum. The LCFA-degrading cultures were subsequently transferred to their parent alkanes, hexadecane and octadecane, in order to see if these cultures maintained the ability to degrade the hydrocarbon substrates present in the original oil degrading culture after long-term incubation on LCFA. All of these cultures (including the whole crude oil-degrading culture) were subjected to pyrotag sequencing of the 16S rRNA gene. We hypothesized that variations in the microbial community composition would be related to the specific carbon substrate supplied, which could provide clues to the identity of hydrocarbon-degraders in the cultures. We expected that the crude oil-degrading culture, which is exposed to a diverse hydrocarbon mixture and is the original parent culture, would be the most biodiverse of the cultures. LCFA- and *n*-alkane-amended cultures were expected to exhibit less diversity due to the restriction of carbon and energy sources within the culture, and the dilution effects of successive transfers. We further postulated that community members that were maintained across the majority of cultures over time likely play fundamental roles in the syntrophic degradation of shared metabolic products such as fatty acids, acetate, and formate. In light of this, we conducted a co-occurrence network analysis including community members that were retained across the different cultures in an attempt to establish an understanding of the syntrophic interactions occurring in the cultures.

A better understanding of methanogenic hydrocarbon metabolism could lead to the improvement of biotechnological applications for *in situ* bioremediation and for the bioconversion of residual oil to methane as a tertiary energy recovery strategy from fossil-energy reservoirs. Furthermore, insight into the syntrophic lifestyle can help shed light on novel mechanisms for interspecies communication or coordination, interspecies electron and metabolite transfer, and energy conservation in low energy-yielding environments.

MATERIALS AND METHODS

Culture Incubations

The inoculum for the cultures described herein was initially derived from gas condensate-contaminated aquifer sediments that were found to biodegrade whole crude oil under methanogenic conditions (Townsend et al., 2003). This original culture was subsequently amended with crude oil-containing crushed sandstone reservoir core material as previously described (Gieg et al., 2008) and was found to utilize *n*-alkanes (C_{12}–C_{29}) in whole crude oil; the culture is referred to herein as the residual oil culture. Based on previous reports suggesting relationships between the degradation of *n*-alkanes and their corresponding fatty acids (e.g., Aeckersberg et al., 1998), we hypothesized that the residual oil culture would have the ability to utilize LCFA. Thus, in 2008 the residual oil culture was used to establish new enrichments amended with palmitate or stearate. These LCFA were selected because they represented the corresponding fatty acids to *n*-alkanes (C_{16} and C_{18}) in the mid-range of the alkane fraction biodegraded by the residual oil culture (Gieg et al., 2008). Initial incubations showed that palmitate and stearate were metabolized based on the visual disappearance of the waxy substrates, and increased methane production relative to controls (not shown). Since then, these LCFA-degrading enrichments have undergone repeated substrate amendment with 30 μmol of stearate or palmitate as needed and had been transferred three times since their establishment

(30–50% v/v transfer) prior to conducting the work described herein. The hexadecane- and octadecane-amended cultures were subsequently established in 2011 from these LCFA-degrading cultures by inoculating substrate-depleted palmitate- and stearate-degrading cultures with 0.03 g (133 μmol) hexadecane (added neat) or 0.03 g (118 μmol) octadecane, dissolved in 2,2,4,4,6,8,8-heptamethylnonane (HMN; 0.5 g/mL). The reason for the use of HMN, an inert hydrocarbon carrier, is that octadecane is a solid at room temperature (unlike hexadecane), and was thus difficult to amend to sealed serum bottles without first being dissolved in a solvent. These *n*-alkane-amended cultures underwent a single transfer to new medium (50% v/v) and substrate amendment as described above prior to the analyses described here. All cultures were established and maintained in glass serum bottles containing anoxically prepared bicarbonate-buffered minimal salts freshwater medium with 0.01% resazurin as a redox indicator and 2.5% v/v cysteine sulfide as the reductant (Fowler et al., 2012). Incubations were sealed with butyl rubber stoppers and aluminum crimps, and contained a CO_2/N_2 (20/80 vol%) headspace. All substrate-amended incubations were established at least in triplicate. Parallel substrate-unamended and sterile substrate-containing controls were also established.

Methane Measurement

Methane was routinely monitored in all cultures and controls (as a surrogate for substrate utilization) using a Hewlett-Packard model 5890 Series gas chromatograph (GC) equipped with a flame ionization detector (200°C) with helium as the carrier gas. Headspace gas was sampled using a sterile 1 mL syringe flushed with 10% CO_2 in N_2 (Fowler et al., 2012). Injections were carried out at 150°C onto a packed stainless steel column (18″ long × 1/8″ i.d., Poropak R 80/100, Supelco) held isothermally at 100°C.

DNA Extraction, PCR, and Pyrotag Sequencing

DNA was extracted using a modified phenol–chloroform method with bead beating. Cells (6 mL total) were repeatedly centrifuged at 18 000 × *g* for 10 min to pellet cells in 2 mL bead beating tubes containing 0.3 g of 0.01 mm and 0.1 g of 0.5 mm zirconia/silica beads (BioSpec Products). Cells were resuspended in 300 μL of lysis buffer (500 mM Tris, 100 mM NaCl, 10% SDS, pH 8) and 300 μL of chloroform-isoamyl alcohol (24:1). Bead beating was carried out at 6.0 m/s for 45 s. DNA was extracted by phenol chloroform-isoamyl alcohol extraction followed by RNAse and proteinase K treatment and a final phenol then chloroform-isoamyl alcohol extraction step. DNA was precipitated in sodium acetate (3 M, pH 7) and cold 100% ethanol at 18 000× *g* for 20 min. Pellets were washed with cold 70% ethanol and resuspended in nuclease-free water (Fowler et al., 2012). Genomic DNA was found to be within the range of 1.41–9.49 ng/μL using Qubit fluorometry (Invitrogen).

Pyrotag sequencing was carried out following a two-step PCR method. In the first PCR step, DNA was amplified using universal primers 926F (AAACTYAAKGAATTGACGG) and 1392R (ACGGGCGGTGTGTRC) targeting the V6, V7, and V8 regions of the 16S rRNA gene in 25 μL reactions containing 2x PCR Master Mix (Fermentas), 0.2 μM of each primer and 1 μL of DNA using the following thermocycling protocol: 95°C 3 min; 25 cycles of 95°C (30 s), 55.0°C (45 s), 72.0°C (90 s); final extension 72°C 10 min. In order to attach barcode and adaptor sequences for 454 multiplex sequencing, a secondary PCR was carried out with primers 454T-FB-926F (which included the 25 bp B-adaptor sequence CTATGCGCCTTGCCAGCCCGCTCAG 5′ to the primer sequence) and 454T-FA-1392R (which included the 25 nt A-adaptor sequence CGTATCGCCTCCCTCGCGCATCAG and a variable 10 nt barcode sequence 5′ to the primer sequence). Reactions were prepared as above with modified thermocycling conditions: 95°C 3 min; 10 cycles of 95°C (30 s), 55.0°C (45 s), 72.0°C (90 s); final extension 72°C 10 min.

All PCR products containing adaptors and barcodes were then purified using a commercially available kit (Qiagen PCR Purification Kit). Amplicons were quantified by Qubit fluorometry (Invitrogen) according to the manufacturer's protocol and were sequenced by 454 sequencing at the McGill University and Genome Quebec Innovation Centre (Montreal, Canada) using a GS FLX Titanium Series Kit XLR70 (Roche). Microbial community sequencing data are available in GenBank under the accession numbers SRR090429–SRR090434 for the residual oil and palmitate/stearate-degrading enrichments and under SRX1585942 (C_{16}) and SRX1585943 (C_{18}) for the *n*-alkane-amended enrichments.

Bioinformatics Analysis

Analysis of 16S rRNA gene pyrotag data was carried out using the Phoenix 2 pipeline (Soh et al., 2013). Briefly, quality control was performed to remove low quality and chimeric sequences. Dereplication was performed (99% identity threshold) and sequences were clustered into OTUs at 3% distance using the average linkage algorithm. OTUs were mapped to taxa using the RDP classification algorithm within the SILVA training dataset (Pruesse et al., 2007). Biodiversity and other statistical measures were generated using mothur commands (Soh et al., 2013). In the results shown here, taxa comprising a single read were excluded from analysis as singleton and rare OTUs can be the result of sequencing errors, and rare taxa were not the focus of this analysis. Within Phoenix 2, Pearson correlations were calculated among shared OTUs within the five cultures. Microbial co-occurrence networks were constructed and visualized in Cytoscape v3.2.1 (Shannon et al., 2003) with OTUs that occurred in a minimum of three of five cultures with a minimum total abundance of 50 reads, and a Pearson correlation of at least 0.75.

Alkylsuccinate Synthase Gene Amplification and Sequencing

Eight established alkylsuccinate synthase (*assA*) and five benzylsuccinate synthase (*bssA*) gene primers, and their respective reaction thermocycling conditions, were used to probe extracted DNA for the presence of fumarate addition enzymes (Washer and Edwards, 2007; Callaghan et al., 2010). PCR reactions were prepared with 12.5 μL 2x PCR Master

Mix (Fermentas), 9 μL RNase free water, 0.5 μL each of a forward gene primer and corresponding reverse primer (10 μM), and 1 μL of template DNA. Amplicons of expected size were verified on a 1% agarose gel and subsequently purified using the QiaQuick PCR Purification Kit (Qiagen) according to the manufacturer's protocol. The resulting purified amplicons were directly sequenced and queried against the NCBI non-redundant nucleotide database using BLASTN to identify homology to known sequences. A single *assA* gene fragment (523 bp; 393 bp after removing low quality nucleotide sequence ends) was amplified from each of the residual oil-, stearate-, and octadecane-amended enrichment cultures using primers 1432F and 1936R described by Callaghan et al. (2010).

Multiple alignments of the amplified sequences and representative sequences covering the same region were generated using the T-Coffee algorithm within the Centre for Genomic Regulation database (Notredame et al., 2000). Bootstrapped maximum likelihood trees (100 replicates) were constructed in MEGA6 (Tamura et al., 2013). A consensus tree was constructed using the Tamura–Nei model (Tamura and Nei, 1993) with complete deletions. Sequences for the *assA* genes reported in this study are available in GenBank under the accession numbers KU094062, KU094063, and KU094064.

RESULTS

Methane Production from LCFA and Hydrocarbons

Methane production was monitored following routine transfer and substrate amendment of the LCFA- and *n*-alkane-amended cultures, shown in **Figure 1**. Over the course of a 340-day incubation, the LCFA-degrading cultures appeared to completely consume their respective substrate based on a visual inspection wherein the waxy white substrate particles completely disappeared (relative to sterile controls). Based on the amounts of methane measured (**Figure 1A**) and the theoretical stoichiometric equations (Eqs. 1 and 2; Symons and Buswell, 1933) for the production of methane from 30 μmol of LCFAs, approximately 84 and 98% of palmitate or stearate, respectively, were metabolized via methanogenesis.

$$C_{16}H_{32}O_2 + 7H_2O \rightarrow 4.5CO_2 + 11.5CH_4 \text{ (Palmitate)} \quad (1)$$

$$C_{18}H_{36}O_2 + 8H_2O \rightarrow 5CO_2 + 13CH_4 \text{ (Stearate)} \quad (2)$$

Similar amounts of methane were produced from these cultures over successive transfers (data not shown). These results are in line with previous work on methanogenic cultures, where about 64–98% of theoretically predicted methane is produced (assuming 100% conversion of substrates to methane). The balance of carbon presumably goes to the production of biomass, with a small amount being lost during headspace sampling and due to adsorption to the stopper (Stadtman and Barker, 1951; Zengler et al., 1999; Fowler et al., 2012). Substrate-unamended cultures produced 0 μmol (palmitate) and 41 μmol (stearate) methane.

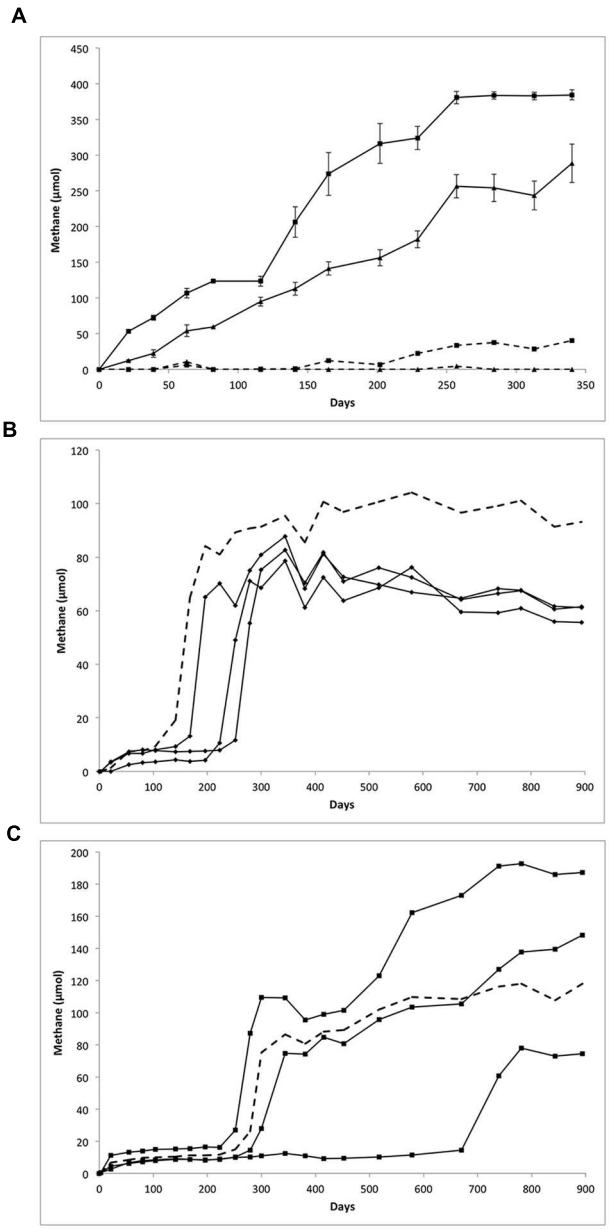

FIGURE 1 | Methane production from the biodegradation of (A) LCFAs, (B) hexadecane, and (C) octadecane. (A) Methane production from palmitate (triangles) and stearate (squares) amended cultures over 340 days. The 30 μmol of palmitate or stearate was added on day 0, resulting in the production of 289 μmol of methane from palmitate (mean of five replicates) and 384 μmol of methane from stearate (mean of six replicates). Error bars show standard error. Dotted line represents substrate unamended control cultures. **(B)** Methane production from hexadecane amended culture over the course of 894 days; replicates were plotted individually (diamonds). Cultures were amended with 133 μmol of hexadecane (0.03 g) resulting in the production of an average of 60 μmol of methane. **(C)** Methane production from octadecane amended cultures over 894 days; replicates were plotted individually (squares). Cultures were amended with 118 μmol of octadecane on day 0 resulting in the production of methane ranging from 75 to 187 μmol. In **(B,C)**, methane production from unamended controls (dotted lines) may be related to the degradation of hydrocarbon substrate carried over during culture transfer or to the presence of cysteine added as a reductant.

The primary transfers of the *n*-alkane amended cultures produced variable amounts of methane in different replicates (**Figures 1B,C**). A long lag period was observed prior to methane production ranging from 141 days up to almost 600 days for one octadecane-amended enrichment. Methane production in the hexadecane-amended replicates was fairly uniform after 894 days of incubation, yielding approximately 60 μmol CH_4 (**Figure 1B**). Methane production in the octadecane-degrading cultures was more varied; 187, 148, or 75 μmol CH_4 were produced over 894 days from each of the three replicates (**Figure 1C**).

Methane production in the corresponding unamended controls incubated alongside the *n*-alkane-amended cultures was substantial (93.1 μmol and 118.0 μmol). This effect may be related to carryover of any undegraded hydrocarbon substrate when the cultures underwent primary transfer. However, a possible alternative carbon source for methane production is the cysteine present in the 2.5% cysteine sulfide added to these cultures as a reductant, which has been shown to be metabolized in other methanogenic hydrocarbon-degrading enrichment cultures (Toth, unpublished results). Based on the theoretical stoichiometric conversion of cysteine to methane ($4C_3H_7NO_2S + 6\ H_2O + 4H^+ \rightarrow 4H_2S + 4NH_4^+ + 5CH_4 + 7CO_2$), the amount of cysteine sulfide added as a reductant could result in the production of up to 107 μmol methane in each of the cultures. Given these calculations, methane production from palmitate-, stearate-, and octadecane-amended cultures exceeded the methane expected from cysteine sulfide alone, strongly suggesting that the LCFAs or octadecane are serving as the substrates for methane production. Methane production in the hexadecane-amended cultures, however, did not exceed 107 μmol, thus it cannot be concluded that hexadecane served as a substrate for methane production in this culture.

Microbial Community Dynamics in Methanogenic Cultures

Pyrotag sequencing of the 16S rRNA gene was carried out to examine and compare the microbial communities in each of the cultures (**Table 1**). The residual oil culture, which served as the inoculum source for the palmitate and stearate cultures, was included in this analysis to determine whether or how transfers onto single carbon sources impact the microbial community composition. Quality controlled reads were clustered into OTUs at a 3% distance. Rarefaction analysis revealed that at a clustering distance of 3%, none of the samples were sequenced to saturation, however, at a clustering distance of 5%, the samples were approaching saturation (Supplementary Figure S1, top). The number of OTUs observed in each of the cultures (3% distance) varied considerably. The residual oil culture harbored the greatest number of OTUs, and observed OTU numbers decreased following the order in which the cultures were successively enriched [e.g., residual oil (297) → LCFA (151/164) → *n*-alkane (114/120); **Table 1**]. The Chao index, which estimates the actual number of OTUs in each sample, also indicated that the residual oil culture was the most diverse, and that diversity decreased with each enrichment step. As expected from the observed OTUs and Chao estimates, as well as the diversity of hydrocarbon substrates and initial culture for enrichment, the Shannon and Simpson diversity indices also indicated that the residual oil culture was the most diverse of the cultures (**Table 1**). The octadecane-amended culture also had high Shannon and low Simpson values, despite comprising lower observed OTUs and Chao values relative to the LCFA and hexadecane-amended cultures. This is due to greater evenness in this culture (**Table 2**). As expected, Bray–Curtis dissimilarity analysis revealed that the microbial communities of the hexadecane- and octadecane-amended cultures were most closely related to each other, and the stearate- and palmitate-degrading cultures were also closely related to one another, while the residual oil degrading culture was the most distantly related culture (Supplementary Figure S1, bottom).

All five cultures were dominated by members of the Firmicutes making up between 37.8 and 69.8% of the sequence reads from each culture (**Figure 2**). The highest abundances of Firmicutes were found in the LCFA-degrading cultures, with 57.9 and 69.8% in the palmitate- and stearate-degrading cultures, respectively (**Figure 2**). Other dominant phyla included the Euryarchaeota and Deltaproteobacteria. Together, members of these three phyla comprised at least 80.2% (residual oil) and up to 96.8% (hexadecane) of each of the microbial communities (**Figure 2**). Euryarchaeota (methanogens) were particularly abundant in the hexadecane- and octadecane-amended cultures where they made up 49.8 and 45.4% of the reads, respectively. Aside from the residual oil culture that exhibited the highest richness and evenness, the only other phylum that made up greater than 1% of the community in any culture was Chloroflexi (**Figure 2**). In all cultures, the Chloroflexi were dominated by a single OTU affiliating with *Anaerolineaceae*. At lower taxonomic levels, *Clostridium* sp. was the most abundant genus in all cultures, comprising between 30.4 and 60.4% of the sequence reads for each culture (**Table 2**). In the residual oil culture, the next most abundant organism was *Smithella* sp. (16.1%). Though *Smithella* sp.

TABLE 1 | Features of 16S rDNA pyrosequencing and alpha diversity statistics based on analysis at 0.03 distance for all methanogenic cultures analyzed in this study.

Feature/Culture	Residual oil	Palmitate	Stearate	Hexadecane	Octadecane
Reads (pre/post QC)	13381/7621	8294/4806	9501/6051	21575/19202	10200/8699
#OTUs observed	297	151	164	114	120
Shannon	3.15	1.92	1.96	1.82	2.28
Simpson	0.12	0.31	0.37	0.28	0.21
Chao	817	313	292	197	185

TABLE 2 | Relative abundance (%) of most abundant taxa in methanogenic cultures as determined by 16S rRNA gene pyrosequencing.

Taxon	Residual oil	Palmitate	Stearate	Hexadecane	Octadecane
Clostridium	30.4	52.1	60.4	43.0	42.0
Methanoculleus	3.2	1.0	2.5	6.1	15.4
Methanolinea	3.5	0	0.2	0	13.3
WCHA1-57	0.2	0.022	0.3	3.5	6.9
Methanosaeta	12.0	8.0	10.9	31.3	6.3
Desulfovibrio	3.7	0.6	0.9	1.0	2.3
Candidatus Methanoregula	0.7	12.9	1.2	8.5	2.2
Ruminococcaceae	0.2	0.7	1.3	0.9	2.1
Anaerolineaceae uncultured	5.7	2.0	8.7	1.4	1.8
Synergistaceae uncultured	0.03	0.23	0.3	0.5	1.6
Geobacter	0.4	0.1	0.3	1.2	0.8
Spirochaetaceae uncultured	2.2	0.6	1.1	0.1	0.6
Lachnospiraceae Incertae Sedis	0.0	1.1	4.4	0.08	0.2
Smithella	16.1	15.4	2.0	0.05	0.3
Anaerobacter	1.0	1.2	1.2	0.3	0.20
Thermosinus	0.04	0.4	1.0	0.005	0.01
Enterobacter	3.4	0	0	0	0
vadinHA17	1.6	0	0	0.9	0.1
Sedimentibacter	1.4	0.8	0.3	0.06	0.09
Proteiniphilum	1.2	0.4	0.4	0.1	0.2
Total	**87.0**	**97.4**	**97.1**	**99.0**	**96.5**

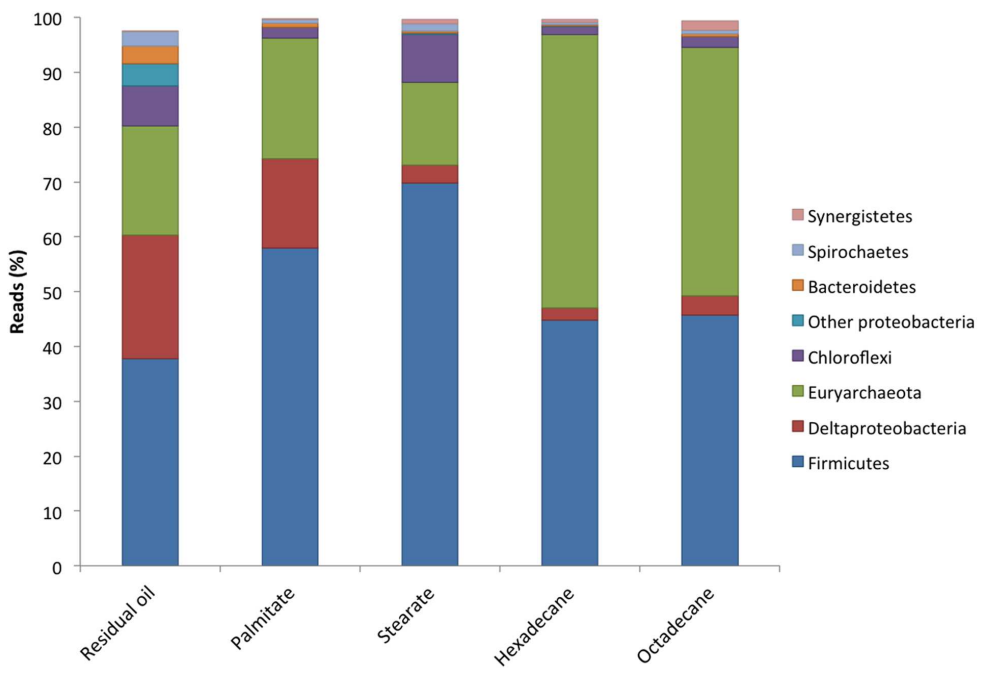

FIGURE 2 | Microbial community compositions at the phylum level of the methanogenic enrichment cultures based on 16S rRNA gene pyrosequencing.

was also abundant in the palmitate-amended culture (15.4%) it was far less abundant in the stearate-, octadecane-, and hexadecane-amended cultures (**Table 2**). Several genera of methanogenic archaea also comprised considerable fractions of the microbial communities, particularly in the *n*-alkane-amended cultures. These included hydrogenotrophic (*Methanoculleus*, *Methanolinea*, and *Methanoregula*) and acetotrophic (*Methanosaeta*) methanogens. Interestingly, the acetotrophic methanogen *Methanosaeta* was the most abundant type of methanogen in the residual oil-, palmitate-, stearate-, and hexadecane-amended enrichments, while hydrogenotrophic methanogens *Methanoculleus* and *Methanolinea* dominated in the octadecane-amended enrichment. A methylotrophic methanogen was also detected at comparatively lower abundance in all cultures (*Methanomethylovorans*), but was most abundant in the *n*-alkane-amended cultures (0.14–0.62% of hexadecane and octadecane communities, respectively). In addition, the archaeon WCHA1-57 (possibly a novel lineage of methanogens, Saito et al., 2015) was also particularly abundant in the octadecane degrading culture (**Table 2**).

Microbial Co-occurrence Network Analysis

Microbial co-occurrence analysis was conducted with OTUs that were present in at least three samples with a total minimum abundance of 50 reads and a positive Pearson correlation greater than 0.75. This included 37 OTUs, with a total of 155 interactions. Co-occurrence analysis revealed the presence of three distinct networks within the samples (**Figure 3**). The first network contains 18 OTUs, and consists primarily of hydrogenotrophic (*Methanoculleus*, two OTUs; *Methanolinea*, two OTUs; *Methanoregula*, one OTU), acetotrophic (*Methanosaeta*, four OTUs) and methylotrophic (*Methanomethylovorans*, one OTU) methanogenic archaea and one OTU related to the archaeon WCHA1-57 of the *Thermoplasmata*. This network also includes seven syntrophic bacteria including a highly abundant OTU corresponding to *Clostridium* sp. as well as a *Clostridiaceae*, *Geobacter* spp. (three OTUs), and OTUs most closely related to members of the *Synergistaceae* and *Ruminococcaceae*. Some of the strongest interactions in this network occur between methanogens and syntrophic bacteria, likely related to the transfer of hydrogen or formate, acetate and/or electrons from the syntroph to the methanogen. However, there are also interactions between methanogenic OTUs with particularly strong correlations between an OTU related to *Methanomethylovorans* with two *Methanoculleus* OTUs. Interestingly, this network actually consists of two interconnected networks linked by the two *Clostridium/Clostridiaceae* OTUs, suggesting a central role for *Clostridiaceae* in the interaction with methanogens in these cultures.

The second network consists primarily of syntrophic bacteria from diverse phyla, as well as a single methanogen OTU affiliating with the hydrogenotroph *Methanolinea* (**Figure 3**). The 14 bacterial OTUs identified are members of the Deltaproteobacteria (five OTUs), Chloroflexi (three OTUs), Firmicutes (two OTUs), Bacteroidetes (two OTUs), Actinobacteria (one OTU), and Spirochaetes (one OTU). With the exception of one *Smithella* OTU, which is only connected to one other *Smithella* OTU, there is dense and strong connectivity within this network and all other OTUs have between 9 and 13 connections. The number and the strength of the interactions within this network suggest that these OTUs play central and cooperative roles in the degradation of hydrocarbon or LCFA substrates in these cultures. The third network consists of four bacterial OTUs comprising three OTUs affiliated with *Clostridiales* (two uncultured *Lachnospiraceae* OTUs, and one *Ruminococcaceae*), as well as one OTU affiliated with *Anaerolineaceae* (Chloroflexi). The organisms in this network were not highly abundant (60–277 reads) but all of the OTUs were found to strongly co-occur with one another, indicating the presence of a second bacterial cooperative metabolic network, this time with the complete absence of methanogens (**Figure 3**).

Detection of Fumarate Addition Genes

Established alkylsuccinate synthase (*assA*) and benzylsuccinate synthase (*bssA*) primer sets were used to probe extracted metagenomic DNA from all cultures (including the residual oil culture) for the presence of fumarate addition genes (Washer and Edwards, 2007; Callaghan et al., 2010). A single *assA* gene fragment was amplified from each of the residual oil-, stearate-, and octadecane-amended enrichment cultures. There was 99.4% nucleotide sequence identity among the gene fragments, suggesting that all three amplicons belong to the same species. These results show that alkane biodegradation potential (via fumarate addition) was maintained across culture transfers despite changes in carbon substrate. Notably, the *assA* gene was undetectable in palmitate- and hexadecane-enriched microcosms; these findings substantiate the observation that hexadecane did not appear to be metabolized to methane in this study (**Figure 1B**). Further, *bssA* could not be detected in any sample with the evaluated primer sets.

Maximum likelihood trees of *assA* gene fragments revealed that sequenced amplicons were most closely related to three identical uncultured prokaryote clones (99% sequence similarity) isolated from sulfate-reducing, alkane-degrading River Tyne sediments (Sherry et al., 2013) (**Figure 4**). The *assA* gene fragments also clustered closely to those retrieved from other anaerobic long chain alkane-degrading enrichment cultures (C_{15}–C_{20}; SL34 enrichment OTUs, Mbadinga et al., 2012) and an oil seep (von Netzer et al., 2013).

DISCUSSION

Reports of methane generation from *n*-alkanes, and the description of the communities mediating these transformations have become increasingly widespread in recent years (e.g., Zengler et al., 1999; Gieg et al., 2008; Jones et al., 2008; Callaghan et al., 2010; Sherry et al., 2013; Berdugo-Clavijo and Gieg, 2014; Abu Laban et al., 2015; Bian et al., 2015; Tan et al., 2015b). Alkanes comprise an abundant fraction of many crude oils, thus their biodegradation under anaerobic conditions is of practical relevance to biotechnological applications in fossil

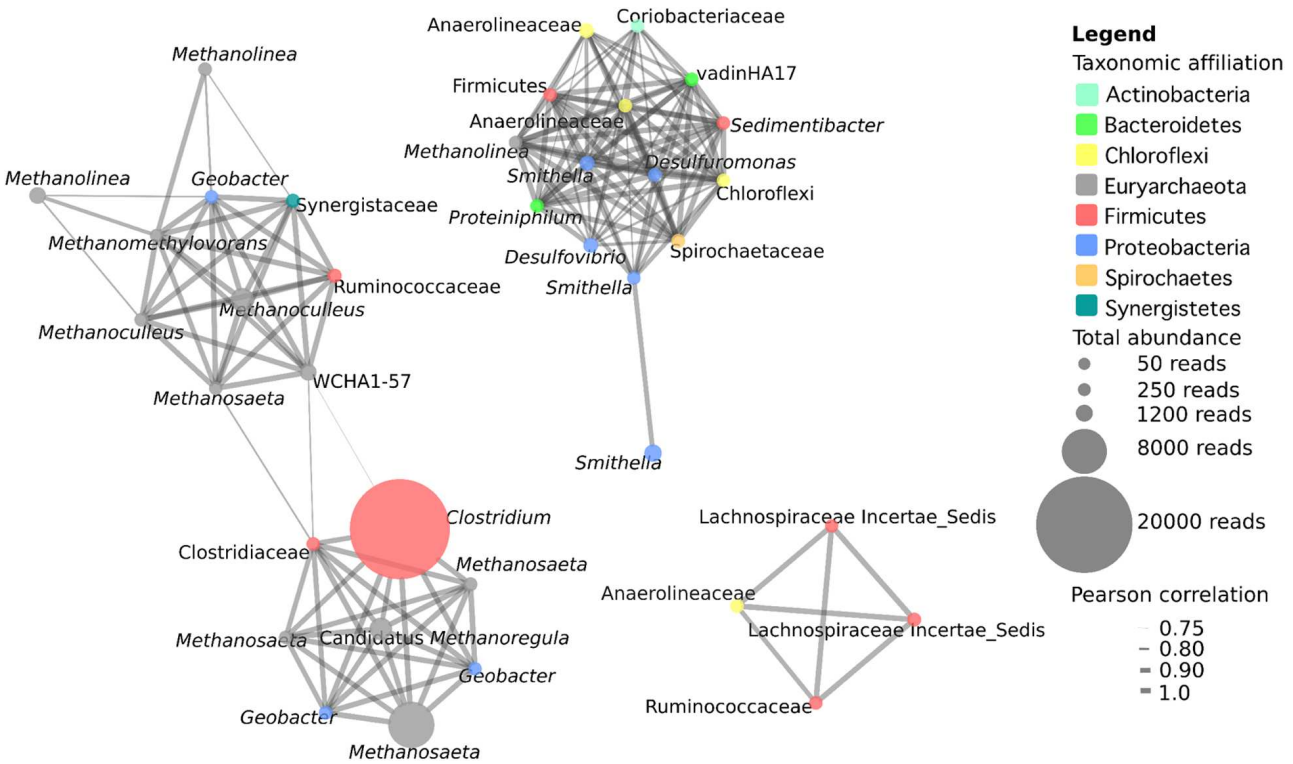

FIGURE 3 | Microbial co-occurrence network from five methanogenic enrichment cultures. OTUs present in a minimum of three cultures at a minimum abundance of 50 reads with a positive Pearson correlation of at least 0.75 were included in the analysis. The size of a node represents the abundance of the OTU across the five samples, and the width of the edges represents the strength of the correlation.

energy reservoirs and fuel-contaminated sites. There remains much to be learned with regards to the pathways, enzymes, and genes involved in strictly anaerobic alkane degradation, as well as the organisms and interactions amongst organisms that methanogenically metabolize hydrocarbons.

In this study, we describe new methanogenic LCFA- and *n*-alkane-degrading cultures, including analysis of their community structure and amplification of known biodegradative genes. Co-occurrence network analysis of the microbial communities of the five related cultures was conducted in order to make a first step in unraveling syntrophic interactions in methanogenic hydrocarbon-degrading systems. As the downstream degradation of both alkanes and fatty acids proceed via a common pathway – β-oxidation, followed by conversion to methanogenic substrates and methane production (Callaghan, 2013) – syntrophic interactions are expected to be similar regardless of the hydrocarbon or fatty acid substrate being degraded. We propose that over time, stable and efficient syntrophic interactions have evolved within the microbial community and that these interactions are fairly resilient to the substrate being degraded.

Degradation of crude oil components was previously demonstrated by the source inoculum for the cultures described herein (Gieg et al., 2008). This culture was subsequently transferred to the LCFAs palmitate and stearate, and then these cultures were transferred to their respective parent alkanes, hexadecane and octadecane, to examine the effects of different substrates on microbial community structure and to determine whether the ability to degrade hydrocarbons was maintained following long-term incubation on LCFAs. Over several years of routine culture transfer and substrate amendment, the LCFA-degrading cultures typically converted approximately 84–98% of their fatty acid substrates to methane (plus CO_2 or H_2O; **Figure 1A**). Alkane cultures were subject to a single culture transfer, after which methane production was monitored for close to 900 days. After an extended lag period, methane generation from octadecane became apparent (**Figure 1C**), while the degradation of hexadecane could not be confirmed (**Figure 1B**) because the methane production did not exceed the maximum amount of methane that could be derived from the reductant, cysteine sulfide. Lag periods exceeding several weeks or months have been reported for other methanogenic hydrocarbon-degrading cultures (Edwards and Grbić-Galić, 1994; Townsend et al., 2003; Berdugo-Clavijo and Gieg, 2014). This delay may be related to a toxic effect as inhibition of microbes by hydrocarbon substrates has been well documented and is thought to be related to interference with biological membranes (Sikkema et al., 1995).

The methane production observations aligned with the results of fumarate addition gene amplification efforts. Alkylsuccinate synthase, the enzyme involved in anaerobic *n*-alkane activation via addition to fumarate, was previously detected in the

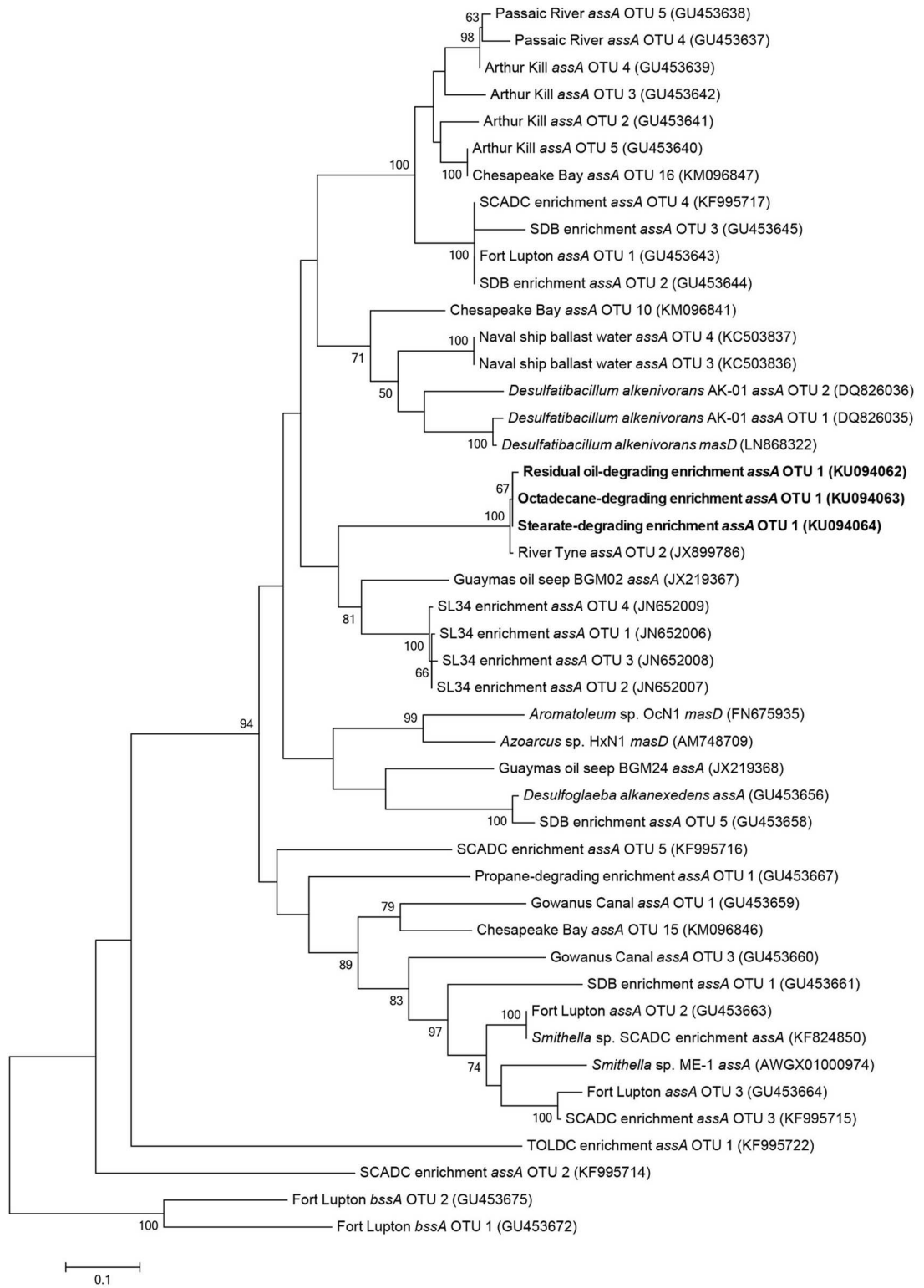

FIGURE 4 | Maximum likelihood tree showing the affiliation of *assA* gene fragments (this study, bold) with previously published reference strains, enrichment cultures, and environmental samples. Evolutionary analyses of aligned nucleotide sequences (320 bp) were conducted in MEGA6 (Tamura et al., 2013). The consensus tree was constructed using the Tamura–Nei model (Tamura and Nei, 1993) with complete deletions (for a total of 288 positions in the final dataset) and performing 100 bootstrap replicates. Bootstrap values below 50% are not shown. Benzylsuccinate synthase (*bssA*) genes cloned from gas condensate-contaminated sediments near Fort Lupton, CO, USA (Callaghan et al., 2010) were used to root the tree.

sediments from which these cultures were initially derived (Callaghan et al., 2010). In the present study, we detected *assA* amplicons, all presumably derived from a single species, in the residual oil-, stearate-, and octadecane-amended cultures. These results show that the potential ability to biodegrade hydrocarbons can be retained within a syntrophic microbial community even following the long-term absence of hydrocarbon exposure. However, gene detection does not indicate actual expression, thus further studies will be required to confirm that this gene is actually expressed during biodegradation under these different substrate conditions. In contrast, the *assA* gene was not detected in the palmitate- or hexadecane-amended cultures (**Figure 4**), for reasons that are not clear given its detection in the stearate and octadecane enrichments. Palmitate metabolism does not require *assA*, thus a simple explanation is that the gene was lost (i.e., the species harboring this gene was lost) upon transfer from the residual oil culture to palmitate. This explains why a subsequent transfer of the palmitate-degrading culture onto hexadecane did not lead to the biodegradation of this *n*-alkane (**Figure 1B**). Another possibility is that the specific *assA*-containing organism in these cultures is involved in the degradation of longer chain alkane substrates, and was thus not capable of hexadecane degradation. If this were true, it would indicate a distinct difference between the fumarate addition genes involved in the degradation of octadecane and higher alkanes, and hexadecane and shorter alkanes. While the necessary evidence to fully test this hypothesis is not yet available due to a shortage of *assA* gene sequences with known substrate range, this idea was previously postulated for the *assA* genes involved in the degradation of short chain alkanes (*n*-C_3-C_{10}; Callaghan et al., 2010; Tan et al., 2015a). A distinction between hexadecane and octadecane would not be particularly surprising, as hexadecane is a liquid at ambient temperature, while octadecane is a solid, making the bioavailability of each different in an aqueous environment under mesophilic conditions. A similar hypothesis was made for gaseous alkane *assA* being phylogenetically distinct from non-gaseous alkane fumarate addition enzymes (Musat, 2015). Phylogenetic analysis in the present study of the amplified *assA* genes with known references and environmental samples did not reliably pinpoint the organism(s) harboring this *assA* gene. While the phylogeny of *bssA* (encoding the alpha subunit of benzylsuccinate synthase) is now generally well enough resolved to infer the clade involved in aromatics activation (von Netzer et al., 2013), this is not yet the case for *assA* (**Figure 4**). In our phylogenetic analysis, as in others (Callaghan et al., 2010), members of the Deltaproteobacteria (*Desulfoglaeba* sp.) grouped more closely with Betaproteobacteria alkane degraders (*Azoarcus* sp., *Aromatoleum* sp.), than with other Deltaproteobacteria (*Desulfatibacillum* sp.) which may indicate that genes encoding alkylsuccinate synthase are more closely related based on the specific alkane substrates being degraded rather than phylogeny or that they are subject to a high degree of horizontal gene transfer (this is currently unknown). It remains to be seen if the purification of additional strictly anaerobic alkane degraders, or the improved description of alkane degraders from the environment or enriched cultures will eventually result in the ability to predict either the taxonomic affiliation of alkane degraders based on phylogenetic analysis of the *assA* gene and/or the substrate range of the degraders. Members of the Deltaproteobacteria are often cited as key alkane and/or fatty acid degraders in methanogenic cultures (e.g., members of the *Syntrophaceae* such as *Syntrophus*/*Smithella* spp., Gray et al., 2011; Cheng et al., 2013; Embree et al., 2014; Tan et al., 2014; Mathai et al., 2015) along with other sulfate-reducing alkane degrading Deltaproteobacterial isolates (Cravo-Laureau et al., 2005; Davidova et al., 2006; Callaghan et al., 2008). While *Smithella* sp. was abundant (16%) in the residual oil culture, it was present at <2% abundance in the stearate- and octadecane-degrading cultures (**Table 2**) and the recovered *assA* gene fragments did not cluster with the *assA* of known *Smithella* sp. (**Figure 4**). These findings suggest that this taxon is not the main stearate- or octadecane-degrading organism in these enrichments. No Deltaproteobacterial OTUs previously associated with hydrocarbon biodegradation were particularly enriched in the octadecane-amended culture (**Table 2**), suggesting that an as of yet unidentified alternate organism(s) catalyzes the activation of this *n*-alkane. The *bssA* gene was not detected in any of the enrichments, which was expected, as the residual oil-amended microcosms contained negligible concentrations of substituted monoaromatic hydrocarbon substrates such as toluene (Gieg et al., 2008).

In comparing the microbial communities of the five different cultures, we found that the microbial richness observed was related to the order in which the cultures were enriched, with the most highly enriched (*n*-alkane-incubated) cultures containing the lowest species richness (**Table 1**). A much greater richness and evenness was observed in the presence of more diverse hydrocarbon substrates as found in the residual oil-containing culture (**Tables 1** and **2**). In addition, there were substrate-specific variations in the microbial communities, with a particular enrichment of methanogens in the *n*-alkane-incubated cultures (**Table 2**). While observed and predicted richness decreased with degree of enrichment, evenness actually increased in both of the alkane-incubated cultures relative to the LCFA-degrading cultures (**Table 1**). Nonetheless, all cultures shared a similar microbial community structure and were dominated by members of the Firmicutes (*Clostridium* sp.), Deltaproteobacteria, and Euryarchaeota (mainly hydrogenotrophic and acetotrophic methanogens; **Figure 2**, **Table 2**). These findings are consistent with the previous clone library analysis of the residual oil culture (Gieg et al., 2008), and also of a toluene-degrading methanogenic culture derived from the same contaminated sediments (Fowler et al., 2012). This is not particularly surprising as the majority of known syntrophic bacteria are members of the Firmicutes or Deltaproteobacteria (Sieber et al., 2012), and the methanogenic archaea are members of the Euryarchaeota. The extremely high abundance of *Clostridium* sp. is also consistent with the community from the toluene degrading enrichment from the same sediments in which 30.7% of the culture was found to consist of *Clostridium* sp. (Fowler et al., 2012). While *Clostridium* sp. is an abundant organism in these new enrichment cultures, it is possible that its extremely high abundance is partly an artifact of PCR as *Clostridium*

spp. often have multiple copies of 16S rRNA genes with 14 copies having been observed in a single genome (Větrovský and Baldrian, 2013). The presence of a large *Clostridium* sp. OTU and several *Clostridiaceae* sp. OTUs in the network analysis, and their connectivity to methanogens in this analysis suggests that, despite their high abundance, this clade is not involved in hydrocarbon activation, but is involved in the downstream conversion of smaller molecules to methanogenic intermediates (**Figure 3**). This is also in agreement with the results from the aforementioned toluene-degrading culture, in which the highly abundant *Clostridium* sp. did not incorporate ^{13}C label from toluene during a 7-day time course experiment (Fowler et al., 2014). While it can not be ruled out that *Clostridium* spp. might be directly involved in hydrocarbon activation, these results collectively point toward a general role for *Clostridium* sp./*Clostridiaceae* in the downstream degradation of hydrocarbons to methanogenic substrates in oil-associated environments, rather than directly activating hydrocarbons in these cultures.

Co-occurrence network analysis revealed the presence of three distinct networks within the cultures (**Figure 3**). The first consisted of diverse methanogenic archaea including hydrogenotrophs, acetotrophs, and methylotrophs. These methanogens were found to co-occur with syntrophic bacteria including *Geobacter* sp., *Ruminococcaceae*, *Synergistetes*, and in particular, two *Clostridiaceae*-affiliated OTUs with a high degree of connectivity that linked the two sections of this network. This network is likely reflecting a number of direct interactions between syntrophic bacteria and methanogenic archaea involving interspecies metabolite transfer, and possibly even direct interspecies electron transfer (DIET) between *Geobacter* sp. and methanogens (Rotaru et al., 2014). The second network consisted of 14 OTUs of diverse syntrophic bacteria and a single *Methanolinea* sp. OTU. The syntrophic bacteria within this network were densely interconnected with a high mean degree of connectivity. Whether these OTUs are all connected due to a highly interactive syntrophic network, or due to the presence of a small number of organisms that interact with a large number of partners (which has previously been observed in these densely connected networks; Berry and Widder, 2014) is unknown. However, this network indicates that it is not only interactions between syntrophic bacteria and methanogens that are important in these communities, but also that interactions amongst syntrophic bacteria are substantial. The third network also emphasizes the importance of interactions amongst syntrophic bacteria as it consists solely of four bacterial OTUs related to obligate anaerobic fermenters (three *Clostridiales*, one *Anaerolineaceae*) that all co-occur. *Lachnospiraceae* and *Ruminococcaceae* are common inhabitants of GI tracts, anaerobic digesters, and other methanogenic environments (Nelson et al., 2011; Biddle et al., 2013). *Anaerolineaceae* are also commonly found in syntrophic environments including the GI tract and anaerobic digestors, where they are typically characterized as secondary fermenters that sequentially degrade fatty acids and/or carbohydrates to methanogenic or other syntrophic intermediates, and are known to associate with hydrogenotrophic methanogens (Yamada et al., 2006; Biddle et al., 2013; St-Pierre and Wright, 2013). *Anaerolineaceae* have also been previously detected in high abundance in alkane-degrading cultures and anaerobic oil-impacted environments (Savage et al., 2010; Sherry et al., 2013; Liang et al., 2015) including methanogenic and non-methanogenic syntrophic hydrocarbon-degrading cultures (Kleinsteuber et al., 2012). Further, *Anaerolineaceae* have previously been postulated to be involved in alkane activation under methanogenic conditions (Sherry et al., 2013; Liang et al., 2015). Thus, an alternative possibility is that *Anaerolineaceae* is involved in alkane activation, and subsequent fatty acid degradation is catalyzed by the *Clostridiales* OTUs, though additional evidence to support *Anaerolineaceae* as alkane degraders in these cultures is currently lacking. Overall, network analysis indicates that there are strong interactions between syntrophic bacteria and methanogens, but the strongest and most abundant interactions we observed in these cultures occurred amongst the bacteria themselves. This suggests the existence of numerous cooperative interactions between groups of bacteria as well as between bacteria and methanogens within syntrophic methanogenic ecosystems. While the use of co-occurrence networks can provide clues as to how organisms interact in syntrophic cultures, they must also be interpreted with caution. Co-occurrence in this analysis merely indicates that the organisms were observed to co-occur repeatedly, but does not preclude the possibility that co-occurring organisms merely share similar niches within these enrichment cultures and do not interact. However, due to the difficulty in elucidating syntrophic interactions in mixed cultures, we believe that co-occurrence network analysis provides a method that can be used to predict syntrophic relationships in complex communities when applied to multiple related communities.

In summary, we demonstrated the methanogenic bio-degradation of palmitate, stearate, and octadecane in cultures derived from a whole crude oil-degrading enrichment culture (Gieg et al., 2008). The fact that octadecane degradation occurred following 3 years of pre-incubation on a non-hydrocarbon substrate (stearate) showed that alkane degraders can persist in environments despite the absence of hydrocarbons. In addition, we described the microbial communities of each of these cultures and a hexadecane-amended culture, and observed an expected diversity reduction when whole crude oil-amended cultures were successively transferred onto single carbon substrates. Confirmation of syntrophic interactions between individual OTUs ultimately requires physiological evidence. However, given the complexity of methanogenic communities, and the difficulty in culturing syntrophic bacteria as individuals or in co-culture, applying microbial co-occurrence network analysis provides a means to predict microbial interactions, enabling insight into potential interspecies interactions and the microbial foodwebs that exist in complex communities. By examining microbial co-occurrence in these cultures, we were able to identify organisms that were insensitive to the carbon substrate being metabolized, and examine their degree of co-occurrence with other community members. While these co-occurrences likely do not all represent syntrophic interactions, this is a first step toward

identifying organisms that form associations within this stable syntrophic community. Our analysis reveals not only stable interactions between syntrophs and methanogens, including possible DIET interactions, but also strong interactions amongst the syntrophic bacteria themselves. These findings emphasize the complex foodwebs existing in methanogenic communities. Furthermore, these predictions can provide preliminary evidence for further hypothesis testing using metagenomic and/or metatranscriptomic data and/or physiological investigations.

AUTHOR CONTRIBUTIONS

SF and LG conceived the research. LG established the original oil and LCFA cultures and SF transferred and maintained subsequent enrichments. SF conducted the methane measurements, the 16s rRNA gene sequencing, and microbial co-occurrence network analysis for all cultures. CT assayed for and interpreted the biodegradation gene results. All authors participated in writing the manuscript.

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SUPPLEMENTARY MATERIAL

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Analysis of microbial communities in the oil reservoir subjected to CO_2-flooding by using functional genes as molecular biomarkers for microbial CO_2 sequestration

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Sequestration of CO_2 in oil reservoirs is considered to be one of the feasible options for mitigating atmospheric CO_2 building up and also for the *in situ* potential bioconversion of stored CO_2 to methane. However, the information on these functional microbial communities and the impact of CO_2 storage on them is hardly available. In this paper a comprehensive molecular survey was performed on microbial communities in production water samples from oil reservoirs experienced CO_2-flooding by analysis of functional genes involved in the process, including *cbbM*, *cbbL*, *fthfs*, [FeFe]-hydrogenase, and *mcrA*. As a comparison, these functional genes in the production water samples from oil reservoir only experienced water-flooding in areas of the same oil bearing bed were also analyzed. It showed that these functional genes were all of rich diversity in these samples, and the functional microbial communities and their diversity were strongly affected by a long-term exposure to injected CO_2. More interestingly, microorganisms affiliated with members of the genera *Methanothemobacter*, *Acetobacterium*, and *Halothiobacillus* as well as hydrogen producers in CO_2 injected area either increased or remained unchanged in relative abundance compared to that in water-flooded area, which implied that these microorganisms could adapt to CO_2 injection and, if so, demonstrated the potential for microbial fixation and conversion of CO_2 into methane in subsurface oil reservoirs.

Keywords: CO_2 fixation, bioconversion, methane, functional genes, oil reservoir, microbial communities

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Introduction

Storage of CO_2 in deep geological formations, such as oil reservoirs, is one of the feasible measures to reducing CO_2 emissions into the atmosphere. Understanding the fate of CO_2 in the subsurface environment is of great scientific interest and significance, and has received increasing attention for more information to assess the feasibility. Due to the fact that abundant microorganisms inhabit in these formations, microbial fixation and conversion of the sequestered CO_2 into CH_4 are becoming an area of active research and development.

After CO_2 injection, characteristics of the formation water may be changed by CO_2 dissolution, including pH, the availability of inorganic and organic components in the brine, microbial

attachment and biofilm formation as well as the microbial activities at *in situ* oil reservoirs. Generally, as CO_2 is also a potential source of carbon of chemolithoautotrophic microorganisms such as methanogens, the injected CO_2 may activate these microorganisms and notably influence the microbial structure and their activity *in situ*. Studies have been performed on the physical and chemical changes in the CO_2 storage sites. The first on-shore CO_2 storage site in Europe was established, and the effects and feasibility of CO_2 injection and storage in a 650 m deep saline aquifer was examined (Wandrey et al., 2011). The potential of microbial conversion of CO_2 into CH_4 by hydrogenotrophic methanogens isolated from oil reservoirs has been evaluated based on laboratory experiments by Sugai et al. (2012). As to the microbial involvement, six autotrophic CO_2 fixation pathways were documented, of which the Calvin–Benson–Bassham (CBB) cycle plays an important role in autotrophic CO_2 fixation (Berg, 2011). The CBB biochemical process was reported to occur in *Proteobacteria*, including some members of *Firmicutes*, *Actinobacteria*, and *Chloroflexi* as well as in plants, algae, and cyanobacteria (Ivanovsky et al., 1999; Zakharchuk et al., 2003; Berg et al., 2005; Caldwell et al., 2007; Lee et al., 2009). Another important pathway of CO_2 fixation is the reductive acetyl-CoA pathway that has documented to occur in acetogenic prokaryotes, ammonium-oxidizing *Planctomycetes* (Strous et al., 2006), sulfidogenic bacteria (Schauder et al., 1988), and autotrophic archaea affiliated with the order *Archaeoglobales* (Vorholt et al., 1995, 1997). This pathway is also utilized by acetogenic prokaryotes for energy conservation (Ragsdale and Pierce, 2008; Thauer et al., 2008; Biegel and Muller, 2010).

Petroleum reservoirs are known to harbor diverse microorganisms including bacteria such as *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Chloroflexi* and archaea such as methanogens and *Archaeoglobales* mentioned above (Magot et al., 2000; Li et al., 2010, 2011; Wang et al., 2011; Mbadinga et al., 2012) and they are expected to fix and/or convert CO_2 into CH_4 more effectively. To investigate whether oil reservoirs have the potential of CO_2 biofixation and bioconversion of CO_2 into CH_4, and to have a better knowledge on microorganisms involved in this process and the impact of long-term CO_2 exposure on them, studies from a viewpoint of functional genes are necessary. Functional genes involved in CO_2 fixation and conversion into CH_4 have been shown to be valuable functional biomarkers for detecting the microbial communities both in environments and enrichment cultures. The genes *cbbL* and *cbbM* respectively encoding the key enzymes ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) form I and II of the CBB cycle for CO_2 fixation have been used to study microbial communities from hydrothermal vents of the Logatchev field (Hugler et al., 2010). The gene *fthfs* encoding formyltetrahydrofolate synthetase, a key enzyme in the reductive acetyl-CoA pathway, has been used to investigate the diversity of homoacetogenic bacteria in thermophilic and mesophilic anaerobic sludge (Ryan et al., 2008). Methyl-Coenzyme M reductase (*mcr*) is vital for CH_4 formation, and the α-subunit of MCR (*mcrA* gene) is commonly used in the detection of specific groups of methanogenic communities (Juottonen et al., 2006). In addition, H_2 should be supplied in the process of CO_2 conversion into CH_4. H_2 can be produced by H_2-producing prokaryotes which are polyphyletic. [Fe-Fe]-hydrogenases are known to catalyze H_2 production in fermentative microorganisms, and thus gene encoding for *[Fe-Fe]*-hydrogenases represent a good marker gene for the detection of H_2-producing anaerobes (Schmidt et al., 2010). These valuable functional biomarkers involved in CO_2 fixation and conversion into CH_4 are shown in **Figure 1**.

The objectives of this study were to evaluate the potential of *in situ* microbial fixation and conversion of CO_2 into CH_4 in subsurface oil reservoir through analysis of functional genes (*cbbM*, *cbbL*, *fthfs*, gene encoding by [FeFe]-hydrogenase, and *mcrA*) by: (1) characterization of the functional microbial communities involved in this process in the production waters from CO_2-flooded and water-flooded areas, respectively, of the same high-temperature oil-bearing bed in Daqing Oilfield; and (2) Analysis of the impact of long-term exposure of CO_2 on these functional microbial communities.

Materials and Methods

Sampling Site and Production Water Samples

Production water samples were collected from production wells (designated as C and W) in YSL block of Daqing oilfield, China. At that time, the water cut of fluid from C and W production wells were 15 and 11%, respectively. The CO_2 injected had been produced from the sampling well about 1 year before, and the ratio of gas (CO_2) to oil was between 22.8 and 145 m^3/m^3 in production wells. The distance between injection well and the sampling production well is about 250 m. These wells produced oil from the same oil-bearing bed but C is located in the area subjected to CO_2 flooding since 2007, whereas W by water-flooding only. To date, about 100,000 m^3 of liquid CO_2 have been injected into the oil-bearing bed with an average injection rate of 10 m^3/d per injection well in a manner of CO_2-H_2O alternate injection for Enhancement of Oil Recovery (EOR). These samples were taken through sampling valves located at the wellhead (average temperature 45°C) and put into 5 L sterile bottles, respectively, to fullness, and then capped and sealed to maintain anoxic conditions. The bottles were kept at 4°C before further treatment. The *in situ* temperature and pressure of the target oil-bearing bed with a depth of about 2000 m were about 90°C and 19 MPa, respectively. The average density of oil in this oil-bearing bed is 0.8581 g/cm^3 and the information of the production water is listed in **Table 1**.

DNA Extraction

DNA was extracted from the oil/water sample according to the method previously described by Wang et al. (2012). Briefly, the water phase was separated from the oil/water mixture by heating the samples to 50°C and by phase separation in sterilized separatory funnels. The microbial biomass in the water fraction was concentrated onto membrane filter (0.22-μm-pore-size). Total genomic DNA of samples was extracted from 2.0 L of production water samples using AxyPrep™ Bacterial Genomic DNA Miniprep Kit (Axygen

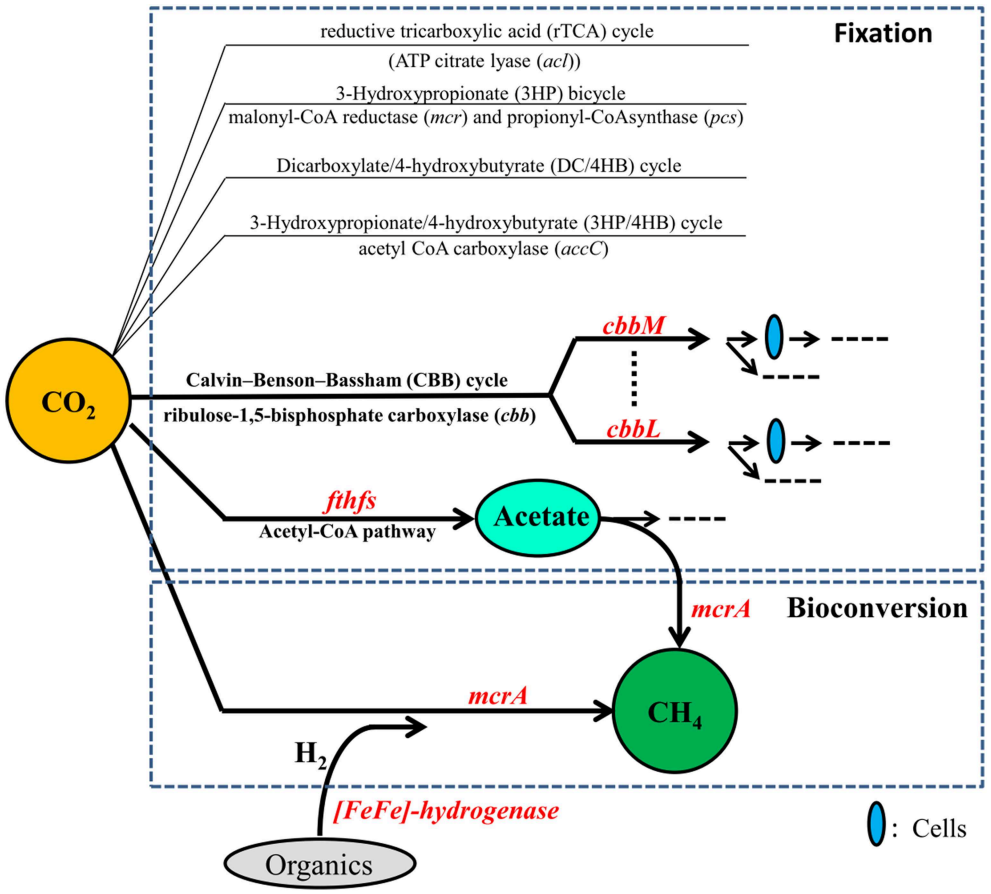

FIGURE 1 | Genes and pathways for CO_2 fixation and bioconversion into CH_4.

Biosciences, Inc., CA, USA) according to the manufacturer's DNA Miniprep spin protocol after concentration onto membrane filters. The genomic DNAs obtained were purified with a DNA purification kit (U-gene, China) according to the manufacturer's instructions. The extracted DNAs were stored at −20°C until PCR amplification of different functional genes (Wang et al., 2012).

PCR Amplifications

Amplifications of the *cbbL* gene fragment (771 bp) and the *cbbM* gene fragment (328 bp) were carried out under the conditions described by Campbell and Cary (2004). For amplification of a portion (1102 bp) of the *fthfs* gene, the PCR conditions used were those described previously by Leaphart and Lovell (2001). For amplification of a fragment (620 bp) of [Fe-Fe]-hydrogenase-encoding gene, the PCR primer set HydH1f/HydH3r was applied using the conditions described by Schmidt et al. (2010). A fragment (470 bp) of the *mcrA* genes was amplified using the primer set MLf/MLr (Luton et al., 2002) with the conditions as reported previously (Galand et al., 2005). Functional genes fragments were all amplified in five parallel PCR reactions in a Peltier thermal cycler (Bio-Rad, USA), which were subsequently pooled for cloning and construction of genes libraries.

Construction of Functional Genes Clone Libraries

The amplified and pooled PCR products were gel-purified using the Gel Extraction Kit (U-gene, China) and then cloned into *Escherichia coli* using a pMD19®-T simple vector kit (Takara, Japan) following the instructions of the manufacturer. For each gene clone library, the white colonies obtained were randomly picked and cultured overnight at 37°C in 0.8 ml Luria broth (LB) medium supplemented with ampicillin (50 μg ml^{-1}). The inserted DNAs were amplified by using M13-47 (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and RV-M (5′-GAGCGGATAACAATTTCACACAGG-3′) primers targeting the flanking vector sequence, followed by agarose gel electrophoresis with ethidium bromide staining (Guan et al., 2013).

Sequencing and Phylogenetic Analyses

Sequencing was carried out with an ABI 377 automated sequencer. After sequencing, reads were first trimmed for vector before subsequent analyses. Bellerophon was used to check for putative chimeric sequences (Huber et al., 2004). DNA sequences with more than 97% similarity were assembled into the same operational taxonomic units (OTUs) using FastGroup II (Yu et al., 2006), and one representative sequence was chosen

TABLE 1 | Characteristics of the production water samples.

Parameter	C	W
pH	6.4	6.0
Salinity (mg L^{-1})	3897	3920
Cl^- (mg L^{-1})	1947	1872
SO_4^{2-} (mg L^{-1})	667	808
PO_4^{3-} (mg L^{-1})	nd	nd
NO_3^- (mg L^{-1})	nd	nd
Na^+ (mg L^{-1})	1110	1115
NH_4^+ (mg L^{-1})	24.6	25.8
K^+ (mg L^{-1})	6.8	6.9
Ca^{2+} (mg L^{-1})	131.5	83.1
Mg^{2+} (mg L^{-1})	10.0	8.9
Mn^{2+} (mg L^{-1})	nd	nd
Formate (mg L^{-1})	nd	nd
Acetate (mg L^{-1})	109.1	7.7
Propionate (mg L^{-1})	nd	nd
Isobutyrate (mg L^{-1})	2.5	2.9
Butyrate (mg L^{-1})	nd	nd

pH, anion, cation were analyzed by pH meter, ion chromatography, and ICP-AES (Inductively Coupled Plasma-Atomic Emission Spectrometry), respectively; Volatile fatty acids were determined by GC-MS after butanol esterification; nd, not detectable.

from each OTU to compare with sequences in the GenBank Database using the BLASTX algorithm to identify nearest related ones (Altschul et al., 1997). Representative OTUs from clone libraries as well as reference sequences from GenBank were translated into corresponding amino acid sequences using EMBOSS Transeq tool (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) with default parameter (Standard Genetic Code) and then aligned using Clustal Omega (Sievers et al., 2011). Phylogenetic trees were generated using MEGA5 software (Tamura et al., 2011). The topology of the trees was obtained by the Neighbor-Joining method (Saitou and Nei, 1987) with the Poisson correction method and 1000 bootstrap replicates were applied to estimate the support for the nodes in the tree.

Nucleotide Sequence Accession Numbers

Gene sequences data reported here are available in GenBank sequence database under the accession numbers KF111435–KF111455, KF111525–KF111548, KF111456–KF111492, KF111493–KF111501, and KF111502–KF111524 for *cbbM* gene, *cbbL gene, mcrA* gene, *fthfs* gene, and gene encoded by [Fe-Fe]-hydrogenase.

Results

Characterization of Clone Libraries

cbbL and *cbbM* Genes

The *cbbL* gene types were positively detected in all two kinds of samples (**Figure 2**). The *cbbL* gene clone libraries from sample C and W resulted in 11 and 13 OTUs, respectively, and the PCR amplified sequences are spread over the entire tree. Phylogenetic analysis indicates that the *cbbL* gene sequences obtained are related to those of *Alpha-*, *Beta-*, and *Gamma-Proteobacteria. One* OTU (*cbbL*-C2-18) is closely related to *Hydrogenophaga* sp. CL3 affiliated to the family *Comamonadaceae* within *Beta-Proteobacteria* (Garcia-Dominguez et al., 2008). The sequences of *cbbL*-C1-13, *cbbL*-C1-17, *and cbbL*-W4-9 all share high similarity with *Cupriavidus metallidurans* CH34 belonging to the family *Burkholderiaceae* within *Beta-Proteobacteria.* The sequence of *cbbL*-W3-24 shares high identity with endosymbiont of *Bathymodiolus azoricus* (Spiridonova et al., 2006), a member of *Gamma-Proteobacteria*. One OTU represented by *cbbL*-W4-12 shows highest identity with an uncultured bacterium from iron-rich environment (Kellermann et al., 2012).

Similarly, the *cbbM* gene types were also detected in these two samples and yielded 10 and 11 OTUs in C and W, respectively (**Figure 3**). The *cbbM* sequences detected are all very similar to those from organisms affiliated with members of *Alpha-*, *Beta-*, and *Gamma-Proteobacteria.* The sequence of *cbbM*-C1-3 is related to an uncultured bacterium from cave water of Romania (Chen et al., 2009). The OTUs represented by *cbbM*-W4-22, *cbbM*-W4-32, *cbbM*-W3-12, and *cbbM*-C2-9 are closely related to uncultured bacterium from tar contaminant aquifer and MTBE and ammonium polluted groundwater (Alfreider et al., 2012). Sequences represented by *cbbM*-C2-21, *cbbM*-C1-13, and *cbbM*-C1-7 all share similarities with those recovered from the East China Sea and basin water and sediment. Interestingly, these sequences are also closely related to *Halothiobacillus* spp., members of sulfur-oxidizing symbionts belonging to *Gamma-Proteobacteria*. Three OTUs (*cbbM*-C2-28, *cbbM*-W3-9, *cbbM*-W4-38, and *cbbM*-C1-16) are similar to an uncultured organism from iron-rich environmental samples (Kojima et al., 2009). Sequences represented by both *cbbM*-W3-14 and *cbbM*-C1-21 are closely related to *Rhodopseudomonas palustris*, a member of the order *Rhizobiales* within the *Alpha-Proteobacteria*. OTUs *cbbM*-W4-14 and *cbbM*-W4-6 representing 29 clones show highest similarities with *Phaeospirillum molischianum*, affiliated with the family *Rhodospirillaceae* within *Alpha-Proteobacteria* and with sequences from methane seep sediment. And *cbbM*-W3-7, which appeared to forms its own cluster, is related to *Thauera* spp. within the *Beta-Proteobacteria* and also to an uncultured bacterium from an environmental sample of paddy soil in China (Yuan et al., 2012).

fthfs Genes

The *fthfs* gene sequences were also detected in both samples. However, it showed a less abundant diversity as depicted in the phylogenetic tree (**Figure 4**) with the screened clones divided into 5 and 4 OTUs in sample C and W, respectively. Phylogenetic analysis shows that most of the *fthfs* gene sequences are related to members of the *Firmicutes.* Three OTUs (*FTHFS*-C2-9, *FTHFS*-C2-12, and *FTHFS*-C2-19) of sample C are all most similar to *Acetobacterium psammolithicum*, a member of the order *Clostridiales* within *Firmicutes* while 2 OTUs (*FTHFS*-C1-7 and *FTHFS*-C1-5) are obtained in sample C and sharing high similarities with *Firmicutes* members of the genus *Acetobacterium* (Xu et al., 2009). OTUs *FTHFS*-W3-24 and *FTHFS*-W3-12 are related to sequences

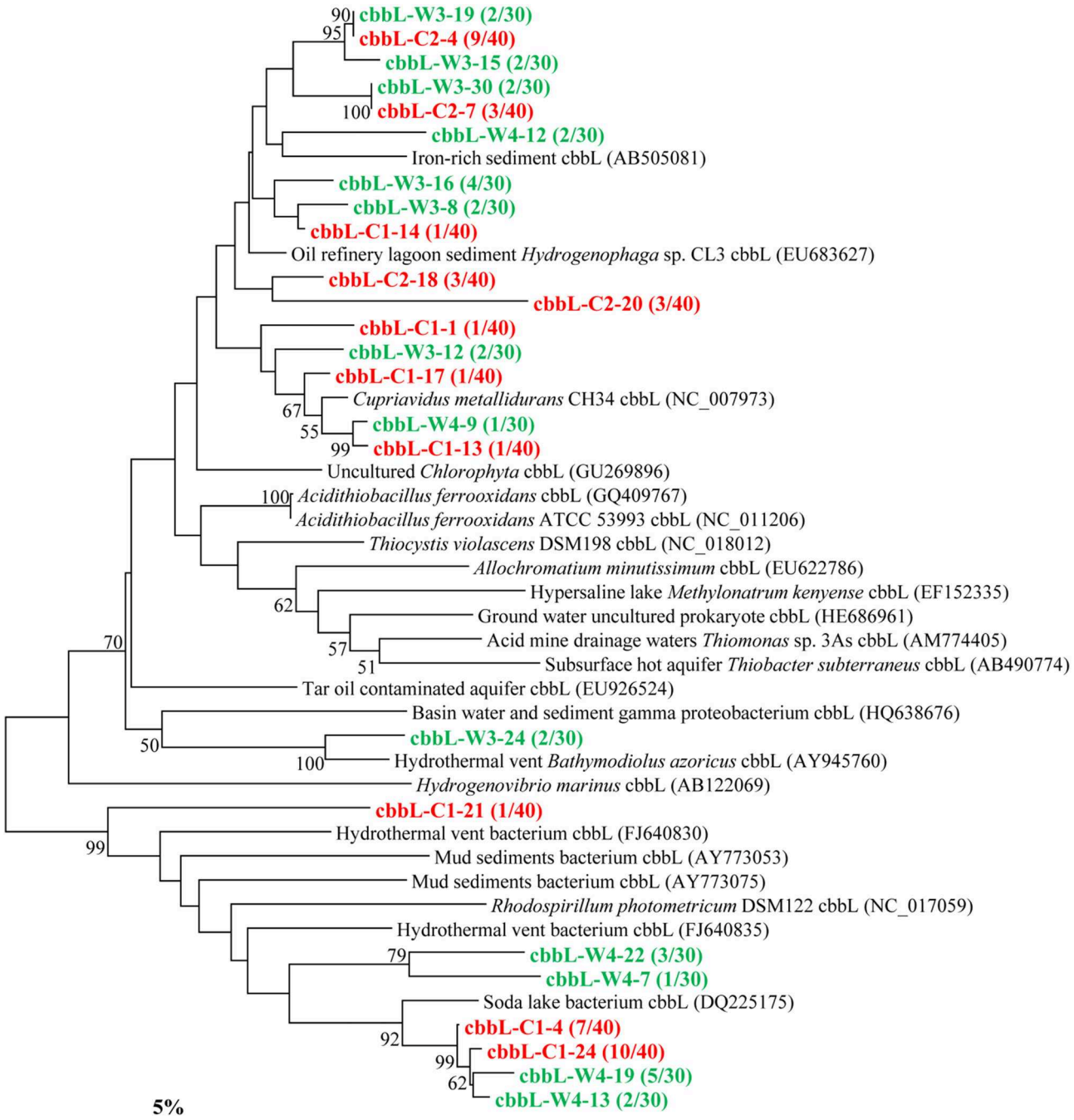

FIGURE 2 | Phylogenetic tree of the *cbbL* gene retrieved from the water samples (shown in colored) and closely related sequences from GenBank database. Alignments to related sequences (shown with accession number) were performed with MEGA 5 software. The topology of the tree was obtained with the neighbor-joining method. Bootstrap values ($n = 1000$ replicates) greater than 50% are reported. Scale bar represents 5% amino acid substitution.

from genera *Moorella*, *Desulfitobacterium*, and *Desulfosporosinus*, also members of the *Firmicutes*. *FTHFS*-W3-4 is similar to uncultured *Alkaliphilus* sp. from anaerobic wastewater of Mesa Northwest Wastewater Reclamation Plant (Parameswaran et al., 2010).

[FeFe]-Hydrogenase-Encoding Gene

The [FeFe]-hydrogenase-encoding gene was detected in both C and W samples, and phylogenetic analysis of the sequenced clones were assembled into 9 and 14 OTUs, respectively (**Figure 5**). The majority of the gene sequences obtained

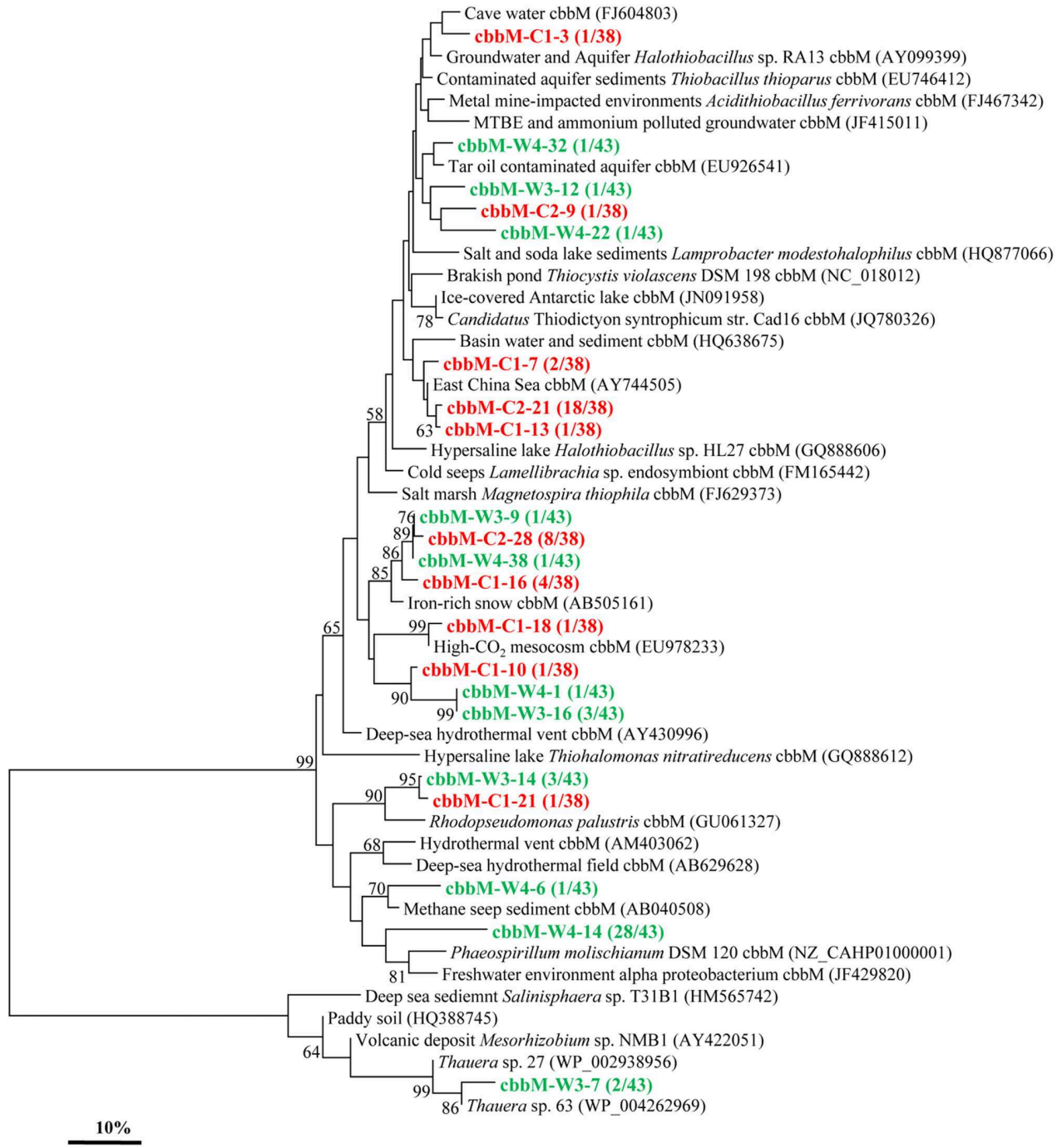

FIGURE 3 | Phylogenetic tree of the *cbbM* gene retrieved from the water samples (colored) and closely related sequences from GenBank database. Alignments to related sequences (shown with accession number) were performed with MEGA 5 software. The topology of the tree was obtained with the neighbor-joining method. Bootstrap values ($n = 1000$ replicates) greater than 50% are reported. Scale bar represents 10% amino acid substitution.

from the two samples cluster with sequences related to *Firmicutes*. One OTU represented by FeFe-Hyd_W3-6 shares similarity with *Syntrophothermus lipocalidus* of the *Firmicutes*. FeFe-Hyd_W4-38 is either related to *Shewanella halifaxensis* HAW-EB4 within the *Gamma-Proteobacteria* or to *Thermodesulfovibrio yellowstonii* within the *Nitrospira* (**Figure 5**). FeFe-Hyd_W4-36 is related to *Thermodesulfobium narugense* belonging to the family *Thermodesulfobiaceae* within

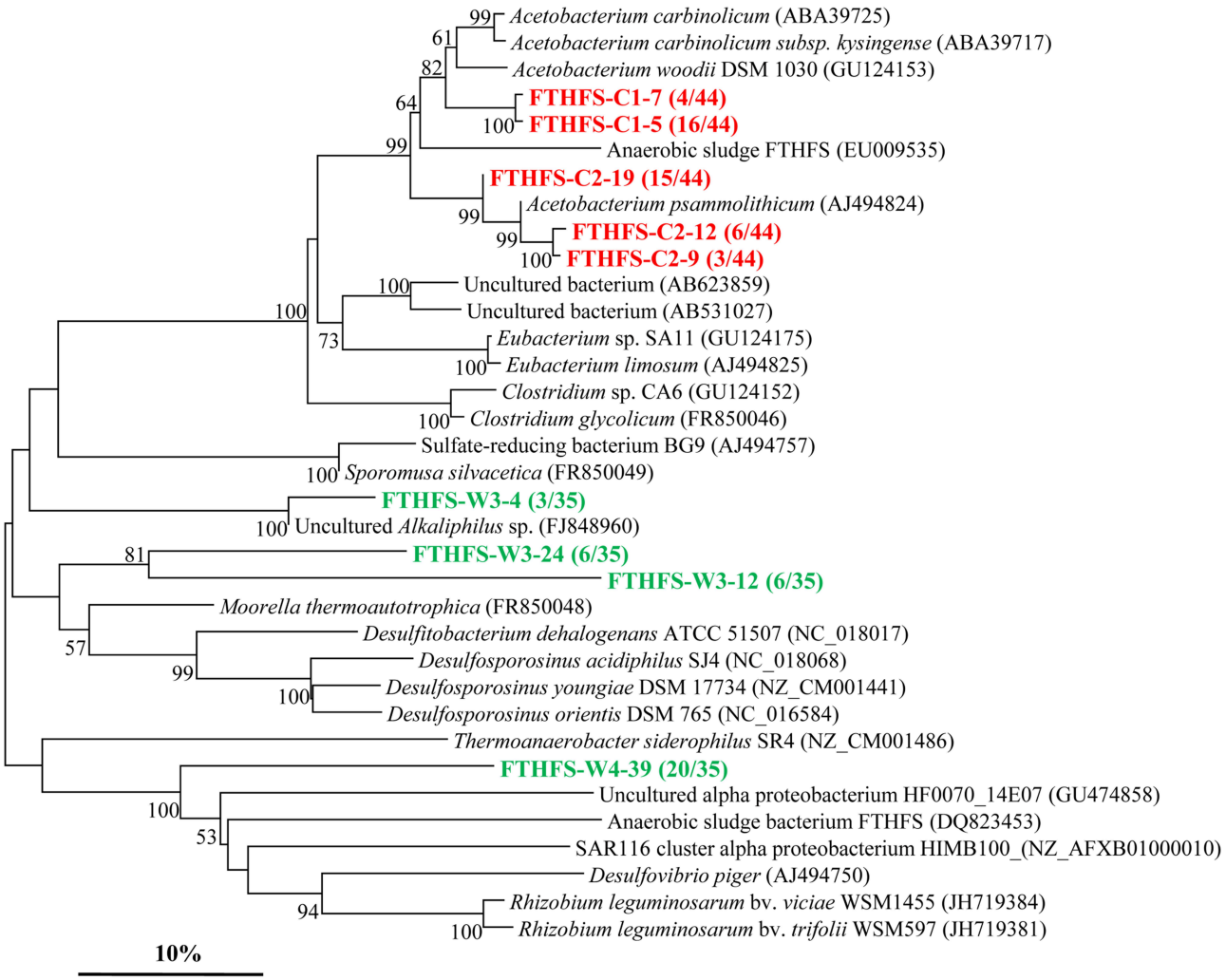

FIGURE 4 | Phylogenetic tree of the *fthfs* gene retrieved from the water samples (colored) and closely related sequences from GenBank database. Alignments to related sequences (shown with accession number) were performed with MEGA 5 software. The topology of the tree was obtained with the neighbor-joining method. Bootstrap values ($n = 1000$ replicates) greater than 50% are reported. Scale bar represents 10% amino acid substitution.

the *Firmicutes*. FeFe-Hyd_W4-22 shares high identity with *Moorella thermoacetica* affiliated to the family *Thermoanaerobacteraceae* of *Firmicutes*. FeFe-Hyd_W4-4, FeFe-Hyd_C2-26, and FeFe-Hyd_W4-32 are all related to *Desulfotomaculum kuznetsovii*, a member of the order *Clostridiales* within *Firmicutes*. FeFe-Hyd_C2-10 and FeFe-Hyd_W4-35 are both similar to *Thermotoga lettingae* TMO affiliated with the family *Thermotogaceae*.

mcrA Genes

By using *mcrA*-targeted specific PCR primers set, 21 and 16 OTUs (37 overall) were obtained in samples C and W, respectively (**Figure 6**). Phylogenetic analysis shows that 21 OTUs (13 in C and 8 in W) are all closely related to sequences from members affiliated to the *Methanobacteriales*, an order known to harbor mostly CO_2-reducing methanogens. A total of 7 OTUs (3 in C and 4 in W) shared high identities with *mcrA* sequences from the *Methanomicrobiales*. And 9 OTUs (5 in C and 4 in W) are closely related to sequences affiliated to methylotrophic and acetoclastic methanogens within the order *Methanosarcinales*.

Characterization of Functional Microbial Communities

Changes in microbial structure were analyzed by their relative abundance calculated from the number of clones and the results were showed in **Figure 7**. The community structure of microorganisms with most similarity to the retrieved amino acid sequences of *cbbM* gene was distinct in W and C samples (**Figure 7A**). The genera *Phaeospirillum* (67.4%), *Leptothrix* (14.0%), *Rhodopseudomonas* (7.0%), and *Thiobacillus* (7.0%) were dominant in W sample, whereas, *Halothiobacillus* (55.3%) and *Leptothrix* (36.8%) were dominant in C sample. In the *cbbL*

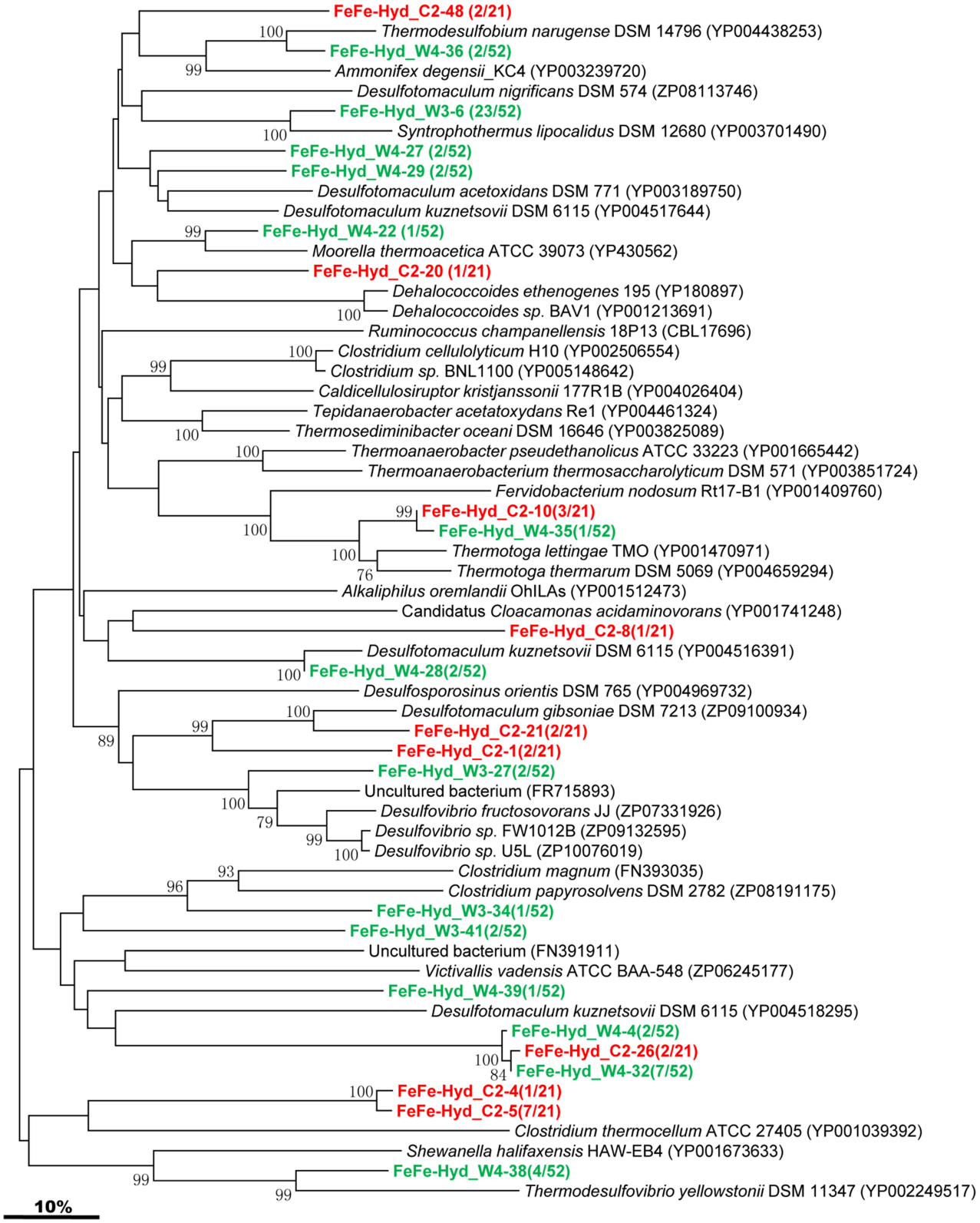

FIGURE 5 | Phylogenetic tree of the *[FeFe]-Hydrogenase* gene retrieved from the water samples (colored) and closely related sequences from GenBank database. Alignments to related sequences (shown with accession number) were performed with MEGA 5 software. The topology of the tree was obtained with the neighbor-joining method. Bootstrap values ($n = 1000$ replicates) greater than 50% are reported. Scale bar represents 10% amino acid substitution.

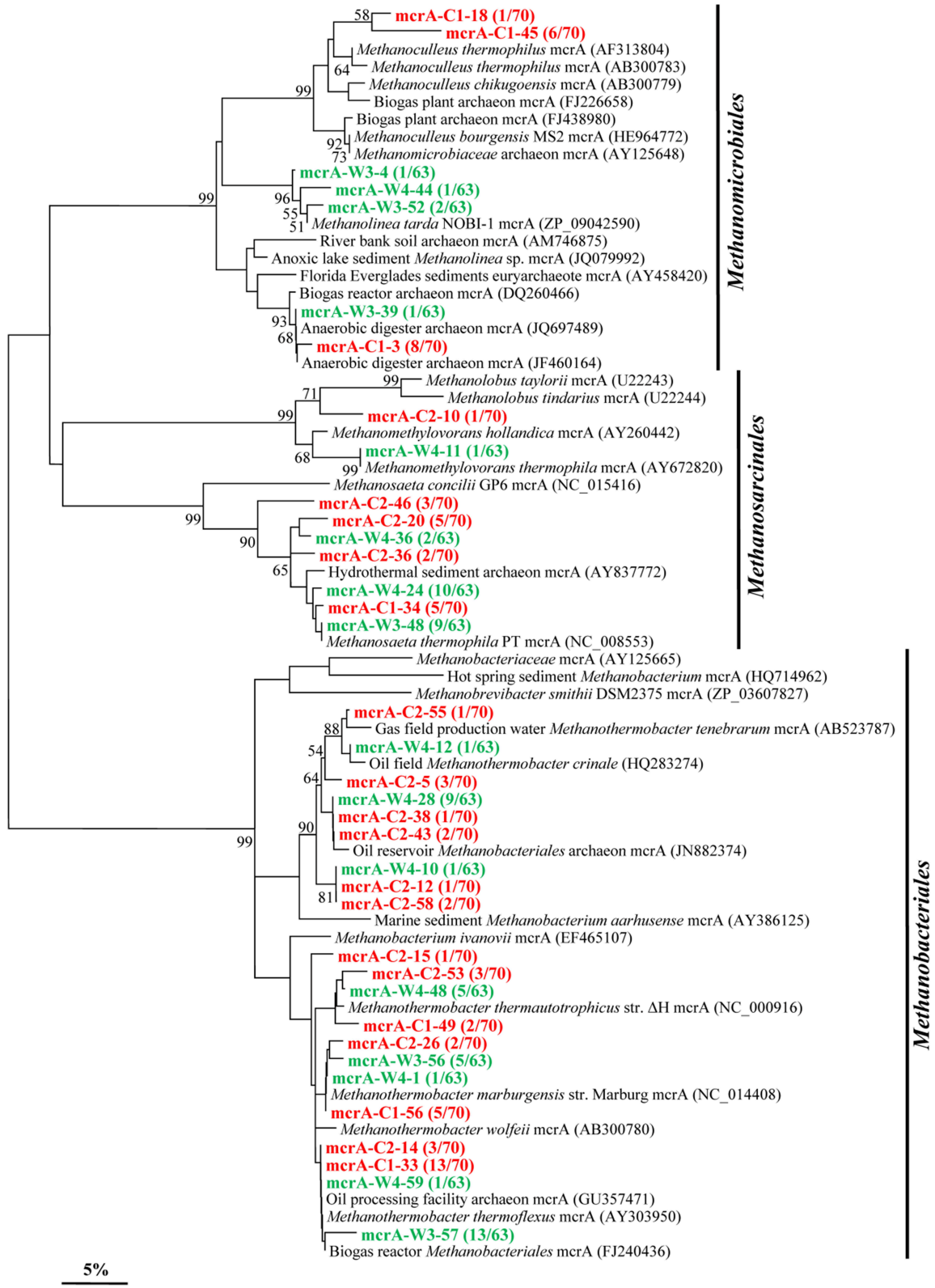

FIGURE 6 | Phylogenetic tree of the *mcrA* gene retrieved from the water samples (colored) and closely related sequences from GenBank database. Alignments to related sequences (shown with accession number) were performed with MEGA 5 software. The topology of the tree was obtained with the neighbor-joining method. Bootstrap values ($n = 1000$ replicates) greater than 50% are reported. Scale bar represents 5% amino acid substitution.

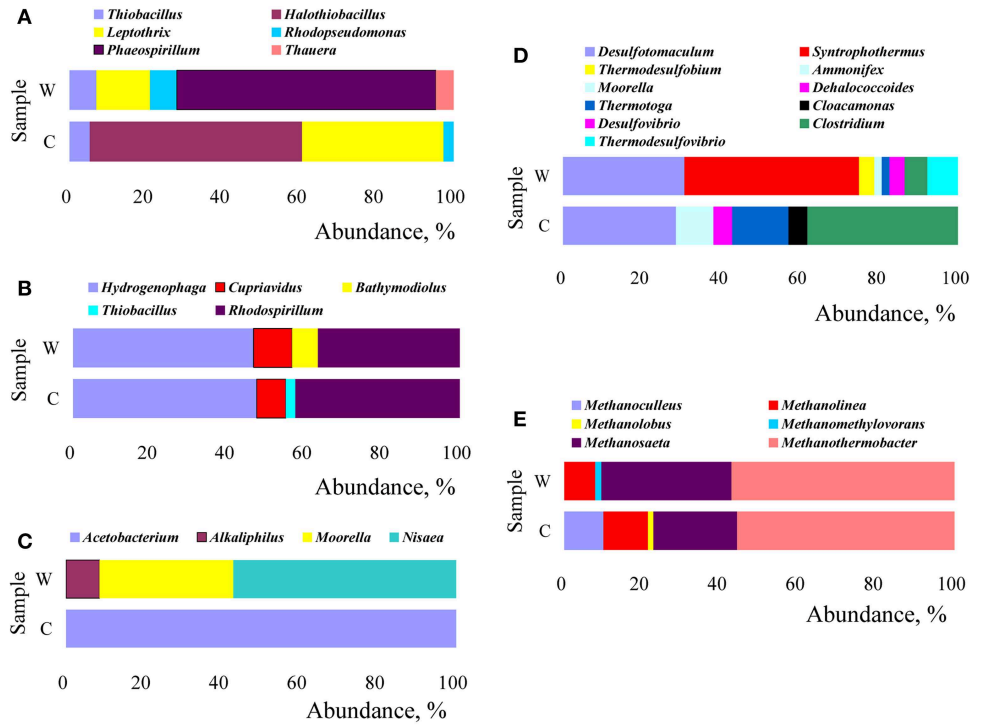

FIGURE 7 | Relative abundance of functional microbes (at the genus level) with respect to the sequences retrieved by functional marker genes of *cbbM* (A), *cbbL* (B), *fthfs* (C), *FeFe*-hydrogenase(D), and *mcrA* (E).

clones libraries (**Figure 7B**), the genera *Rhodospirillum* 36.7% and 42.5%, *Hydrogenophaga* 46.7% and 47.5%, *Cupriavidus* 10.0% and 7.5% were dominant in W and C sample, respectively. As for the composition of *fthfs* communities (**Figure 7C**), in W sample, the community was mainly composed by microorganisms related to genera *Nisaea* (57.1%), *Moorella* (34.3%), and *Alkaliphilus* (8.6%), however, only by microorganisms related to genus *Acetobacterium* (100%) in C sample. It can be seen from **Figure 7D** that C sample was dominantly composed by microbes related to members of genera *Clostridium* (38.1%), *Desulfotomaculum* (28.6%), and *Thermotoga* (14.3%), while the W sample by *Syntrophothermus* (44.2%) and *Desulfotomaculum* (30.8%). Meanwhile, those related to *Ammonifex*, *Dehalococcoides*, and *Cloacamonas* all rose in relative abundance from undetectable in W sample to 4.8% in C sample. The methanogen community was demonstrated in **Figure 7E**. As shown in **Figure 7E**, thermophilic *Methanothermobacter* (55.7% and 57.1% in C and W sample, respectively), *Methanolinea* (11.4% and 7.9% in C and W sample, respectively), and *Methanosaeta* (21.4% and 33.3% in C and W sample, respectively) were the predominant methanogens. *Methanoculleus* (10.0%) were only detected in the C sample.

Discussion

Occurrence of Microorganisms Associated with CO_2 Sequestration in Oil Reservoirs

The microbial community structure in production water samples in Daqing oilfield of China was analyzed by means of a suite of functional genes as biomarkers. Our results indicate that members of the *Proteobacteria* (*Halothiobacillus*, *Leptothrix*, *Hydrogenophaga*, and *Rhodospirillum*) were the predominant ones with the ability of fixation of CO_2 in *in situ* oil reservoirs. It has been reported that the CBB cycle for CO_2 fixation operates in *Proteobacteria* belonging to the *alpha-*, *beta-*, and *gamma*-subgroups, and some members of the *Firmicutes* (Zakharchuk et al., 2003; Caldwell et al., 2007). In addition, the acetogens belonging to *Clostridiaceae* within *Firmicutes* can use the reductive acetyl-CoA pathway not only for CO_2 fixation but also for the production of acetic acid, which is substrate for methanogenesis. Other major bacterial sequences in the clone libraries of sample W are related to those of *Hydrogenophilaceae,* and similar microorganisms were reported to use the rTCA cycle for autotrophic CO_2 fixation (Schauder et al., 1987; Thauer et al., 1989). For the archaeal *mcrA* gene clone libraries, the predominance of the genus *Methanothermobacter* belonging to hydrogenotrophic methanogens is notable.

The majority of *cbbL* gene types obtained were very similar to the microorganisms belonging to *Alpha-*, *Beta-*, and *Gamma-Proteobacteria*. And some members of these phyla have been reported in previous studies, but of which *Hydrogenophaga* sp. and *Cupriavidus* sp. were rarely documented (Alfreider et al., 2003). The *cbbM* gene types detected are also related to those of *Alpha-*, *Beta-*, and *Gamma-Proteobacteria*, and this is consistent with the research results of Hugler et al. (2010). All above data suggest that microorganisms within *Proteobacteria* mainly use the CBB cycle for CO_2 fixation in the oil reservoirs studied.

Acetogenic bacteria are among the most phylogenetically diverse bacterial functional groups. To date, approximately hundreds of homoacetogenic species have been identified and phylogenetically classified into 21 different genera. The *fthfs* gene sequences obtained from CO_2-flooded fraction of the reservoir shared high similarities with those from members of the *Firmicutes* with most of the sequences related to the order *Clostridiales*, deducing that microorganisms affiliated with *Firmicutes* inhabiting the herein investigated oil reservoirs have the ability to fix CO_2 as well as convert CO_2 into acetic acid via the acetyl-CoA pathway.

H_2 is necessary to *in situ* CH_4 production by hydrogenotrophic methanogens in oil reservoirs. In the present study, we found that sequences from microorganisms similar with those from the *Firmicutes*, *Gamma-Proteobacteria,* and *Thermotogae* were the most encountered in clone libraries established for [FeFe]-hydrogenase-encoding gene, and these results are consistent with those of Schmidt et al. (2010), who found that members of the order *Clostridiales* and *Thermoanaerobacter* sp. were likewise all capable of fermentative production of H_2 (Schmidt et al., 2010).

Methanogenesis is the terminal step of organic compound degradation and plays a major role in the global carbon cycle (Garrity and Holt, 2001; Liu and Whitman, 2008). The most important precursors for methane production during anaerobic digestion of organic matter are H_2-CO_2 and acetate, which are converted into methane by hydrogenotrophic and aceticlastic methanogens (Mayumi et al., 2011), respectively. Interestingly, it is proposed that syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis is an alternative methanogenic pathway in petroleum reservoirs (Mayumi et al., 2011). Analysis based on the *mcrA* gene types indicates 12 OTUs detected share high identity with those of the genus *Methanothermobacter*.

To the best of our knowledge, the collection of functional genes described in the present work has not yet been investigated in oil reservoir systems, although some of them have been reported in geothermal environments. The detection of CO_2 fixation genes as well as hydrogenases-encoding and *fthfs* genes in production fluids of high temperature oil reservoirs provides new insights on the diversity and composition of microorganisms involved in the microbial fixation of CO_2 and its subsequent conversion to methane.

Impact of CO_2 Injection on Specific Microbial Communities with Respect to Microbial Fixation and Bioconversion of CO_2

Microbial fixation and conversion of CO_2 into methane in oil reservoir by indigenous microorganisms is one of the most promising solutions to the mitigation of CO_2 emission. We explored the potential for autotrophic CO_2 fixation and bioconversion with microbial communities in oil reservoir by detection of relative functional biomarker genes such as CO_2 fixation (*cbbM*, *cbbL*), acetogenesis (*fthfs*), hydrogen formation ([FeFe]-hydrogenase-encoding gene), and methanogenesis (*mcrA*). Microbial fixation and conversion of CO_2 are usually implemented by chemolithoautotrophic microorganisms, which usually obtain their energy through the oxidation of inorganic compounds and utilization of CO_2 as their sole source of carbon. Thus, the CO_2 injected as well as the subsequent changes in pH and other geochemical parameters induced by CO_2 have an influence on the metabolism of the both heterotrophic and lithoautotrophic microorganisms (Ramos, 2003). Therefore, injection of CO_2 may cause some changes in microbial populations as well as their activities, and it is important to characterize these changes with respect to CO_2 fixation and bioconversion to methane.

Methanogens use molecular hydrogen (H_2) anaerobically by transferring electrons from H_2 to CO_2 to form methane. As demonstrated in **Figure 7E**, Thermophilic *Methanothermobacter*, *Methanolinea,* and *Methanosaeta* were predominant methanogens both in W and C samples. With comparison to W sample, the promotion in relative abundance of *Methanolinea* (from 7.9 to 11.4%) and *Methanoculleus* (from undetectable to 10.0%) as well as the reduction in relative abundance of *Methanosaeta* (from 33.3 to 21.4%) were observed, which implied that the injected CO_2 influenced negatively on *Methanosaeta* but positively on *Methanoculleus* and *Methanolinea*. Considering that *Methanothermobacter*, *Methanolinea*, and *Methanoculleus* are known to be hydrogenotrophic methanogens, *Methanosaeta* to aceticlastic methanogens, and *Methanomethylovorans* to methylotrophic methanogens, it is reasonable to conclude that injection of CO_2 either increase or maintain the relative abundance of hydrogentropic methanogens, but it decreases that of aceticlastic methanogens and methylotrophic methanogens.

More interestingly, *Methanoculleus* was detected only in C sample. The genus has been found in different habitats including oil reservoir (Berdugo-Clavijo and Gieg, 2014), deep marine sediments (Mikucki et al., 2003), and swine manure storage tank (Barret et al., 2012, 2013). The occurrence of this genus in C sample implies that it may be related to CO_2 injection driven high acetate concentration. This assumption is consistent with the fact that *Methanoculleus* spp. consume acetate while carrying out hydrogenotrophic methanogenesis and the growth of some *Methanoculleus* members requires acetate even though they do not convert it to methane (Mikucki et al., 2003; Barret et al., 2013, 2015). Also, Berdugo-Clavijo and Gieg found that the relative abundance of *Methanoculleus* decreased substantially with acetate (Berdugo-Clavijo and Gieg, 2014). In this study, the C-water is highly enriched in acetate relative to W, which one might normally assume favors aceticlastic methanogens. Based on the known properties of *Methanoculleus* spp., it seems that the acetate is favoring acetate assimilating methanogens.

Ribulose 1, 5-bisphosphate carboxylase (Rubisco, specifically, *cbbL*, *cbbM*) are usually used as a biomarker for the CBB CO_2 fixation pathway (Campbell and Cary, 2004). Specifically, in subsurface environments, CO_2 fixation is usually conducted by chemolithotrophs through the CBB pathway (Kellermann et al., 2012). As **Figure 7A** showed, the most dominant genus *Phaeospirillum*(67.4%) in W sample was not detected in C sample and the abundance of *Thiobacillus* and *Rhodopseudomonas* in C sample decreased notably while compared to W sample. In addition, the *Halothiobacillus* (undetected in W sample) appeared to be the most prevalent in C sample. Also, the relative percentage

of *Leptothrix* in C sample increased compared to that in W sample. In the *cbbL* clones libraries, the abundance of *Rhodospirillum* increased in abundance from 36.7% in W sample to 42.5% in C sample, members of the genus *Hydrogenophaga* increased in abundance slightly in C sample compared to that in W sample, while those affiliated to genus *Cupriavidus* decraed from 10.0% in W sample to 6.5% in C sample (shown in **Figure 7B**). Alfreider et al. (2003) also detected *Hydrogenophaga*, *Thiobacillus*, and others related *cbb* sequences in a contaminated aquifer. The abundance and diversity of the detected *cbb* genes hint at a significant potential for CO_2 fixation via the Calvin cycle within oil reservoir microbial communities.

Most acetogens are obligate anaerobic bacteria that use the reductive acetyl-CoA pathway as their main mechanism for energy conservation and for synthesis of acetyl-CoA and cell carbon from CO_2. Formyltetrahydrofolate synthetase (*fthfs*) is used to detect acetogenic, fermentative bacteria (Leaphart and Lovell, 2001). In the present work, notable changes were observed in the composition of *fthfs* communities (**Figure 7C**). The community dominated by microorganisms related to genera *Nisaea*, *Moorella*, and *Alkaliphilus* in W sample was changed completely to be dominated only by microorganisms related to genus *Acetobacterium* in C sample. The mechanism for the change of *Alkaliphilus* from dominance in sample W to undetectable in sample C is not very clear. Generally, this genus is known to be extremely alkaliphilic and thus would not be prone to survive in the acidic conditions caused by the injection of CO_2. Although the ability of acetate production on CO_2+H_2 by *Acetobacterium woodii* and *Moorella* were systematically studied (Ragsdale and Pierce, 2008; Demler and Weuster-Botz, 2011), surprisingly, *Moorella*-like microorganisms were not detected in C sample. This observation implies that *Acetobacterium*-like microbes are probably more suitable for acetogenesis in CO_2-injected oil reservoirs.

Hydrogen is an alternative energy source for autotrophic microbes in a variety of subsurface environments. When hydrogen and carbon dioxide are present, development of autotrophic microorganisms would be possible. For example, methanogens and acetogens may produce organic matter from hydrogen by means of respiring carbon dioxide. As it can be seen from our study (**Figure 7D**), the composition of [FeFe]-hydrogenase-encoding gene clones libraries at the genus level shows interesting differences in relative abundance between W and C samples. The microbes related to *Syntrophothermus* predominated in W sample (44.2%) disappeared in C sample, and *Desulfotomaculum* decreased from 30.8% in W sample to 28.6% in C sample. The relative abundance of members of genera *Clostridium*, *Thermotoga*, and *Ammonifex* increased from 5.8%, 1.9% and undetectable (0.0%) in W sample to 38.1%, 14.3% and 9.5% in C sample, respectively. Interestingly, all the three sulfate reducing bacteria were influenced very markedly as either decreased in relative abundance (*Desulfotomaculum*) or became undetectable in C sample (*Thermodesulfobium* and *Thermodesulfovibrio*). Meanwhile, those related to *Dehalococcoides* and *Cloacamonas* all rose in relative abundance from undetectable in W sample to 4.8% in C sample. Morozova et al. (2010) also showed that CO_2 injection caused a decrease in the diversity of microorganisms and revealed temporal out-competition of sulfate-reducing bacteria by methanogenic bacteria. Morozova's experiments showed that after CO_2 injection the SRB population declined until it was no longer detected while the archaeal population increased, which indicates that archaea may be able to adapt more readily to the more acidic conditions after CO_2 injection. Our results reached the same conclusion. But, Morozova found that after a 5 month period of exposure to CO_2, the SRB population returned in numbers greater than that prior to CO_2 injection. This phenomenon was not observed in our study at present. The reason for this was not quite clear although it was assumed to be resulted partly from the water–gas alternative injection and long-term exposure of CO_2 (about 5 years) in our study which were quite different from that in Morozova's experiments.

We found great differences in relative abundance among all the five functional gene clone libraries established from W and C samples, as showed in **Figure 7**. This phenomenon of previously undetectable and/or rare members of microbial communities becoming dominant after exposure to CO_2 has been reported previously (Gulliver and Gregory, 2011). Microorganisms with increasing abundance implies that they may be better withstanding or adapting to exposure to CO_2 and subsequent changes in physical and biochemical conditions resulted by CO_2 injection.

Analysis of functional genes shows that microbial communities were strongly influenced and the diversity reduced by CO_2 injection. For example, there were eight different genera in W sample whereas only six were retrieved from C sample for [FeFe]-hydrogenase-encoding gene library. Also, for *fthfs* library, three different genera were detected in W sample but only one was found in C sample. Our data agree with Gulliver and Gregory (2011) which showed that different families of bacteria presided with variation in CO_2 partial pressure. Knowledge of surviving and thriving microbial populations may help in better understanding of the fate of CO_2 following injection and to make better strategy for use of microorganisms in subsurface environments for improving the efficiency of injection and microbial fixation of CO_2, and hence ensuring the security for long-term CO_2 storage in subsurface petroleum reservoirs.

Primers used for *mcr*A amplification are divided into different groups: MCR, ME, ML, and these primers are able to amplify most methanogens. It has been reported that the ME-related primers are also able to amplify anaerobic methane-oxidizing archaea (ANME) (Narihiro and Sekiguchi, 2011). The primers used for *mcr*A amplification to target the methanogenic communities in the samples investigated in the present study were described by Luton et al. (2002) which belonged to the ML group. To the best of our knowledge, the ML group ability to amplify ANME's remains to be demonstrated.

Due to the fact that the CO_2 injected had been produced about 1 year before the collection of these samples when the ratio of gas (CO_2) to oil was between 22.8 and 145 m^3/m^3 in production wells, the changes in the relative abundance of five genes relevant to CO_2 utilization and methane production by microorganisms can be considered mainly attributed to CO_2 injection. The small size of the clone library and the number of clones sequenced would influence, to some extent, on the

analysis of microorganisms with low frequency. Nevertheless, the major functional microorganisms and their changes in relative abundance can still be recognized, even with certain biases, as demonstrated in the present study. The analysis of the changes in microbial community may be influenced by the following factors: (1) The samples were all collected from the sampling valve located at the wellhead of production well and hence, these samples may contain microbes from oil reservoir as well as that survived in oil tubes between the well bottoms to the sampling valve; (2) The sampling water may be produced both from oil-bearing layers or sub-layers with CO_2 production (CO_2-impacted water) and that with no CO_2 production even they received CO_2(non CO_2-impacted water); (3) The retention time of CO_2 in oil reservoir is relatively short, i.e., while CO_2 was injected through injection wells into target oil reservoir, part of them would be produced afterwards from the production well about 250–300 m away from the injector; (4) CO_2 was injected into the target oil reservoir with water-CO_2 alternative injection manner.

For a more accurate characterization of microbial community and their changes caused by CO_2 injection in oil reservoir, the collection of produced water from only the CO_2-impacted zones, the qualitative and quantitative analysis of microbial community, the physiochemical changes of subsurface water such as pH, volatile acids over time, as well as the analysis of the origin of volatile acids (by isotopic analysis) and etc. are very important.

Methane Formation Potential from Injected CO_2 in Oil Reservoirs

Bioconversion of CO_2 into CH_4 *in situ* oil reservoirs by indigenous methanogens is an area of active research and development. Hydrogenotrophic methanogens need not only CO_2 but also H_2 to produce CH_4; therefore, H_2 should be supplied to them in reservoirs for this process. It has been reported that there are several kinds of microorganisms capable of producing H_2 by degrading crude oil in reservoir environments. The potential of the microbial conversion of CO_2 into CH_4 by enrichment culture experiments using microorganisms indigenous to oil reservoirs has been studied (Sugai et al., 2012). Different from that mentioned above, we evaluated the potential of this process from the viewpoint of functional genes. In our study, both the functional genes of H_2-producing and CH_4-producing were detected in the CO_2-flooding oil reservoirs, and the water-flooding oil reservoirs as well. Furthermore, some H_2-producing microorganisms (e.g., *Clostridium* and *Thermotoga*) and hydrogenotrophic methanogens such as *Methanothermobacter* and *Methanolinea* as well as *Methanoculleus* remained or evolving to be predominant after long term exposure to CO_2 in CO_2-flooding area compared to that in water-flooding area. Meanwhile, these H_2-producing bacteria and hydrogenotrophic methanogens were both identified in the 16S rRNA genes cloning libraries (data not shown in this paper). It is assumed that these hydrogenotrophic methanogens live in symbiosis with hydrogen-producing bacteria and convert CO_2 into CH_4 in oil reservoirs. These results indicate that indigenous microbial conversion process of CO_2 into CH_4 has high potential.

The detection of CO_2 fixation potential is alternative evidence to autotrophic activity *in situ* oil reservoirs. Therefore, attentions should be further paid on the evaluation of the activities of those microorganisms in subsurface ecosystems with the potential of microbial fixation of CO_2 and its subsequent bioconversion into methane. Once those microorganisms are activated by means of nutrient injection and etc., taking into consideration of the tremendous capacity of CO_2 sequestration in oil and gas reservoir (totally about 9×10^{11} tons in the world), it seems more reasonable to believe that the *in situ* fixation and reclamation of CO_2 sequestrated in oil reservoir will play an notable role in mitigating atmospheric CO_2 building up as well as energy shortage.

Conclusions

Analysis of a suite of functional genes shows that a diverse microbial community with potential for fixation and conversion of CO_2 into methane inhabits oil reservoir. Microorganisms affiliated with members of the genera *Methanothemobacter* (hydrogenotrophic CO_2-reducing methanogens), *Acetobacterium* and *Halothiobacillus* as well as hydrogen producers (*Firmicutes*) seem to be more adaptable to CO_2 injection and present the potential for microbial fixation and bioconversion of CO_2 into methane in subsurface oil reservoirs. Due to the limitation of clone numbers and the co-production nature of CO_2-impacted and non-impacted water in the C sampling well, the impact of CO_2 injection on microbial community may be not fully characterized and presented in this study. Even so, the present results showing the response, to some extent, of microbial community on the CO_2 injection are of some help in predicting the fate of CO_2 following injection and making better strategies for use of microorganisms in subsurface environments for microbial CO_2 fixation and bioconversion of CO_2 into sustainable energy in subsurface oil reservoirs.

Author Contributions

This study was designed by JL and BM. XS and GY performed all the laboratory experiments. SM analyzed the functional genes data and constructed the phylogenetic trees of these functional genes. JG provided valuable suggestions in the design of the experiments and the preparation of the manuscript. The manuscript was written by JL, assisted by all co-authors. All authors reviewed the final manuscript.

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New Bio-Indicators for Long Term Natural Attenuation of Monoaromatic Compounds in Deep Terrestrial Aquifers

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Deep subsurface aquifers despite difficult access, represent important water resources and, at the same time, are key locations for subsurface engineering activities for the oil and gas industries, geothermal energy, and CO_2 or energy storage. Formation water originating from a 760 m-deep geological gas storage aquifer was sampled and microcosms were set up to test the biodegradation potential of BTEX by indigenous microorganisms. The microbial community diversity was studied using molecular approaches based on 16S rRNA genes. After a long incubation period, with several subcultures, a sulfate-reducing consortium composed of only two *Desulfotomaculum* populations was observed able to degrade benzene, toluene, and ethylbenzene, extending the number of hydrocarbonoclastic–related species among the *Desulfotomaculum* genus. Furthermore, we were able to couple specific carbon and hydrogen isotopic fractionation during benzene removal and the results obtained by dual compound specific isotope analysis ($\varepsilon_C = -2.4‰ \pm 0.3‰$; $\varepsilon_H = -57‰ \pm 0.98‰$; $AKIE_C$: 1.0146 ± 0.0009, and $AKIE_H$: 1.5184 ± 0.0283) were close to those obtained previously in sulfate-reducing conditions: this finding could confirm the existence of a common enzymatic reaction involving sulfate-reducers to activate benzene anaerobically. Although we cannot assign the role of each population of *Desulfotomaculum* in the mono-aromatic hydrocarbon degradation, this study suggests an important role of the genus *Desulfotomaculum* as potential biodegrader among indigenous populations in subsurface habitats. This community represents the simplest model of benzene-degrading anaerobes originating from the deepest subterranean settings ever described. As *Desulfotomaculum* species are often encountered in subsurface environments, this study provides some interesting results for assessing the natural response of these specific hydrologic systems in response to BTEX contamination during remediation projects.

Keywords: natural attenuation, deep aquifer, BTEX, sulfate-reduction, *Desulfotomaculum*

INTRODUCTION

Deep subterranean ecosystems have been described during the last decades as a key living earth component for global carbon cycling and geo-engineering system (Pedersen, 2000; Griebler et al., 2014; Wilkins et al., 2014). This is explained by the unexpected microbial biomass discovered leading to the presumption that these ecosystems potentially host an estimated biomass equivalent to about 40–60% of the terrestrial surface biomass (Whitman et al., 1998; McMahon and Parnell, 2014). However, the available ecological data concerning subterranean environments remain limited in literature, mainly because of the difficulties in collecting representative samples, especially in deep confined aquifers. Here, we imply deep confined aquifers to be geological formations located 100s of meters deep and isolated from surface interaction by an impermeable geological layer. These geological formations are often associated with active petroleum reservoirs, or depleted oil fields used for underground gas storage.

The biodegradation of hydrocarbons has long been regarded as a strictly aerobic process, depending on oxygen availability and the presence of oxygen-respiring bacteria. But during the last few decades, anaerobic hydrocarbon degradation has been described in anaerobic surface or shallow subsurface environments (Barker et al., 1987; Ball and Reinhard, 1996; Head et al., 2003, 2010; Widdel et al., 2010) by numerous consortia and several original bacterial strains (Heider et al., 1999; Widdel and Rabus, 2001; Weelink et al., 2010; Kuppardt et al., 2014). It has been shown that BTEX (Benzene, Toluene, Ethylbenzene, and Xylenes isomers) could be anaerobically degraded using a variety of terminal electron acceptors such as sulfate, nitrate, ferric iron, and CO_2 (Anderson and Lovley, 2000; Chakraborty and Coates, 2004; Weelink et al., 2010; Holmes et al., 2011; Vogt et al., 2011). However, benzene and ethylbenzene are the most recalcitrant of these hydrocarbons. No pure strain able to degrade benzene in sulfate-reducing conditions has been isolated, and only one regarding ethylbenzene (Kniemeyer et al., 2003).

Oil biodegradation in petroleum reservoirs, and by extension in all deep subsurface environments, is an anaerobic process (Magot et al., 2000; Head et al., 2003; Widdel et al., 2010). Very little is known about bacterial species involved in similar processes in the terrestrial subsurfaces, despite the importance of oil biodegradation to the oil industry. Some studies have reported direct or indirect evidences of oil biodegradation by molecular ecology studies (Nazina et al., 2006), thermodynamic calculations (Dolfing et al., 2008; Onstott et al., 2010), isotopic fractionation and ^{13}C tracer-labeled experiments in microcosms (Mancini et al., 2003; Elsner et al., 2005; Fischer et al., 2008; Jones et al., 2008; Imfeld et al., 2014) and by cultural approaches under high temperature and pressure mimicking petroleum reservoir conditions (Mayumi et al., 2011; for a review, see Head et al., 2010).

Studying the microbial ecology and microbial activities of the deep subsurface is difficult, mainly because representative samples are technically very challenging to collect in these deep environments and microbiological studies are scarce (Greksák et al., 1990; Ivanova et al., 2007; Balk et al., 2008; Balk et al., 2010). The opportunity to collect representative fluids from the deep subsurface occurred for a few years with a specific sampling protocol set up for control wells of natural gas storage in deep aquifers (Basso et al., 2005, 2009). This led to the recent observation that original anaerobic microbial communities collected from a 830 m deep gas storage aquifer were involved in the natural attenuation of BTEX in these hydrocarbon-impacted environments (Berlendis et al., 2010). Different microbial consortia were obtained depending on the culture conditions and hydrocarbons used as carbon sources. *Proteobacteria, Firmicutes* related to the *Desulfotomaculum, Chlorobi, Thermotogales, Bacteroidetes, Synergistes,* and Euryarchaeota were shown to be present in the BTEX-degrading consortia by 16S rRNA gene studies, but specific hydrocarbon degradation activity has not been linked to any specific bacterial or archaeal group. Microbial inventory investigations of potential biodegraders isolated from deep confined subterranean environments are still of crucial interest for geo-engineering activities from oil production to bioremediation strategies. Therefore, we investigated the selection of potential biodegraders from another deep gas-storage subterranean aquifer. The microbial community strongly differs from that previously reported (Berlendis et al., 2010) and was able to anaerobically degrade benzene, ethylbenzene, and toluene during 10 years of culturing. It confirms the presence of spore-forming bacteria belonging to the genus *Desulfotomaculum* as another set of autochthonous mono-aromatic hydrocarbons biodegraders in deep subsurface environments under sulfate-reducing conditions.

MATERIALS AND METHODS

Samples

Formation water was sampled from a deep subterranean aquifer (Parisian basin, France) located at 760 m of depth in a Jurassic superior geological formation (Lusitanien, calcareous oolites). This aquifer is confined in a poorly carbonated sandstone formation by an overlaying impermeable geological layer. The aquifer is used for geological storage of natural gas. *In situ* temperature and pH were 37°C and 8.2, respectively. The total salinity of water was 1.6 $g.L^{-1}$. Formation water and concentrated biomass (Sterivex Filter units, EMD Millipore) were collected anoxically from the wellhead of a peripheral monitoring well after a specific cleaning procedure as previously described (Basso et al., 2005). The samples filtered on-site used in this study were transported to the laboratory under anoxic conditions (GasPak^{TM} EZ, BD), stored at 4°C to avoid microbial growth and processed the day after.

Microcosm Experiments

The concentrated microflora collected on site on 78 0.2-μm-pore-size Sterivex^{TM} filters (Millipore) were resuspended in 2.6 L of anoxic formation water. The 102-fold concentrated bacterial suspension was used as inoculum in several flasks with formation water supplemented with 0.5 $g.L^{-1}$ NH_4Cl, 0.3 $g.L^{-1}$ K_2HPO_4, 0.3 $g.L^{-1}$ KH_2PO_4, and 2 $g.L^{-1}$ Na_2SO_4 for sulfate-reducing conditions, or 0.085 $g.L^{-1}$ $NaNO_3$ for

nitrate-reducing conditions, or 0.3 g.L^{-1} FeIII-citrate for iron-reducing conditions, or flushed with CO_2/H_2 (20/80) for methanogenic/fermentative conditions. For each condition, the media were supplemented with 1 mL.L^{-1} of trace-elements solution and 1 mL.L^{-1} of vitamin solution from a sterile anoxic stock solution prepared under N_2 (Pfennig et al., 1981; Eichler and Pfennig, 1986). One milliliter per liter of dithionite solution (0.2% w/v) was added to the media as a reducing agent and resazurine (1 mg.L^{-1}) was used as a redox indicator. From all the initial conditions (sulfate-, nitrate-, iron-reducing, and methanogenesis/fermentation media), three microcosms were prepared including one with 5% (v/v) of 1 M HCl added in order to create abiotic control conditions. Fifty milliliters aliquots were distributed in 100-mL Wheaton serum bottles sealed with butyl rubber stoppers (Bellco Glass, Inc). Benzene, toluene, ethylbenzene, o-, m-, and p-xylenes were finally added (100 ppm final concentrations; Sigma–Aldrich). All manipulations were done in an anaerobic glove box (Getinge La Calhene, France) under an atmosphere of 95% N_2 and 5% H_2. Incubations were performed under static conditions at the deep aquifer *in situ* temperature of 37°C in the dark.

Subcultures in sulfate reducing conditions were prepared outside the glove box using synthetic water (0.5 g.L^{-1} NH_4Cl, 0.1 g.L^{-1} $MgCl_2.6H_2O$, 2 g.L^{-1} Na_2SO_4, 0.06 g.L^{-1} $CaCl_2,2H_2O$, 0.5 g.L^{-1} NaCl, 0.3 g.L^{-1} KH_2PO_4, 0.3 g.L^{-1} K_2HPO_4, 1 mL.L^{-1} of an anoxic solution of trace-elements (Eichler and Pfennig, 1986), the composition of which mimicked that of the formation water. Culture media were sterilized by autoclaving for 20 min at 120°C and immediately flushed under a stream of O_2-free N_2 gas and cooled to room temperature prior to the addition of sterile and anoxic solutions of 1 mL.$L^{-1}$of vitamins, 2 g.L^{-1} $FeCl_2.4H_2O$, and 2.55 g.L^{-1} $Na_2S.H_2O$. The medium was adjusted to pH 8 and 50 mL aliquots were distributed in Wheaton serum bottles sealed with butyl rubber stoppers under a stream of O_2-free N_2. A 10% inoculum and BTEX (100 ppm) were finally added. Chronology of the different experiments during this study is provided in **Figure 2**.

Analytical Procedure

Aqueous samples (0.3 mL) were collected by syringe through the stoppers, transferred to chromatographic vials and acidified (10 μL of 3 N HCl) for monitoring BTEX degradation periodically by SPME/GC/FID with an autosampler Combi Pal (CTC Analytics) coupled with a gas chromatograph 7890A (Agilent Technologies) equipped with a flame ionization detector. BTEX was absorbed in headspace vials during 10 s with a micro-extraction fiber (SPME, Supelco 75 μm carboxen-PDMS). Desorption time inside the GC injector was 100 s. Compounds were separated through an Optima Wax (Macherey–Nagel) column (30 m × 0.32 mm × 0.50 μm). Helium was used as a carrier gas with a constant flow rate of 1 mL min^{-1}. Results were processed as the residual percentage of BTEX as (*Ct*/*Cc*) × 100, where *Ct* is the hydrocarbon concentration in the microcosm, and *Cc* the hydrocarbon concentration in the abiotic control microcosm. The resulting values were normalized to o-xylene as an internal standard.

DNA Extraction, PCR and ssu rRNA Clone Libraries for Taxonomic Assignment

From the fifth to eighth subcultures, genomic DNA was extracted in duplicate from the microbial communities using the Powersoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA). For the construction of 16S bacterial rRNA gene libraries, targeted genes were amplified using the PCR Core Kit Plus (Roche Diagnostics) with the primer sets 8F/1492R or 8F/B926R (Lane, 1991; Weisburg et al., 1991). DNA amplicons were purified, cloned, sequenced, and analyzed as previously described (Stackebrandt and Goebel, 1994; Cole et al., 2003; Berlendis et al., 2010). From the different clone libraries, 226 clones were randomly sequenced. Phylogenetic analyses were carried out after aligning related sequences using the Muscle program (Edgar, 2004). Ambiguous regions were removed using Gblocks (Castresana, 2000) and phylogenetic trees were constructed using the maximum likelihood method implemented in the phyML program v 3.0 (Guindon and Gascuel, 2003). Reliability for internal branch was assessed using the aLRT test (Anisimova and Gascuel, 2006). The 16S rRNA gene sequences reported in this study were deposited in GeneBank database with accession No. KR061296 to KR061298. Archaeal primers tested were primer couples A9F (Vetriani et al., 1999)-U1492R, A9F/A958R (DeLong, 1992), and A109F (Grobkopf et al., 1998)-A958R. Amplification of the gene coding the α-subunit of benzylsuccinate synthase was carried out using the "semi-nested" protocol described previously for *Desulfotomaculum* sp. OX39 (Winderl et al., 2007) or with the primers set 7768F/8543R designed by von Netzer et al. (2013).

Determination of Isotopic Fractionation of ^{13}C-Benzene

For the determination of isotopic fractionation, the bacterial activity was stopped in four microcosms on the seventh subculture at different stages of ^{13}C-benzene (SIGMA) biodegradation by acidification to pH 2 with 15% HCl. Flasks were conserved at −20°C until analysis.

Hydrogen isotope analyses were performed in duplicate by SPME-GC-TC-IRMS using a Hewlett Packard 6890 gas chromatograph connected to a Delta plus XL^{TM} mass spectrometer with a GC/TC interface (Finnigan MAT). The gas chromatograph was equipped with a DB-PETRO column (100 m × 0.25 mm × 0.5 μm film, J&W Scientific). Helium was used as a carrier gas with a flow rate of 1.7 mL.min^{-1} for hydrogen isotope analysis. The temperature program started at 35°C for 20 min isothermally, was increased at a rate of 2°C.min^{-1} to 315°C and maintained isothermally during 50 min. Vienna Standard Mean Ocean Water (VSMOW) was used as the standard for the detection of hydrogen isotope ratios and results were reproducible within $\pm 2.5^0/_{00}$.

Carbon isotope analyses were performed in duplicate by SPME-GC-C-IRMS using the same GC as described previously connected to a Delta plus XL^{TM} mass spectrometer with a GC/C interface (Analytical Precision). The column, carrier-gas and temperature program used were the same as for hydrogen isotope analyses. Vienna Pee Dee Belemnite (VPDB) was used as the standard for the analysis of carbon isotope ratios (Coplen et al., 2006) and results were reproducible within $\pm 0.3^0/_{00}$.

Element isotope ratios "$\delta^h E$" (where h is atomic number) are expressed in delta notation in per mil (‰) and will be specifically designed for hydrogen and carbon, δH and δC, respectively: $\delta^h E\ [^0/_{00}]$ (δH or δC) = $[R_{sample}-R_{standard}/R_{standard}]$ * 1000. R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ or $^2H/^1H$ ratios of the sample and of the internal standard, respectively. Enrichment factors for hydrogen and carbon were determined according to the logarithmic form of the Rayleigh equation using delta notation (‰) as previously described (Elsner et al., 2005; Fischer et al., 2008). $\ln (R_t/R_0) = (\varepsilon /1000) * \ln(C_t/C_0)$ where $R_t/R_0 = (\delta^h E_t + 1000)/(\delta^h E_0 + 1000)$. R_t and C_t are, respectively, the isotopic composition and the concentration of the compound at a given time t; R_0 and C_0 the same variable at the starting point of the reaction. ε $[^0/_{00}]$ represents an isotopic enrichment factor calculated from the slope of the plot $\ln (C_t/C_0)$ versus $\ln (R_t/R_0)$ multiplied by 1000 giving the ε-value in per mil. The factor Λ_{bulk} expresses the slope of the linear regression for carbon and hydrogen discrimination: $\wedge_{bulk} = \Delta\delta^2 H_{bulk}/\Delta\delta^{13} C_{bulk}$. In agreement with previous studies, C_t/C_0, which is the residual concentration of the compound at the time t, is called *f* and *B* [%] is a parameter expressing the extent of the benzene biodegradation such as $B\ [\%] = (1-f) * 100$.

Enrichment factors were then corrected by considering enrichment factors specific for the reactive position ($\varepsilon_{\text{reactive position}}$). As benzene is a symmetrical molecule with potentially six reactive carbons and six hydrogen atoms (Fischer et al., 2008), the AKIE (Apparent Kinetic Isotope Effect) considering the intramolecular competition of carbon and hydrogen atoms, is calculated according the following equation (Elsner et al., 2005): $AKIE = 1/(1 + z * \varepsilon_{\text{reactive position}}/1000)$ with $z = 6$ (number of atoms of an element in identical reactive positions).

Microscopic Observation and Cell Counts

Throughout the study, microcosms were sampled and observed under phase contrast microscopy. Total cell counts were performed by DAPI-staining (4′,6′-diamidino-2-phenylindole, Sigma–Aldrich) with an Olympus BX60 epifluorescence microscope equipped with a monochrome camera (12 bits, QIClick) and with a mercury light source. Formation water (18 mL) was fixed on-site with 2 mL of 10% borax-buffered formaldehyde (37%, Sigma–Aldrich) and stored at 4°C. Ten milliliters of sample were stained with 0.5 mL DAPI stock solution (200 μg.mL^{-1}) then filtered onto 0.2 μm pore-size black polycarbonate filters (Millipore) under vacuum. For the eighth subculture, counterstaining was done by filtering a mixture of 22 μL of culture with 1 μL of DAPI stock solution onto 0.2 μm pore-size black polycarbonate filters (Millipore) under vacuum. For each filter, 10 randomly selected fields were counted.

Inhibition Tests

On the eighth subcultures, BTE degradation inhibition tests were carried out by monitoring the biodegradation periodically and injecting sodium molybdate, Na_2MoO_4 (10 mM final concentration) and sodium 2-bromoethanesulfonate or BES, $BrCH_2CH_2SO_3Na$ (2 mM final concentration) through butyl stoppers just after the beginning of the biodegradation.

RESULTS

Benzene, Ethylbenzene, and Toluene Removal Kinetics Along Successive Enrichments

Deep aquifer water from the original microcosm (November 2000) showed degradation of ethylbenzene after 900 days incubation under sulfate-reducing conditions. The first subculture (February 2002) was able to degrade ethylbenzene in 100 days and showed the beginning of toluene degradation whereas the second subculture (July 2002) degraded ethylbenzene in 100 days, toluene in 150 days and benzene in 270 days (**Figures 1A** and **2**). The ability to sequentially degrade ethylbenzene, toluene, and benzene (BTE) was observed for the next enrichments under sulfate-reducing conditions (from the second subcultures to the eighth ones), but no removal of xylene isomers was observed (**Figures 1** and **2**). Degradation was not observed in the abiotic controls, although there was slow mono-aromatic hydrocarbon absorption by the butyl septa as reported in similar studies (Shen and Sewell, 2005; Holmes et al., 2011). After 38 months of incubation, no BTEX removal was detected in the presence of the electron acceptors: nitrate, iron III, and CO_2. Eight successive enrichments were achieved under sulfate-reducing conditions during the eight following years. The initial observation of the biomass in the formation water collected in November 2000 and before any transfer, showed a low bacterial biomass (8.5×10^3 cells.mL^{-1}) with apparent low morphological cell diversity. Subcultures of the BTE-degrading microcosms under sulfate-reducing conditions lead to a significant gain in biomass along the successive enrichments obtained years after years (**Figure 1B**). Degradation started after a lag phase lasting from 50 to 120 days and benzene degradation was complete approximately 4 months later. Whereas BES, an inhibitor of methanogenesis, addition did not show any effect, BTE degradation was significantly stopped as soon as sodium molybdate ($NaMoO_4$), a specific inhibitor of sulfate reduction, was injected as shown in **Figure 1C**. It was particularly apparent with ethylbenzene where the removal process was stopped at 60% immediately molybdate was introduced. No further BTE disappearance was then observed once MoO_4 was introduced. We also tested the BTE-degrading microbial community with ethylbenzene (100 ppm), or toluene (100 ppm), or benzene (100 ppm) as the sole carbon and energy sources. In these

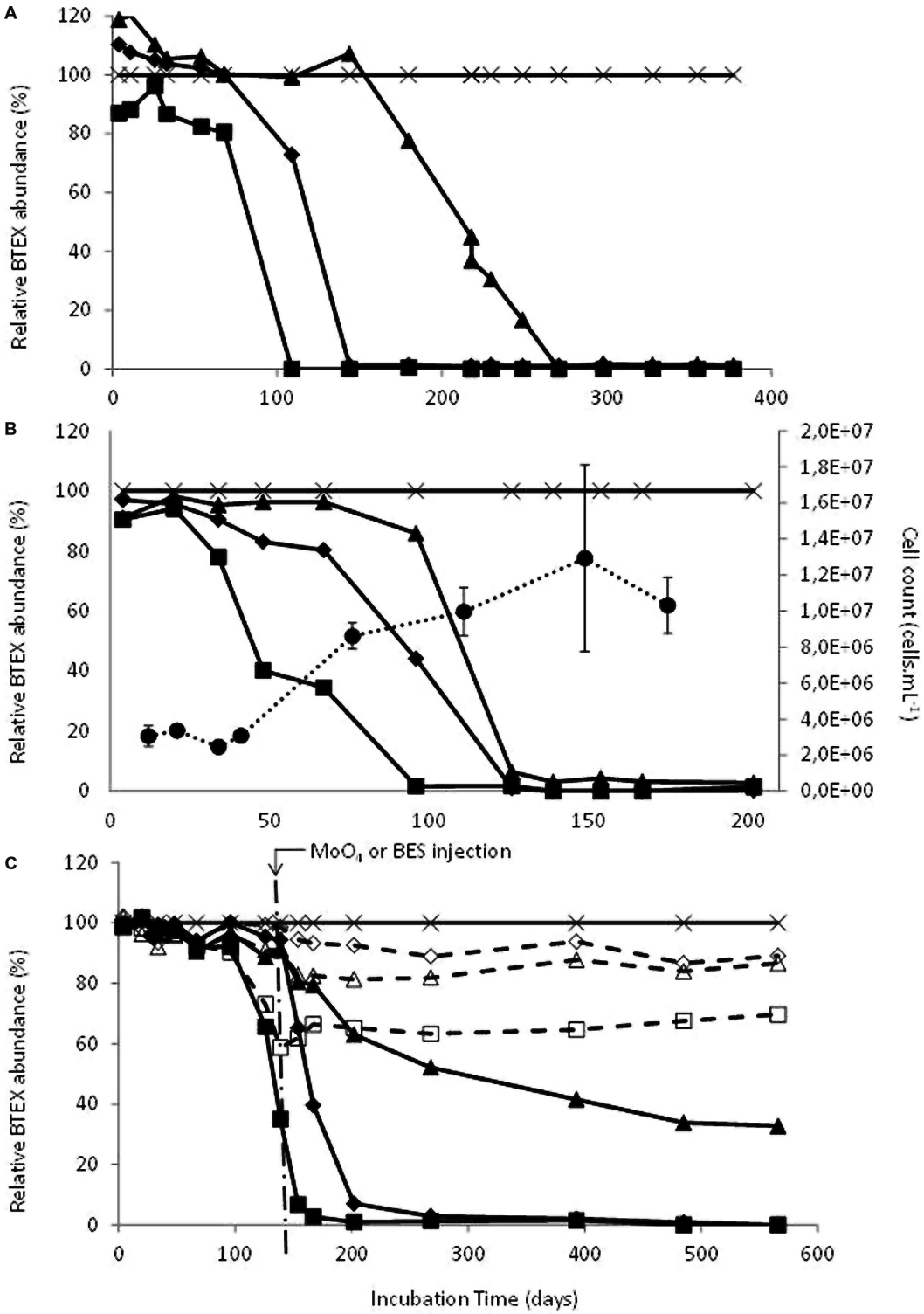

FIGURE 1 | (A) Degradation of benzene, toluene, and ethylbenzene (BTE) during the second subculture (07/2002); *Filled squares* : ethylbenzene, *filled diamonds* : toluene, *filled triangles* : benzene, *cross* : o-xylene as internal standard. **(B)** Degradation of BTE along increase of observable biomass during the eighth subculture (04/2008); *Filled squares* : ethylbenzene, *filled diamonds* : toluene, *filled triangles* : benzene, *cross* : o-xylene as internal standard and *filled circles*: cells.mL^{-1}. **(C)** Effects of inhibitors addition on BTE biodegradation during the eighth subculture (04/2008); Arrow indicates the addition at day 139 of sodium molybdate (MoO_4, 10 mM) or bromoethanesulfonate (BES, 2 mM); *Filled squares* : ethylbenzene + BES, *filled diamonds* : toluene + BES, *filled triangles* : benzene +BES, *cross* : o-xylene as internal standard + BES or + MoO4, *open squares* : ethylbenzene + MoO_4, *open diamonds* : toluene + MoO_4, *open triangles* : benzene + MoO_4. Start levels of BTEX were 100 ppm.

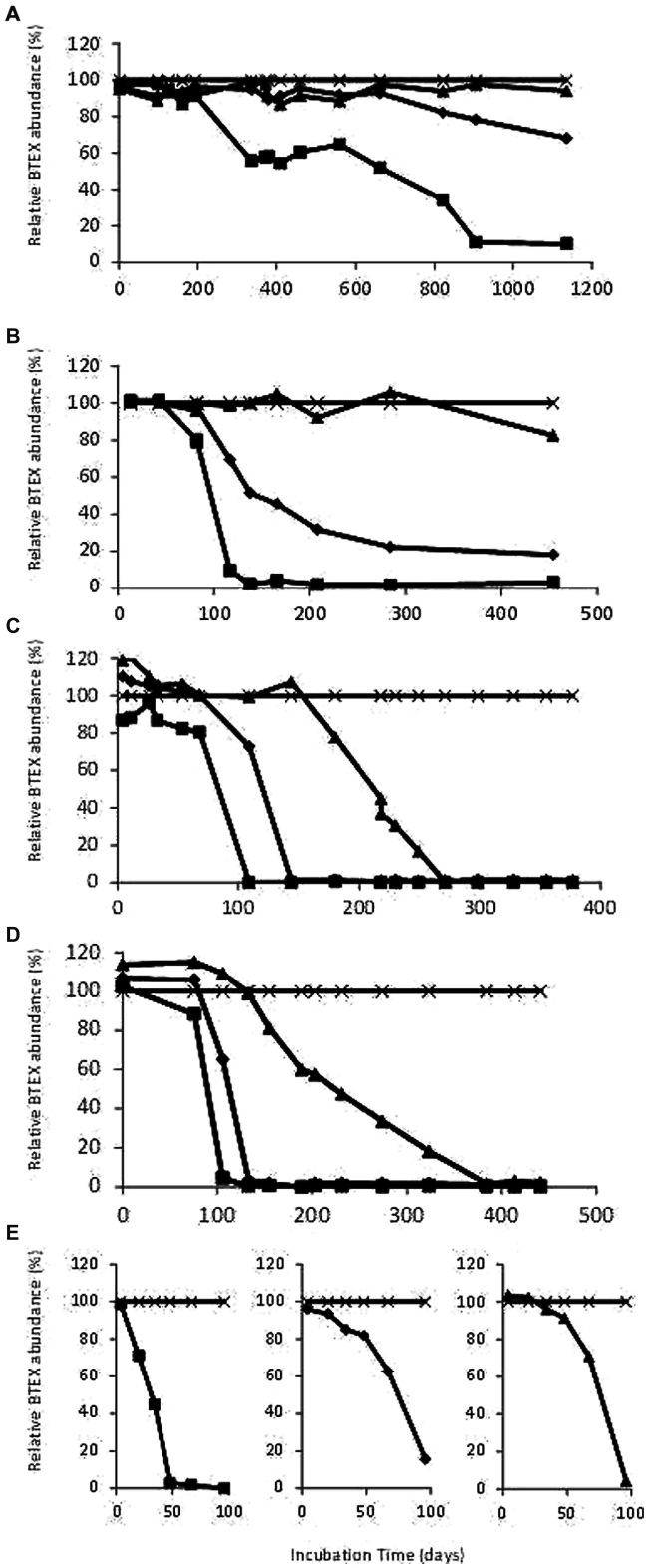

FIGURE 2 | (A) Degradation of benzene, toluene, and ethylbenzene (BTE) during the original microcosm (10/2000) incubation; **(B)** Degradation of BTE during the first subcultures (02/2002); **(C)** Degradation of BTE during the second subcultures (07/2002); **(D)** Degradation of BTE during the fifth subcultures (01/2005). **(E)** Degradation of ethylbenzene, or toluene, or benzene during the sixth subcultures (04/2008). *Filled squares*: ethylbenzene, *filled diamonds*: toluene, *filled triangles*: benzene, *cross*: o-xylene as internal standard. Start levels of BTEX were 100 ppm.

last assays, the toluene and the benzene removal rates were higher than in the BTEX mixture enrichment with a complete disappearance within 3 months (**Figure 2**).

Isotopic Fractionation Associated to Anaerobic Benzene Degradation

Further investigation performed in the benzene-only amended microcosm showed that organic chemistry of the residual benzene was also affected along the degradation (noted *B* [%]). The initial $\delta^{13}C$ and $\delta^{2}H$ values of the labeled benzene were $-25.2 \pm 0.1‰$ and $-43.5 \pm 0.7‰$, respectively. The analysis of the residual benzene fraction revealed a significant and regular increase of $\delta^{13}C$ up to $-21.2 \pm 0.1‰$ and $\delta^{2}H$ up to $58.0 \pm 1.4‰$ linked to the extent of benzene degradation (**Figure 3**). Sterile controls under sulfate-reducing conditions with different benzene concentrations added showed no isotopic fractionation for carbon and hydrogen, with stable carbon and hydrogen isotopes signatures over time (data not shown). The specific apparent kinetic effect of the isotopic fractionation for carbon and hydrogen ($AKIE_C$ and $AKIE_H$) along the benzene removal were, respectively, 1.0146 ± 0.0009 and 1.5184 ± 0.0283 and were derived from enrichment factors (ε_C and ε_H) given in **Table 1**; **Figures 4A,B** and **5**. The general combined effect of carbon and hydrogen isotopic fractionations was gaged by the ratio $\wedge$ ($\wedge = 23.8 \pm 0.4$) obtained by the dual plot analysis of the carbon vs. hydrogen isotope fractionation range along the degradation (**Figure 4C**).

Microbial Characterization of the Hydrocarbonoclastic Enrichment

Cell counts in the BTE-degrading enrichments showed that BTE removal was linked to a fourfold increase of cells in microcosms increasing from 3.6×10^6 to 1.2×10^7 cells.mL^{-1} (**Figure 1B**). However, the shortest doubling time about approximately 20 days was extremely low. Spores were observed from the initial enrichments (November 2000) and were still observed along the enrichments. No evidence of archaeal populations could be detected by biomolecular approaches (Archaeal 16S rRNA gene amplification). The clone libraries analyses based on 8F-926R or 8F-1492R amplicons revealed only two distinct phylotypes, both affiliated to the genus *Desulfotomaculum* (**Figure 6**). The comparison of the two related nucleic sequences based on 1492 nucleotides exhibited divergence above 5.7% between these two phylotypes. The phylotype Bc107 covered 98% of the clone libraries from the both clone libraries obtained by both primers couple, the second one, so-called, Bc105 clustered only 2% of the total clone library. Complementary microscopic observations and biomolecular approaches by t-RFLP analysis confirmed the low diversity obtained with only two distinct peaks (data not shown). The dominant phylotype Bc107 shared the closest affiliation with environmental sequences obtained from borehole water in a deep South African gold mine (clone TTMF126, accession number AY741686). The more closely related environmental sequences for the minor phylotype Bc105 were derived from a 896 m-deep aquifer linked to the South African gold mine (96% similarity with the clone DR9IPCB16SCT7, accession number AY604051) and a petroleum-contaminated soil (96% similarity with the clone EK CK572, accession number JN038217). Bc107 population shared the closest sequence similarity with thermophilic and moderate thermophilic strains in the cluster Ia of the *Desulfotomaculum* genus (Gram-positive Bacteria), such as *D. putei* isolated from the deep subterranean biosphere at 2.7 km depth (Liu et al., 1997), *D. hydrothermale* isolated from a terrestrial hot spring (Haouari et al., 2008), or *D. varum* a moderately thermophilic bacterium from a 66°C- Great Artesian Basin (Ogg and Patel, 2011).

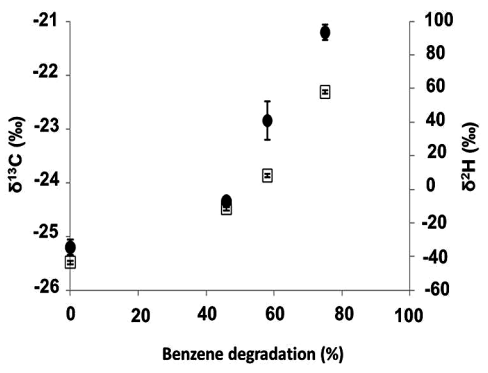

FIGURE 3 | $\delta^{13}C$ and $\delta^{2}H$ of residual benzene fraction versus benzene degradation rate under sulfate-reduction. *Filled circle*: $\delta^{13}C$; *clear square*: $\delta^{2}H$. Benzene degradation refers to *B* [%], see Experimental procedures section. Start level of benzene was 12 ppm.

DISCUSSION

Assessment of Biodegradation Process at the Origin of the BTE-and Benzene Removal Under Sulfate-Reducing Condition

The natural gas stored in underground gas storage aquifers is mainly composed of methane but also contains traces of other compounds, which include BTEX, with concentrations in parts per billion (ppb). The majority of BTEX is withdrawn at the same time as the natural gas is extracted from the underground reservoir; however, during storage a part of the BTEX dissolves in the formation water where these compounds are undesirable. In surface environments, or in shallow aquifers with direct influences from surface environments, microorganisms are exposed to hydrocarbons naturally present in the environment (alcanes, terpenoïds) or introduced by human activities (oil spill). In the case of very deep environments (below −100 m), ecosystems have remained remarkably stable over geological time. After 150 million years isolated from the surface, gas injection with the input of a trace amount of organic matter represents an unknown stress to indigenous microorganisms which could lead to the selection of specific populations among the indigenous microbial community.

TABLE 1 | Carbon and hydrogen isotope enrichment factors retrieved in this study and compared with literature data obtained during anaerobic degradation of benzene.

	Initial benzene added [μM]	εC bulk [‰] ± 95 % CI [‰]	R^2	εH bulk [‰] ± 95 % CI [‰]	R^2	Reference
Pure culture						
Ralstonia picketti VKOl(omc)	885	−1.7 ± 0.2	0.98	−11 ± 4	0.86	Fischer et al., 2008
Cupriavidus necator ATCC 17697 (oxic)	1180	−4.3 ± 0.4	0.99	−17 ± 11	0.89	Fischer et al., 2008
Burkholderia sp. (oxic)	700	−3.5 ± 0.3	0.97	−11 ± 2	0.91	Hunkeler et al., 2001
Acinetobacter sp. (oxic)	700	−1.5 ± 0.8	0.99	−13 ± 1	0.99	Hunkeler et al., 2001
Azoarcus denitrificans strain BC (oxic)	603	−2.6 ± 0.8	0.97	−16 ± 4	0.97	Fischer et al., 2008
A. denitrifans strain BC (Chlorate-reducing)	462	−1.5 ± 0.5	0.86	−28 ± 6	0.98	Fischer et al., 2008
Mixed cultures						
Nitrate-reducing, mixed negative	250	−2.2 ± 0.4	0.98	−35 ± 6	0.91	Mancini et al., 2003
Sulfate-reducing, mixed negative	192	−3.6 ± 0.3	0.92	−79 ± 4	0.79	Mancini et al., 2003
Sulfate-reducing, mixed negative	192	−1.9 ± 0.3	0.97	−59 ± 10	0.99	Fischer et al., 2008
Methanogenic, mixed negative	750	−1.9 ± 0.1	0.98	−60 ± 3	0.92	Mancini et al., 2003
Methanogenic, mixed negative	900	−0.8 ± 0.2	0.93	−34 ± 8	0.88	Mancini et al., 2008
Methanogenic, mixed negative	450	−1.1 ± 0.1	0.88	−38 ± 6	0.80	Mancini et al., 2008
Sulfate-reducing, enriched positive	450	−2.5 ± 0.2	0.97	−55 ± 4	0.93	Bergmann et al., 2011
Iron-reducing, enriched positive	200	−3.0 ± 0.5	0.93	−56 ± 8	0.93	Bergmann et al., 2011
Sulfate−reducing, enriched positive	**400**	**−2.4 ± 0.3**	**0.91**	**−57 ± 0.0**	**0.98**	**This study**

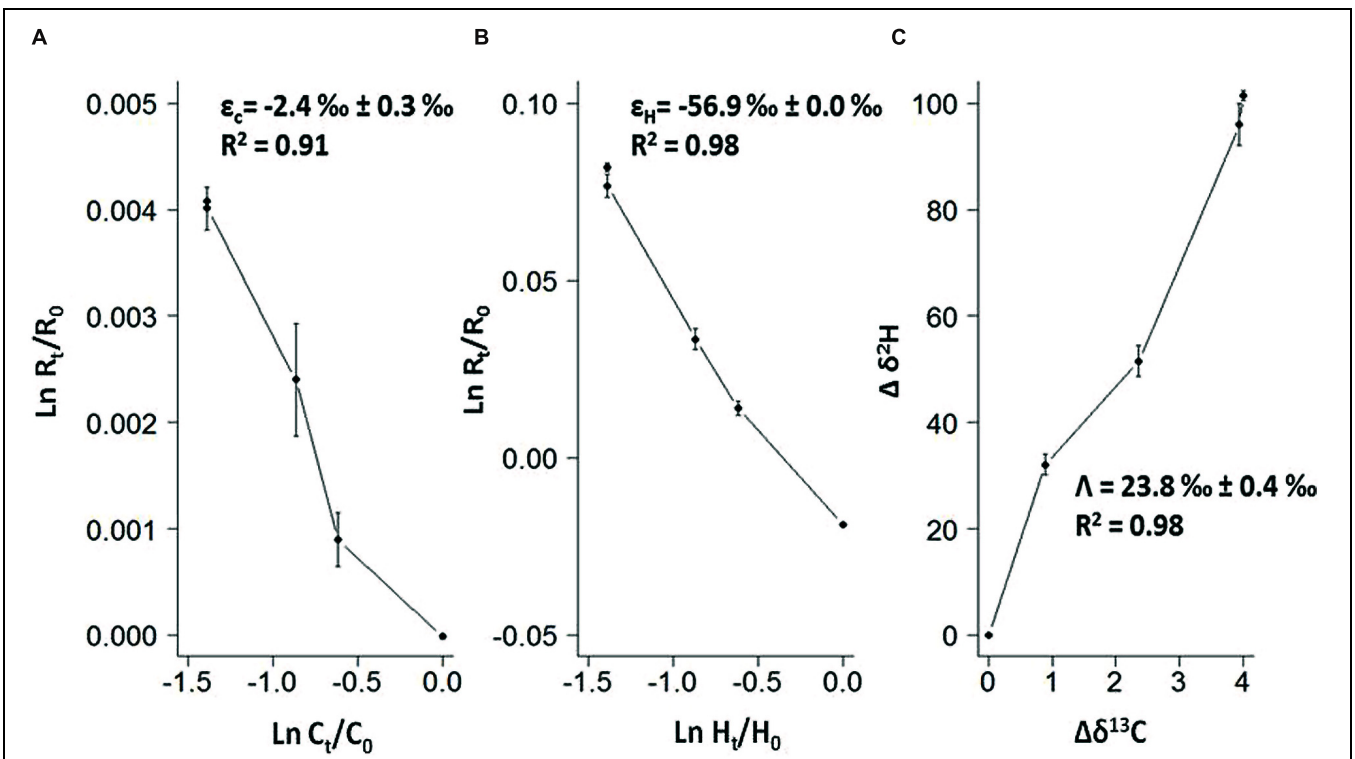

FIGURE 4 | (A,B) Double logarithmic plot according to the Rayleigh equation expressing changes in isotopic composition and compounds concentration along time for carbon and hydrogen during anaerobic degradation of benzene; **(C)** Dual isotope plots of $\Delta\delta^2$H versus $\Delta\delta^{13}$C for anaerobic benzene biodegradation giving the Λ values as the slope of the regression. Dashed lines in all graphes represent the corresponding 95% confidence intervals from duplicate analysis.

Our results showed that a deep subsurface confined pristine aquifer can hold microbial communities acting as a key player in the natural attenuation of benzene and alkylbenzenes. Our evidence is consistent with a biological, rather than a physical–chemical process, causing the disappearance of benzene, toluene and ethylbenzene (BTE): (i) repeatability

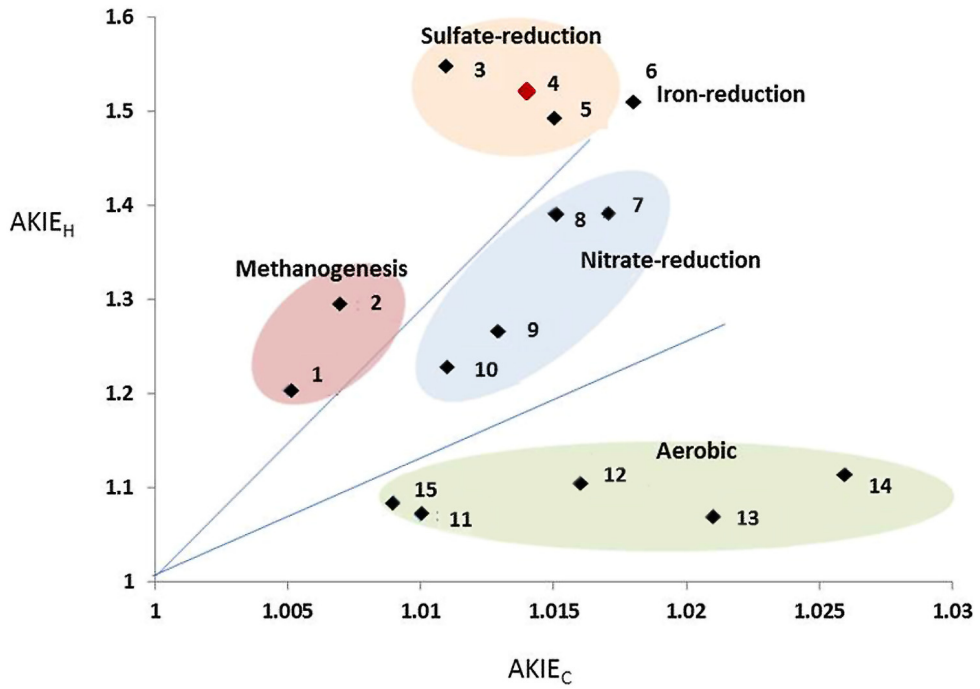

FIGURE 5 | Biplot of AKIE$_C$ vs. AKIE$_H$ values from data retrieved in this study and in literature dedicaced to benzene biodegradation. Redox condition are deduced from the culture condition where the biodegradation was observed. 1: methanogenic consortium (Mancini et al., 2008); 2: methanogenic consortium (Mancini et al., 2008); 3: sulfate-reducing consortium (Fischer et al., 2008); 4: sulfate-reducing consortium, this study; 5: sulfate-reducing consortium (Bergmann et al., 2011); 6: iron-reducing consortium (Bergmann et al., 2011); 7: nitrate-reducing consortium (Mancini et al., 2003); 8: Nitrate-reducing consortium (Mancini et al., 2008); 9: nitrate-reducing consortium (Mancini et al., 2008);10: nitrate-reducing consortium (Mancini et al., 2003); 11: *Ralstonia Pickettii* (Fischer et al., 2008); 12: *Azoarcus denitriificans* (Fischer et al., 2008); 13: *Burkholderia* sp. (Hunkeler et al., 2001); 14: *Cupriavidus necator* ATCC 17697 (Fischer et al., 2008); 15: *Acinetobacter* sp. (Hunkeler et al., 2001).

over time (eight subcultures); (ii) degradation of benzene, or toluene, or ethylbenzene as sole carbon sources but no xylene degradation; (iii) an increase of biomass linked to the degradation of the monoaromatic hydrocarbons, in particular the ethylbenzene (**Figures 1** and **2**). Isotopic fractionation studies along with biological processes have been reported for decades as a promising technique for *in situ* direct evidences of biodegradations (Borden et al., 1995; Kelley et al., 1997; Ahad et al., 2000; Meckenstock et al., 2004; Zwank et al., 2004; Bergmann et al., 2011; Braeckevelt et al., 2012). Here, the extent of carbon isotope fractionation was about 4‰ which is in agreement with the extent of isotopic fractionation expected for this compound for biodegradation (>2‰); for hydrogen isotope fractionation, the change was about 100‰ which is in the same range as reported in the review by Braeckevelt et al. (2012) with values >20‰ as a proof of biodegradation process.

Other evidence, strongly suggests that sulfate-reducing microorganisms are involved in the BTE degradation. Such as the lack of degradation with electron acceptors other than sulfate (nitrate, iron(III), carbon dioxide). Archeal 16S rRNA gene was not amplified suggesting the absence of this domain in this community, in particular methanogenic archaea. This was confirmed by the inhibition of degradation with molybdate but not with BES. Additionally, the dual plot analysis of CSIA with C and H showed that our $\wedge$ value (23.8 $\pm$ 0.4 with R^2 = 0.98; **Figure 4C**) was well integrated with literature values obtained in low redox potential conditions including fermenting, methanogenic and sulfate-reducing benzene-degrading enrichments ($\wedge$ = 22–28). In contrast, all these values strongly differ with $\wedge$ reported in literature for benzene degradation in higher redox conditions like in nitrate-reducing condition ($\wedge$ = 12–16) (Mancini et al., 2003, 2008; Fischer et al., 2007, 2008; Bergmann et al., 2011; Gieg et al., 2014). Moreover, enrichment factors obtained during anaerobic benzene degradation under sulfate-reducing conditions showed the influence of microbial composition on the extent of the isotopic fractionation of C and H among sulfate-reducing benzene degrading enrichments: our results agree with values obtained by Bergmann et al. (2011) with a Gram-positive enriched benzene degrading community ($\varepsilon_C = -2.5 \pm 0.2$‰ with R^2 = 0.97; $\varepsilon_H = -55 \pm 4$‰ with R^2 = 0.93) but are different from values obtained with mixed Gram-negative sulfate-reducing enrichments ($\varepsilon_C = -3.6 \pm 0.3$‰ with $R^2 = 0.92$; $\varepsilon_H = -79 \pm 4$‰ with R^2 = 0.79 [Mancini et al., 2003) and $\varepsilon_C = -1.9 \pm 0.3$‰ with R^2 = 0.97; $\varepsilon_H = -59 \pm 10$‰ with R^2 = 0.99 (Fischer et al., 2008)]. Our unsuccessful attempt to isolate a pure strain able to degrade at least one of the BTE compounds could be due to the existence of an obligatory syntrophism between the two detected populations at the origin of the BTE degradation

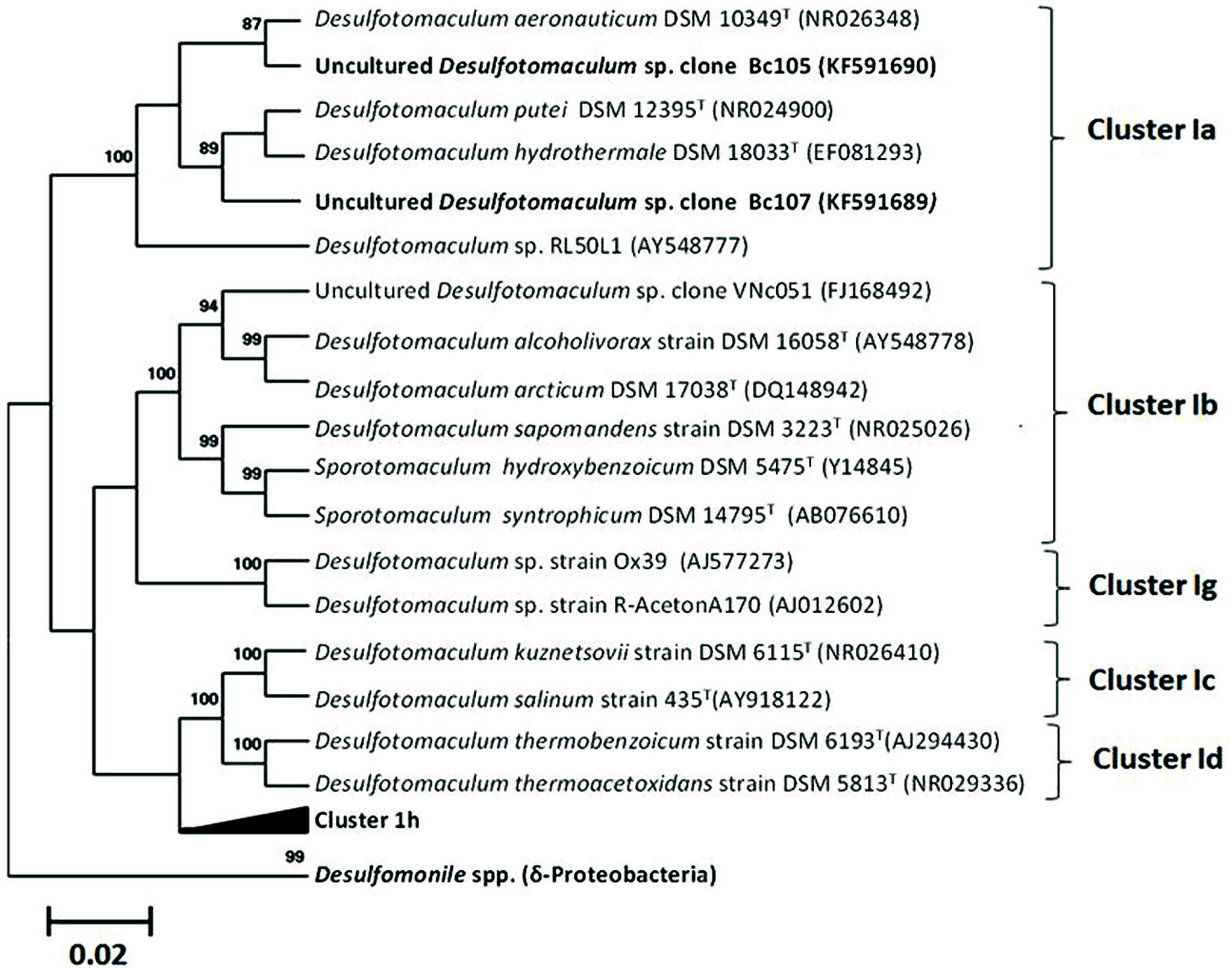

FIGURE 6 | Maximum-likelihood tree based on 16s rRNA gene (1115 bases) showing the phylogenetic relationship between the both sequences detected in microcosms (clone Bc105: minor phylotype and clone Bc107: dominant phylotype) and closest relatives. Reliability values (aLRT values) greater than 50% are given at nodes.

as it has been previously described for the anaerobic degradation of toluene by syntrophic fermentative oxidation with a co-culture containing a sulfate-reducing bacterium (Meckenstock, 1999).

The addition of fumarate has previously been shown to be the mechanism of activation of toluene and ethylbenzene under sulfate-reducing conditions (Rabus and Heider, 1998; Kniemeyer et al., 2003). These reactions are catalyzed by fumarate-adding enzymes (FAEs) which encompass benzylsuccinate-synthase (Bss) and alkylsuccinate-synthase (Ass). PCR amplifications targeting *bssA* gene were unsuccessful using previously described specific primers (Winderl et al., 2007; von Netzer et al., 2013). The absence of known metabolic genes has also been reported in other BTEX degrading consortia dominated by Gram-positive bacteria (Hermann et al., 2008; Abu Laban et al., 2009; Bergmann et al., 2011). For two axenic BTEX degrading nitrate-reducing strains: *Dechloromonas aromatica* RCB and *Dechloromonas* sp. JJ (Coates et al., 2001; Chakraborty et al., 2005), functional genes research investigations and genomic analyses did not highlight any known pathways for anaerobic degradation of aromatics such as the central benzoyl-CoA pathway for monoaromatics and benzylsuccinate synthase (bssABC) genes for toluene and m-xylene degradation (Salinero et al., 2009). In our study, the absence of benzylsuccinate-synthase activity remains unclear. It can be postulated that if present in our community, this enzyme and its genes are different enough from those previously described to prevent gene amplification by known primers. Similar difficulties have already been reported for the *bssA* gene in toluene-degrading communities (Sun et al., 2013) and with the toluene-degrading strain *Desulfotomaculum* sp. Ox39 (Winderl et al., 2007). Alternatively, the isotopic signature of carbon and hydrogen obtained both here and previously by Bergmann et al. (2011) could be related to an unknown pathway for the anaerobic biodegradation of monoaromatic

compounds and possibly specifically to a lineage among sulfate-reducing Gram-positive biodegraders. Derived values from isotopic fractionation of carbon and hydrogen, especially $AKIE_C$ and $AKIE_H$, published here in **Figure 5** (1.0146 ± 0.0009 and 1.5184 ± 0.0283, respectively) were perfectly integrated with other AKIE indices available in literature and showed a specific clustering among other benzene-degrading conditions with sulfate-respiring cultures. Our data will reinforce the limited existing research in term of anaerobic biodegradation of benzene available in the literature (Mancini et al., 2003; Fischer et al., 2008; Bergmann et al., 2011). Dual plot carbon specific isotope analysis, especially AKIE indices, initially suggested by Elsner et al. (2005) and reviewed by Braeckevelt et al. (2012) enables a wider range of comparisons across the biodegradation kinetics of different hydrocarbons and remains one of the more promising monitoring techniques which may, in the long term, discriminate the biochemical pathway and energy sources involved during *in situ* natural attenuation in subterranean environments.

A New Hydrocarbonoclastic Microbial Population Representative of the Deep Subsurface Confined Aquifer

The regular observation of spores along the successive BTE-degrading enrichments and benzene-degrading enrichments strongly suggest that spore-forming microorganisms play an important role in the BTE degradation. Molecular biology approaches revealed the presence of only two different species and both belonging to *Desulfotomaculum* genus (*Firmicutes*/*Clostridia*/*Clostridiales*/*Peptococcaceae*, Widdel, 2006). *Desulfotomaculum* sp. and related Gram-positive sulfate-reducing bacteria such as *Desulfosporosinus* sp. are frequently encountered in deep environments (Daumas et al., 1988; Nilsen et al., 1996; Ehinger et al., 2009; Aüllo et al., 2013) and are sometimes the main representatives of these communities (Baker et al., 2003; Moser et al., 2003; Detmers et al., 2004; Moser et al., 2005), especially in several oilfields over the world with generally a positive correlation of cell abundance with increasing temperature with depth (Aüllo et al., 2013; Guan et al., 2013). Their presence in these environments could be linked to their ability to sporulate allowing them to withstand adverse periods during burial such as lack of nutrients or heat (O'Sullivan et al., 2015), and their metabolic versatility as demonstrated by their ability to respire sulfate, thiosulfate, sulfur, sulfite, metals, and metalloids. Available data on microbial diversity in deep aquifers used as natural gas storage are scarce. Nevertheless, others studies on other deep aquifers revealed the presence of this genus sometimes abundantly (Basso et al., 2009; Ehinger et al., 2009; Berlendis et al., 2010). Although members of *Desulfotomaculum* can also be found in surface ecosystems, both populations identified in this study are close to environmental sequences detected in deep subsurface environments supporting their ecological relevance (Moser et al., 2005; Gihring et al., 2006; Baito et al., 2015) (AY604051, AY741686, AB910321). On the basis of their sequences encoding the 16S rRNA gene, we assume that we are dealing with two different species. Some members of *Desulfotomaculum* and *Desulfosporosinus* have the ability to degrade hydrocarbons, in particular mono-aromatic hydrocarbons such as toluene, m-xylene and o-xylene (Robertson et al., 2000; Liu et al., 2004; Morasch et al., 2004; Abu Laban et al., 2015). Until now, bacterial isolates have shown the ability to degrade benzene either in iron-reduction (Holmes et al., 2011; Zhang et al., 2012) or in nitrate-reduction (Coates et al., 2001; Chakraborty et al., 2005; Kasai et al., 2007). Here, we demonstrate that some members of *Desulfotomaculum* are also able to degrade benzene and ethylbenzene under sulfate-reducing conditions. Despite multiple assays, no pure isolates able to degrade benzene were obtained confirming the difficulty to obtain a pure sulfate-reducing strain able to degrade anaerobically benzene (Vogt et al., 2011; van der Zaan et al., 2012), which requires us to hypothesize a possible synergy between these two populations of *Desulfotomaculum*. However, why have these organisms the capacity to degrade BTE in a poorly carbonated sandstone deep aquifer? What we know about this type of aquifers implies that the indigenous microbial communities before gas storage had to be essentially based on using CO_2 and H_2 on the principle of a subsurface lithoautoautrophic microbial ecosystem (SLiME) as mentioned by Stevens and McKinley (1995) and Basso et al. (2009). Having a hydrocarbon biodegradation ability could provide a benefit to *Desulfotomaculum* which can switch to various energy sources along with post-diagenetic environmental changes and consume this type of molecule potentially present as residues in a fossil organic matter entrapped in rocks. Alternatively, these microorganisms may have the same origin as the injected gas and would therefore be derived from an oil reservoir. This hypothesis would explain the ability of these organisms to degrade hydrocarbons, in particular BTEX. The natural gas treatment process after extraction from the oil reservoir, and especially the dehydration steps would allow only few spores to resist. Recently, it has been shown that *Desulfotomaculum* spores could resist triple autoclaving processes (O'Sullivan et al., 2015). Spores could then be transported thousands of kilometers in pipelines and co-injected with natural gas into deep aquifers. A previous report of the isolation of *Desufotomaculum thermocisternum* from the North sea oil reservoir able to grow syntrophically with a methanogen (Nilsen et al., 1996), the presence of *Desulfotomaculum* and methanogen species dominating the microbial diversity of a deep gold mine at 4–6 km of depth (Moser et al., 2005) support the hypothesis of a versatile metabolism in various subsurface habitats.

Many studies suggest a possible key role of Gram-positive of members of *Clostridia* (Winderl et al., 2010) and the family *Peptococcaceae* in BTEX biodegradation (Phelps et al., 1998; Da Silva and Alvarez, 2007; Kunapuli et al., 2007; Kleinsteuber et al., 2008; Musat and Widdel, 2008; Oka et al., 2008; Abu Laban et al., 2009; Berlendis et al., 2010; Taubert et al., 2012; van der Zaan et al., 2012; Kuppardt et al., 2014). This study demonstrates the key role of sulfate-reduction in this community (**Figure 1C**) and we could deduce that *Desulfotomaculum* from the community (Bc105 or Bc107 or both) degrade the aromatic hydrocarbons by direct oxidation. This BTE degradation would be similar to those described by Abu Laban et al. (2009). These authors clustered the highly abundant sequences between the

genera *Desulfotomaculum* and *Pelotomaculum* and postulated these organisms were key players in benzene degradation with sulfate as the electron acceptor. The slow kinetic removal of monoaromatic hydrocarbons, and benzene in particular, observed in our study compared to kinetics reported in literature (Oka et al., 2008; Abu Laban et al., 2009; van der Zaan et al., 2012) could be linked to (i) an inhibition of anaerobic benzene degradation by co-contaminants (Edwards and Grbić-Galić, 1992; Cunningham et al., 2001; Ruiz-Aguilar et al., 2003; Da Silva and Alvarez, 2007; Vogt et al., 2011); (ii) microbial metabolism under extreme energy limitation (Hoehler and Jørgensen, 2013) and (iii) syntrophic consortia requiring optimal conditions (Vogt et al., 2011). Syntrophism between a fermentative and a by-products utilizer could be a logical adaptive strategy of indigenous lithoautotrophic communities to the presence of recalcitrant hydrocarbons. This is supported by a previous investigation using DNA-SIP techniques from a sulfate-reducing community degrading benzene enriched from contaminated groundwater, showed the likely dominance of still undescribed Gram-positive species seemingly active at the early stage of the benzene degradation followed by an undescribed Epsilon-proteobacteria assumed to be a hydrogen scavenger (Hermann et al., 2008). In the same way, a study by protein-SIP applied on a benzene-degrading microbial consortium from a shallow aquifer, Taubert et al. (2012) hypothesized the key role of *Clostridiales*, in particular *Desulfotomaculum* and *Pelotomaculum* genera, for benzene degradation. These authors and others (Kleinsteuber et al., 2008) suggested *Peptococcaceae* could putatively ferment benzene and excrete by-products such as acetate and hydrogen which would be used by the whole community, in particular Delta-proteobacteria (sulfate-reducers). If this hypothesis is true, the inhibition of sulfate-respiration after adding molybdate could stop the benzene degradation since the reaction would be thermodynamically unfavorable.

New Bio-Indicator Parameters (AKIE Values, Phylotypes) for Benzene Biodegradation: Field Applicability in Deep Subterranean Environments

The selection of an active benzene and alkylbenzenes-degrading community shows that these hydrocarbonoclastic populations could be persistent and certainly able to sustain *in situ* biodegradation as long as a substrate is available. Deep subsurface confined aquifers despite restricted access remain key location points for subsurface engineering activity (water resources, oil, and gas industries, geothermal energy, bioremediation, fundamental research interests). However, field biodegradation studies are currently mandatory for bioremediation, oil recovery or geological exploration. Indeed, in the context of hydrocarbon biodegradation strongly linked to aquifers properties (Warren et al., 2004), petroleum industries, bioremediation experts, and environmental ecologists point out the need to systematically collect and share various technical information requiring a strong fundamental research background such as microbial ecology, isotopic studies, and biogeochemistry (Scow and Hicks, 2005; Declercq et al., 2012; Hubbard et al., 2014). Because of the higher benzene persistence in anoxic environments than its others alkylated derivatives, we focused our study on this hydrocarbon. Sequences of both phylotypes showed no close affiliation with any *Desulfotomaculum* species detected in biphenyl or benzene-degrading consortia reported in literature (Abu Laban et al., 2009; Selesi and Meckenstock, 2009). In addition, both phylotypes were loosely affiliated to any known hydrocarbonoclastic microorganisms, such as *Desulfotomaculum* sp. Ox39 (Morasch et al., 2004). Hence the results presented here obtained with this new benzene-degrading enrichment significantly extent the diversity of Gram-positive biodegraders from a deep subterranean aquifer (Abu Laban et al., 2009; Bergmann et al., 2011: for the last review about microorganisms involved in anaerobic biodegradation of petroleum, see Widdel et al., 2010). As microbial diversity in low-energy environments is known to contain only a few cultivated microorganisms, with their biochemistry and physiology largely unknown (Hoehler and Jørgensen, 2013), the additional information provided by DNA-based microbial identification such as AKIE may enable further studies with similar results to bring new evidences and new insights about these biodegradation processes. For example, a conceptual model of syntrophic biodegradation of hydrocarbons has been initially suggested by Head et al. (2010) and recently supported again by Gieg et al. (2014) where several species could be involved.

Although anaerobic benzene biodegradation has been clearly demonstrated in various conditions, the genetic pathways involved are still unclear, despite the finding of a putative gene cluster (Abu Laban et al., 2010; Vogt et al., 2011). So far, no genetic probe targeting functional genes exists to show direct evidence of anaerobic benzene biodegradation. Therefore, field applications of compound-specific isotope analysis (CSIA) and comparison with literature data from well-known biodegradation cases are currently our most powerful diagnostic tools. Significant outputs of this work would concern petroleum companies not only in the context of bioremediation of deep confined aquifers (Declercq et al., 2012) but also for understanding microbial-induced souring in the oil and gas producing reservoirs (Sun et al., 2005; Wilkes et al., 2008; Gieg et al., 2011).

CONCLUSION

In this study, it was shown for the first time that a bacterial community composed of only two *Desulfotomaculum* populations can use toluene, ethylbenzene and benzene as sole carbon and energy sources in sulfate-reducing conditions. They constitute the simplest model of anaerobic sulfate-reducing hydrocarbon-degrading anaerobes originating from the deep subterranean environments ever described. While many studies have shown *Pelotomaculum* sp., another genus of *Peptococcaceae*, as key-players in BTEX biodegradation, this work highlights the important role of the genus *Desulfotomaculum* as significant indigenous populations of subsurface habitats, but also as an important agent in the anaerobic degradation of hydrocarbons. In the deep subterranean biosphere, complex litho-autotrophic microbial network promotes reactions for

hydrogen interspecies electron transfer. We hypothesize selection of microbial populations able to conduct syntrophic oxidation of organic carbon could be a logical adaptive response of originally litho-autotrophic indigenous microbial communities to the presence of recalcitrant hydrocarbons. Yet, at a time when the exploitation of shale gas and oil is quickly increasing globally, it is necessary to know whether these still poorly undescribed deep subterranean environments have the potential of hydrocarbon degradation, and particularly BTEX. Field monitored natural attenuation (MNA) approaches for subterranean environments should be favored by updating the existing database grouping the identified microbial phylotypes and isotopic values in the context of BTEX biodegradation.

AUTHOR CONTRIBUTIONS

TA, SB, and J-F L designed, performed experiments, analyzed data and wrote the paper; DD and DD performed isotopic fractionation experiments; SS-L, BS, and JM performed cultural experiments; DP and CB critically reviewed this paper; MM and ARP designed, analyzed data, supervised the project and wrote the paper; TA, SB, and J-FL are co-first authors.

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Effect of Biostimulation Using Sewage Sludge, Soybean Meal, and Wheat Straw on Oil Degradation and Bacterial Community Composition in a Contaminated Desert Soil

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Waste materials have a strong potential in the bioremediation of oil-contaminated sites, because of their richness in nutrients and their economical feasibility. We used sewage sludge, soybean meal, and wheat straw to biostimulate oil degradation in a heavily contaminated desert soil. While oil degradation was assessed by following the produced CO_2 and by using gas chromatography–mass spectrometry (GC–MS), shifts in bacterial community composition were monitored using illumina MiSeq. The addition of sewage sludge and wheat straw to the desert soil stimulated the respiration activities to reach 3.2–3.4 times higher than in the untreated soil, whereas the addition of soybean meal resulted in an insignificant change in the produced CO_2, given the high respiration activities of the soybean meal alone. GC–MS analysis revealed that the addition of sewage sludge and wheat straw resulted in 1.7–1.8 fold increase in the degraded C_{14} to C_{30} alkanes, compared to only 1.3 fold increase in the case of soybean meal addition. The degradation of ≥90% of the C_{14} to C_{30} alkanes was measured in the soils treated with sewage sludge and wheat straw. MiSeq sequencing revealed that the majority (76.5–86.4% of total sequences) of acquired sequences from the untreated soil belonged to Alphaproteobacteria, Gammaproteobacteria, and Firmicutes. Multivariate analysis of operational taxonomic units placed the bacterial communities of the soils after the treatments in separate clusters (ANOSIM $R = 0.66$, $P = 0.0001$). The most remarkable shift in bacterial communities was in the wheat straw treatment, where 95–98% of the total sequences were affiliated to Bacilli. We conclude that sewage sludge and wheat straw are useful biostimulating agents for the cleanup of oil-contaminated desert soils.

Keywords: desert soil, oil, illumina, bioremediation, sewage sludge, soybean meal, wheat straw

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INTRODUCTION

Oil contamination results in a dramatic increase in carbon sources in affected soils and a depletion of important nutrients such as nitrogen and phosphorus (Boufadel et al., 1999; Hazen, 2010). This imbalance in carbon–nitrogen ratio and the nitrogen deficiency hamper the biodegradation process (Chorom et al., 2010). Waste materials, such as sewage sludge and soybean meal (termed

hereafter as SG and SB, respectively), are potential stimulating agents for bioremediation as they are economically feasible and rich in nutrients such as phosphorus, nitrogen and carbon (Cui et al., 2008; Agamuthu et al., 2013). SG has been used as an organic fertilizer, a soil ventilator and a bioaugmentation agent in the bioremediation of oil- and metal-contaminated soils (Gallego et al., 2001; Hamdi et al., 2006; Chorom and Hosseini, 2011; Park et al., 2011). The addition of SG reduced up to 45% of polyaromatic hydrocarbons (PAHs) and 43–98% of total petroleum hydrocarbons (TPHs) in different contaminated soils (Hur and Park, 2003; Chorom et al., 2010; Ros et al., 2010; Zhang et al., 2012). SB has also been used as a source of organic nitrogen in the bioremediation of several oil-contaminated soils (Diab, 2013). The addition of SB has been shown to enhance biosurfactant production and to increase hydrocarbons bioavailability (Diab and Sandouka, 2012).

Besides nutrients, oxygen is another limiting factor that influences the efficiency of bioremediation processes especially that the first step in the breakdown of hydrocarbons under aerobic conditions relies on oxygen-dependent enzymes (Gallego et al., 2001). Different aeration methods, such as titling, forced aeration and addition of bulking agents, were employed to accelerate bioremediation (Rhykerd et al., 1999; Odokuma and Dickson, 2003; Marin et al., 2006; Hu et al., 2012). The function of bulking agents is to increase soil porosity, increase oxygen diffusion, lower soil's bulk density and provides a valuable carbon and energy source for microorganisms (Rhykerd et al., 1999; Lang et al., 2000; Huang et al., 2006). Wheat straw (termed hereafter as WS), wood chips, post-peelings, peanut powder were successfully used as aerating agents to biostimulate the growth and activity of microorganisms in PAH-contaminated soils (Llado et al., 2015).

The bioremediation of desert soils is challenging, mainly because of the harsh environmental conditions. The diversity of microorganisms in oil-contaminated arid deserts and their response to different bioremediation treatments have been relatively much less studied than marine sediments. In the Arabian Peninsula, oil spills are very common in deserts, nevertheless little research has been performed to study their environmental impacts and remediation (Radwan, 2008, 2009). Here, we investigate the effect of the biostimulators SG, SB, and WS on respiration activities, hydrocarbons degradation and bacterial community changes in oil-contaminated soils from the desert of Oman. To the best of our knowledge, the response of bacterial activity and diversity in oil-contaminated desert soils to three biostimulating agents has been rarely compared in a single study and using next generation high throughput sequencing (NGS). Recently, NGS has become a robust and a straightforward technology with the ability to generate large sequence databases in a massively parallel fashion (Shokralla et al., 2012). This technique has yielded comprehensive information on the structure of microbial communities and their shifts in contaminated sites (Singleton et al., 2013; Lamendekka et al., 2014; Sun et al., 2015).

MATERIALS AND METHODS

Collection of Soils and Organic Wastes

Oil-contaminated soils were collected on May, 2013 from a dumping area in the deserts of Marmul, Southwest of Muscat, Sultanate of Oman (lat. 18° 10′ 01.3″N; long. 55° 14′ 32.9″E). The area is located close to an oil exploration field and used to collect the oil-contaminated soils, so crude oil was the sole contaminant in the region. Approximately 500 g of the top 1 cm of the soils were collected in triplicates from three locations that were 50–100 m apart. TPH in the soils were gravimetrically measured after extraction of 5 g of soil using 15 ml of dichloromethane (DCM, Sigma-Aldrich, Germany) for 2–3 times. The extracts were filtered by non-absorbent cotton and then subjected to evaporation by rotary evaporator to quantify TPH (Weisman, 1998). The pH and electrical conductivity (EC) were measured using the filtrates of 10 g soil mixed with 50 ml of deionized water, using calibrated YSI instruments. Anions were extracted and evaluated using ion chromatography (IC, Metrohm AG, Herisau, Switzerland). The percent of sand, silt and clay in the soil was determined by a standard hydrometer method (Klute, 1986), and this percent was used to categorize the soil texture from a soil triangle (Brady, 1984).

SB and WS were collected from the Gulf Mushroom Products Company (S.A.O.G) in Barka, Oman. The average moisture and nitrogen contents of the SB were 10 and 8%, whereas these values were 12 and 0.5% for the WS, respectively. SG was collected from an aeration tank in the wastewater treatment plant at Sultan Qaboos University (SQU).

Biostimulation Experiment in Glass Bottles

Biostimulation experiments were conducted in closed glass bottles (termed hereafter as bottle experiment) in order to follow respiration activities of untreated and treated soils through measuring the amount of produced CO_2 and to measure oil degradation at the end of the treatments using gas chromatography–mass spectrometry (GC–MS). One gram of oil-contaminated soil was placed in 165 ml serum glass bottles. To this, 50 mg of each of the biostimulating agents (i.e., SG, SB, and WS) were added. Five ml of sterilized water were added to each vial. Two controls were maintained; one with contaminated soil but without any treatment (untreated soil) and the other with only the biostimulating agent without soil. All treatments and controls were maintained in triplicates. All vials were sealed with a butyl rubber stopper and an aluminum crimp cap to ensure no gas leakage and were incubated for 110 days in the oven at 30°C without shaking. The produced CO_2 was measured by withdrawing 250 μl from the headspace of the bottle using a gas-tight glass syringe and injecting manually into gas chromatography (GC, Agilent model 6890N). The GC was equipped with a thermal conductivity detector and a 30 m × 250 μm capillary column (HP-PLOT Q). Helium was used as a carrier gas at a flow rate of 4 ml min^{-1} and the injector and detector temperatures were maintained at 200 and 210°C, respectively. The oven temperature was programmed from 50 to 80°C (final hold time 3 min) at a rate of 20°C min^{-1}.

Carbon dioxide (CO_2) evolution data were statistically analyzed by one-way ANOVA using the SPSS software (10th edition, Chicago, IL, USA). *P*-values were adjusted using the sequential Bonferroni (Quinn and Keough, 2002) and Tukey's test was used to determine differences between individual means. The degradation of the oil in the bottle experiment was assessed using GC–MS analysis of the alkanes (see below). The ratios of degraded alkanes to the amount of produced CO_2 in the SG and WS treated soils were calculated, after subtracting the CO_2 produced from soil-free SG and WS.

Biostimulation Experiment in Microcosms

Since the laboratory experiment was performed in closed bottles to enable the measurement of CO_2 gas in the headspace, oxygen could become limited and this could slow down biodegradation processes. Therefore, another experiment was performed in open-air glass aquaria (termed hereafter as microcosm experiment) in order to follow the degradation using GC–MS and to monitor shifts in the bacterial community structure. Here, 100 g of soil were placed in glass aquaria (15 cm diameter). Five grams of each of the biostimulating agents (i.e., SG, SB, and WS) were added individually to each aquarium. Untreated soils were kept as controls. All treatments and controls were maintained in triplicates. The biostimulating agents were mixed with the soil, and the mixture was tilled twice a week using a sterile spatula. The mixture was always kept wet by adding 10 ml of sterile water every 2 days. All incubations were kept for 64 days at ca. 30 ± 3°C in a greenhouse. Samples (i.e., 1 g soil each) were collected for GC–MS analysis and MiSeq illumina (see below).

Oil biodegradation (mainly C_{11} to C_{30} alkanes) from the microcosm experiments (and from the bottle experiments) was followed using GC–MS analysis of the soils at the end of the experiment. One gram of each soil was extracted three times in 5 ml dichloromethane (DCM, Sigma-Aldrich, Germany) and then sonicated for 25 min at 10°C. The extracted supernatant was mixed with sodium sulfate and was filtered with non-absorbent cotton to remove solid particles. The filtrate was then evaporated using a rotary evaporator. The dry extract was re-dissolved into DCM and passed through silica gel prior to injection into GC–MS (Perkin Elmer Clarus 600GC/MS). The Perkin Elmer Clarus 600C MS was coupled with Rtx®-5MS capillary column (30 m × 0.25 mm I.D. × 0.25 μm film thickness; maximum temperature, 350°C). Ultra-high purity helium was used as a carrier gas at a constant flow of 1.0 ml/min. The ionizing energy was 70 eV. Electron multiplier (EM) voltage was obtained from autotune. The injection, transfer line and ion source temperatures were 290, 280, and 280°C, respectively. The oven temperature program was held at 80°C for 5 min and then accelerated at a rate of 10°C/min to 280°C (hold for 30 min). The volume of injected sample was 1 μl with a split ratio of 10:1. The standard mix solution (C_7 to C_{30}) of concentrations 10, 20, 30, 40, and 50 ppm were used for confirmation and quantification purposes. A calibration curve conformed by the standard mixtures was established and quantification of the analyzed compounds was performed in the linear range of the calibration curve. The alkanes were identified based on GC retention times of the standard, injected and analyzed under the same conditions as samples and by comparing the spectra obtained with mass spectrum libraries (NIST 2011 v.2.3 and Wiley, 9th edition).

Bacterial Community Analysis using Illumina

DNA was extracted from the soils at the end of the microcosm experiment using skim milk protocol (Volossiouk et al., 1995). Purified DNA extracts were then submitted to Molecular Research MR DNA laboratory (www.mrdnalab.com, Shallowater, TX, USA) for illumina MiSeq sequencing of the bacterial 16S rRNA genes using the primers 341F (5′-CCT ACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGG TATCTAATCC-3′) with barcode on the forward primer (Klindworth et al., 2013). After amplification, PCR products were checked in 2% agarose gel to determine the success of amplification and the relative intensity of bands. Multiple samples were pooled together in equal proportions based on their molecular weight and DNA concentrations. Pooled samples were purified using calibrated AMPure XP beads. Then, the pooled and purified PCR products were used to prepare a DNA library by following illumina TruSeq DNA library preparation protocol. Sequence analysis was carried out using the Mothur MiSeq SOP pipeline (Schloss et al., 2009). Briefly, barcodes were removed and sequences <200 bp and sequences with ambiguous base calls were eliminated. Sequences were denoised, operational taxonomic units (OTUs) generated and chimeras removed. OTUs were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated GreenGenes database (De Santis et al., 2006). Rarefaction curves and diversity indices (OTU richness, Chao-1, and ACE) were calculated using the Mothur software. A multivariate analysis of all samples was performed to examine for significant changes in soil communities after biostimulation treatments using non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarities as described in Ramette and Tiedje (2007). Analysis of similarities (ANOSIMs) was carried out to test for significant differences in bacterial communities. ANOSIM produces a sample statistic (*R*), which represents the degree of separation between test groups (Clarke, 1993).

RESULTS

Physicochemical Characteristics of the Desert Soil

The TPH content in the studied soil was 41.71 mg g^{-1} soil (**Table 1**). The soil had a neutral pH. Soil texture was classified as silt loam as it contains more silt than sand and clay. The concentrations of nitrate and phosphate were 0.04 and 0.16 mg g^{-1} soil, respectively (**Table 1**). Chloride, bromide, fluoride, and sulfate were measured at detectable amounts (**Table 1**). This desert soil contained no or very little amounts of biogenic matter.

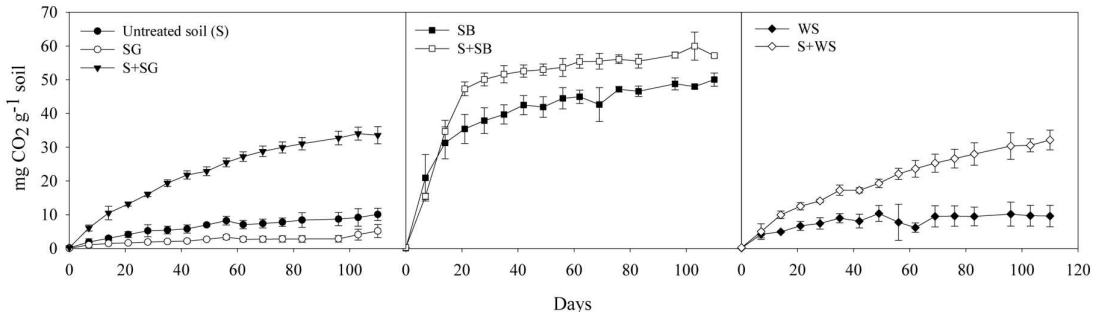

FIGURE 1 | The cumulative amount of CO_2 (in mg g^{-1} soil) produced from the untreated and the biostimulated soils. Note that the produced CO_2 from the soybean alone (SB) was much higher than from the sewage sludge (SG) alone and the wheat straw (WS) alone. Error bars represent ± standard deviation ($n = 3$).

Changes in Respiration Activities

The produced CO_2 in the untreated desert soil without any amendment reached 10.1 ± 1.8 mg CO_2 g^{-1} soil after 110 days of incubation (**Figure 1**; **Table 2**). While the SG and the WS alone produced CO_2 amounts lower than 10 mg CO_2 g^{-1} soil, the SB alone yielded a total amount of 50.0 ± 1.9 mg CO_2 g^{-1} soil (**Figure 1**). The addition of SG and WS to the desert soil significantly ($P < 0.001$) stimulated the respiration activities to reach the values 33.6 ± 2.5 and 32.1 ± 2.9 mg CO_2 g^{-1} treated soil at the end of the incubation period, respectively (**Table 2**; **Figure 1**). In the case of SB, the produced CO_2 of the treated soil displayed the highest value of 57.1 ± 0.5 mg CO_2 g^{-1} soil, however, this value accounts for an insignificant ($P > 0.05$) increase in CO_2 from the untreated soil, given the high respiration activities of the SB alone.

TABLE 1 | Physicochemical properties of the studied oil-contaminated desert soil.

		Unit	Desert soil
Parameters			
TPH		mg g^{-1}	41.71
pH			7.50
EC		mS g^{-1}	1.52
Fluoride		mg g^{-1}	0.03
Chloride		mg g^{-1}	32.52
Bromide		mg g^{-1}	0.15
Nitrate		mg g^{-1}	0.04
Phosphate		mg g^{-1}	0.16
Sulfate		mg g^{-1}	31.83
Soil texture			
	Sand	%	27.00
	Clay	%	23.00
	Silt	%	50.00
	Type		Silt loam

TPH, Total petroleum hydrocarbons; EC, electrical conductivity at 20°C.

Oil Degradation as Revealed by GC–MS

In the bottle experiment, GC–MS revealed that around 10.9 ± 0.1 mg of the C_{14} to C_{30} alkanes g^{-1} soil were degraded in the untreated soil after 110 days of incubation (**Table 2**; **Figure 2**). The lighter fraction of alkanes ($<C_{14}$) completely disappeared from the GC chromatograms (**Appendix Figure A1**). The addition of SG and WS resulted in the degradation of 18.3–19.0 mg of the alkanes g^{-1} soil at the end of the experiment. These values correspond to the degradation of >90% of the total alkanes at a degradation rate of 0.28–0.29 mg of the alkanes g^{-1} soil day^{-1}. The addition of SB resulted in the degradation of 14.3 ± 0.4 mg g^{-1} of the alkanes in 110 days (**Table 2**; **Figure 1**). The degradation of C_{14} to C_{30} alkanes in the microcosm experiment exhibited a similar pattern to that of the bottle experiment. The maximum amount of alkane degradation

TABLE 2 | The total produced CO_2 after 110 days of incubation (without any subtraction) and the amount of degraded alkanes (C_{14} to C_{30}) in the bottle and the microcosm experiments (in mg g^{-1} soil and in % of initial alkane concentrations in the control). All values represent mean ± standard deviation.

Type of treatment	Total evolved CO_2 after 110 days (mg-CO_2 g^{-1} soil)	Alkane (Ci_4 to C_{30}) degradation as measured by GC–MS			
		Bottle experiment (110 days)		Mcrocosom experiment (64 days)	
		(mg g^{-1})	(%)	(mg g^{-1})	(%)
Untreated soil (S)	10.1 ± 1.8	10.9 ± 0.1	55 ± 0.5	9.7 ± 0.6	50 ± 3.0
S+SG	33.6 ± 2.5	19.0 ± 0.7	95 ± 3.5	18.3 ± 0.7	92 ± 3.5
S+SB	57.1 ± 0.5	14.3 ± 0.4	72 ± 2.0	12.4 ± 0.5	63 ± 2.5
S+WS	32.1 ± 2.9	18.3 ± 0.6	92 ± 3.0	17.9 ± 0.1	90 ± 0.5

SG, Sludge; SB, soybean meal; WS, wheat straw.

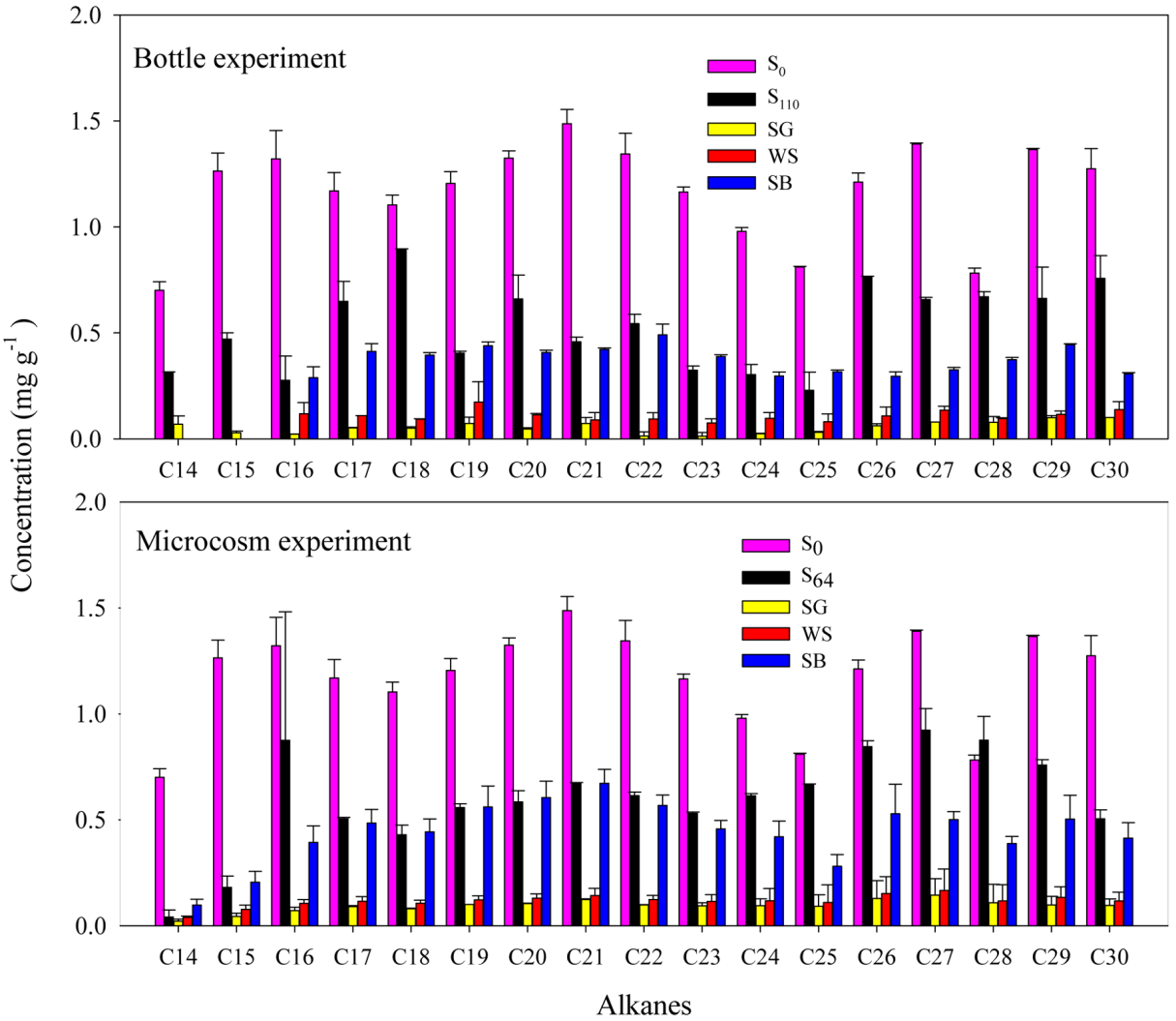

FIGURE 2 | Concentration of individual alkanes (C_{14} to C_{30}) before and after biostimulation with sewage sludge (SG), soybean meal (SB), and wheat straw (WS) in the bottle and the microcosm experiments. Error bars represent ± standard deviation ($n = 3$).

was 18.3 ± 0.7 mg g^{-1} soil after 64 days of treatment with SG (**Table 2**). This amount corresponds to a degradation rate of 0.29 mg of the alkanes g^{-1} soil day^{-1}.

Bacterial Community Changes

The total number of generated 16S rRNA gene sequences by illumina MiSeq was 1,395,417 reads. The lowest number of sequences per sample was 27585 and the highest was 295156 (**Table 3**). Rarefaction curves showed that no samples, regardless of the number of sequences, reached a maximum yield of OTUs (**Figure 3A**). The number of OTUs, calculated using subsets with the same number of sequences for all samples, was in the range of 240–400 OTUs in the untreated soil (**Table 3**; **Figure 3A**). The OTU richness decreased in the untreated soil at the end of the experiment after 64 days of incubation to reach 176–266 OTUs (**Figure 3B**). In the treated soils, the number of OTUs was lowest in the case of the SG with an average of 199 ± 6, whereas the average reached 252 ± 6 and 249 ± 12 in the case of the SB and the WS, respectively (**Figure 3B**). When variations in bacterial community composition were visualized in a two-dimensional space using multivariate analyses of OTUs (**Figure 3C**), the bacterial communities of the triplicates of each treatment were placed in separate clusters (**Figure 3C**, ANOSIM $R = 0.66$, $P = 0.0001$).

MiSeq data showed noticeable changes in bacterial community composition after 64 days of incubation, both in the treated and untreated soils. At the beginning of the experiment, the original soil was dominated by sequences related to the bacterial groups Alphaproteobacteria, Gammaproteobacteria, and Firmicutes (76.5–86.4% of total sequences; **Figure 4**). Sequences belonging to Planctomycetes constituted 15% of the total number of sequences in one of the replica, but <2% in the other two (**Figure 4**). After 64 days of incubation, the relative abundance of Alphaproteobacteria exhibited a decrease only in

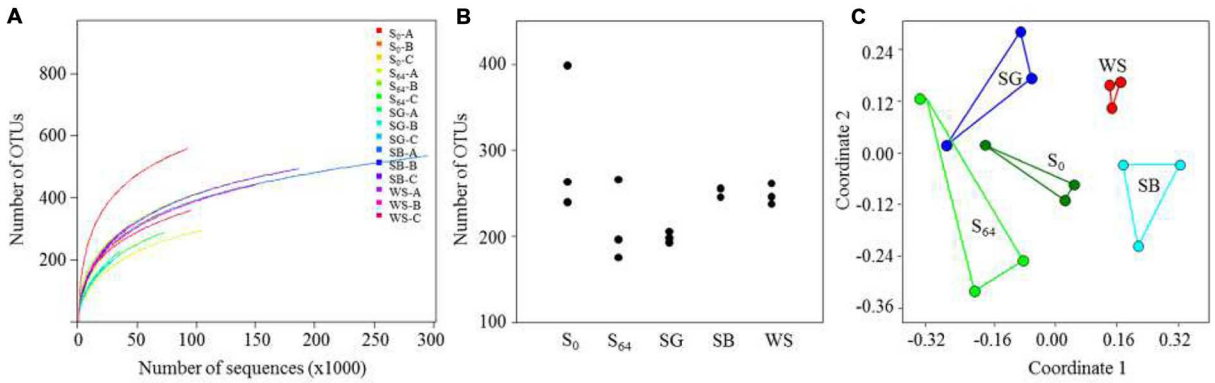

FIGURE 3 | (A) Calculated rarefaction curves of observed OTU (sequences that have 97% similarity are defined as one OTU) richness in the soils; **(B)** number of OTUs calculated for the triplicates of each soil sample, using the same number of sequences for all soils; **(C)** non-metric multidimensional scaling (NMDS) ordination (based on a Bray–Curtis distance matrix) of the triplicates of each treatment.

two replicates to reach 0.6–1.9% of total sequences (**Figure 4**). The bacterial community also showed a decrease in the relative abundance of Gammaproteobacteria from 13–48 to 3–20% of total sequences in all replicates (**Figure 4**). On the other hand, the relative abundance of Firmicutes increased to 46.6–57.8% of total sequences at least in two replicate samples, whereas the third showed an increase in the dominance of Flavobacteria (51.6% of total sequences in S_{64}-A, **Figure 4**). Additionally, sequences belonging to the phylum Actinobacteria increased in abundance in all samples ($\leq$32% of total sequences). There was not much change in the relative abundance of the bacterial phyla Betaproteobacteria, Bacteriodetes, and Chloroflexi (**Figure 4**).

TABLE 3 | MiSeq sequencing and bacterial diversity estimators for the untreated and the biostimulated soils (S_0: original soil; S_{64} untreated soil after 64 days of incubation; SG: soil treated with sewage sludge; SB: soil treated with soybean meal and WS: soil treated with wheat straw).

Sample	Replicate	Total No. of sequences	No. of OTUs*	Chao-1	ACE
S_0	A	92635	399	540.2	533.1
	B	43103	263	384.3	411.7
	C	73774	240	385.6	397.2
S_{64}	A	104773	176	288.8	308.5
	B	108467	266	407.1	414.0
	C	73415	197	315.0	331.2
SG	A	35845	206	307.2	328.0
	B	33379	198	323.1	320.4
	C	29766	193	317.7	344.1
SB	A	295156	246	374.4	391.6
	B	27585	256	451.0	457.7
	C	186157	255	413.4	427.7
WS	A	44936	262	422.7	447.3
	B	150844	246	396.1	384.1
	C	95582	238	354.5	352.1

**Operational taxonomic unit at 3% sequence dissimilarity based on equal subsets of sequences for all samples, Chao-1 is based on rare OTUs in a given sample and ACE is abundance-based coverage.*

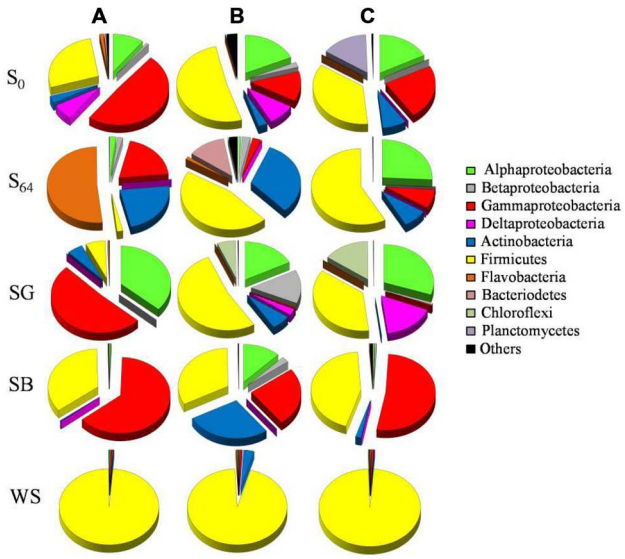

FIGURE 4 | Pie charts depicting the relative abundance (% of total sequences) of bacterial groups in the original soil (S_0) and in the soils at the end of the microcosm experiment (S_{64}: untreated soil after 64 days of incubation; SG: sewage sludge treatment; SB: soybean meal treatment; WS: wheat straw treatment). The triplicates of each soil are denoted as **(A–C)**.

At the genera level, the majority of sequences of Alphaproteobacteria in the untreated soil, at the beginning of the experiment, belonged to the genera *Sphingopyxis*, *Phenylobacterium,* and *Defluviicoccus* (**Figure 5**). However, after 64 days of incubation, only sequences related to *Sphingopyxis* persisted in one of the replicas (i.e., S_{64}-C). Gammaproteobacteria was dominated by *Pseudomonas*, *Halomonas*, *Haemophilus,* and *Alcanivorx* but after incubation, all these genera disappeared and only in one replica, sequences belonging to the genus *Enhydrobacter* appeared (**Figure 5**). Sequences related to *Bacillus* and *Streptococcus* constituted

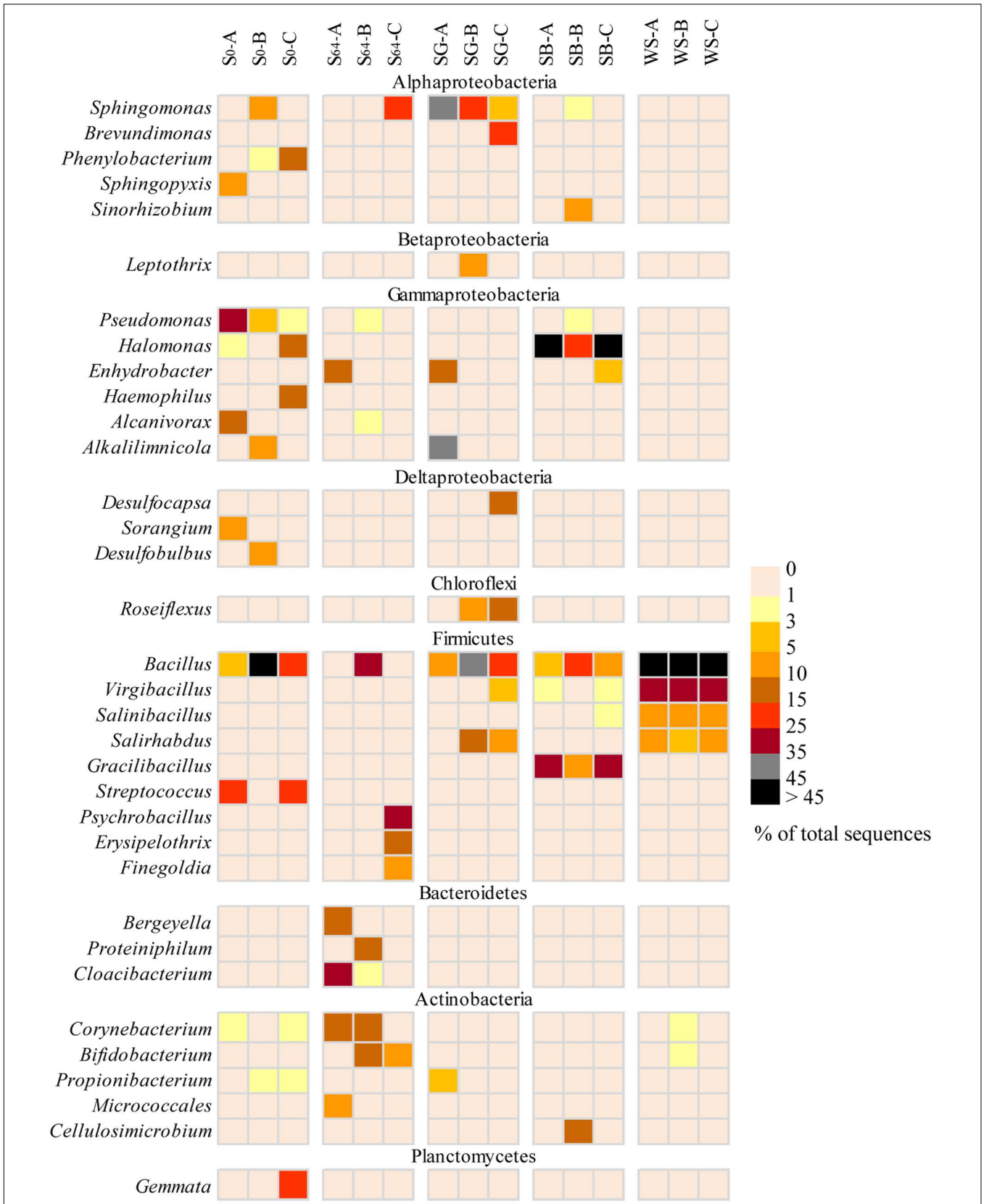

FIGURE 5 | Heatmap representing a comparison of the relative abundance (% of total sequences) of the major bacterial genera in the most dominant bacterial groups in the different soils. The triplicates of each soil are denoted as A–C.

85–98% of total sequences of Firmicutes in the original soil. After incubation, *Bacillus* was still detectable, in addition to two new genera *Psychrobacillus* and *Erysipelothrix*. While the phylum Bacteroidetes was dominated by sequences related to *Bergeyella, Proteiniphilum* and *Cloacibacterium*, Actinobacteria was dominated by sequences affiliated to *Corynebacterium*, *Bifidobacterium,* and *Micrococcales* (**Figure 5**).

The addition of SG to the oil-contaminated desert soils induced variable changes in the triplicate samples. While Alphaproteobacteria increased in abundance in all replicates, Gammaproteobacteria increased only in SG-A (51% of total sequences), Betaproteobacteria increased only in SG-B (14%) and Deltaproteobacteria increased only in SG-C (17%). The majority (>98%) of Aphaproteobacteria belonged to the bacterial genera *Sphingomonas* and *Brevundimonas* (**Figure 5**). Sequences of Gammaproteobacteria were related to the genera *Alkalilimnicola* and *Enhydrobacter* whereas sequences belonging to Betaproteobacteria and Deltaproteobacteria were dominated by the genera of *Leptothrix* and *Desulfocapsa*, respectively (**Figure 5**). Actinobacteria was detectable in all replicates, but at a lower abundance (i.e., 0.2–6% of total sequences) than the untreated soil. In contrast, the relative abundance of Chloroflexi increased at least in two replicates to reach ≤15% of the total sequences and the only detectable genus was *Roseiflexus* (**Figure 5**). Most of the Firmicutes sequences were affiliated to *Bacillus, Salirhabdus,* and *Virgibacillus* and were mainly dominating the samples SG-B and SG-C (**Figure 5**).

In the case of the addition of SB, Gammaproteobacteria and Firmicutes constituted between 58 and 99% of total sequences in all soil samples (**Figure 4**). While 91–99% of the gammaproteobacterial sequences were affiliated to the genus *Halomonas*, 89–90% of the Firmicutes sequences belonged to *Bacillus* and *Gracilibacillus* (**Figure 5**). In one replica, Actinobacteria and Alphaproteobacteria made up ca. 12 and 27% of total sequences, respectively whereas in the other two replicas, both bacterial groups made up ≤2.5% (**Figure 4**). These sequences were mainly affiliated to the genera *Sphingomonas*, *Sinorhizobium,* and *Cellulosimicrobium* (**Figure 5**). The remaining bacterial groups remained at a relative abundance of ≤2% of total sequences each in all samples.

The addition of WS to the contaminated soils induced the most remarkable changes in the bacterial community composition. The whole community shifted in the favor of the Firmicutes group, which made up 95–98% of total sequences in all samples (**Figure 4**). The dominating genera in this group were *Bacillus, Virgibacillus, Salinibacillus,* and *Salirhabdus* (**Figure 5**).

DISCUSSION

Our data demonstrated a greater effect of SG and WS than SB in the stimulation of respiration activities and oil degradation rates in the oil-contaminated desert soil. The addition of these agents has been shown to enrich soils with nutrients, such as phosphorous and nitrogen, whose limitation is known to slow down biodegradation processes (Cui et al., 2008; Chorom et al., 2010; Agamuthu et al., 2013). Although the addition of SB increased oil degradation rates, as revealed by GC–MS analysis, its effect on respiration activities was insignificant. This could be attributed to the active microbial community in the used SB, as suggested by the high amount of produced CO_2 by the SB alone (50.0 ± 1.9 mg CO_2 g^{-1} soil). These exogenous bacteria could compete for the available organics and nutrients with the indigenous bacteria in the contaminated soil. Furthermore, it is not clear whether the measured CO_2, which represents net production, originated from the mineralization of oil components or from other organics in the SB. We speculate that a fraction of the SB and the soil bacterial communities switched between feeding on the SB organics and feeding on hydrocarbons, resulting in the same net production of CO_2. Although, the addition of SB resulted in an increase of only 30% in alkane degradation in our experiments, previous studies showed an increase in the degradation of motor oil and phenol to reach 60–90% upon the addition of SB and soybean seed hull (Flock, 1998; Diab, 2013). SB was even used in the bioremediation of palm oil mill effluent (Ibegbulam-Njoku and Achi, 2014).

The use of CO_2 evolution as a measure of oil mineralization has been successfully used in several reports (Boufadel et al., 1999; Kim et al., 2005; Morais and Tauk-Tornisielo, 2009; Abed et al., 2014; Personna et al., 2014). However, in our experiments, we could not reliably calculate oil mineralization from the produced CO_2, since the added biostimulating agents could act as an additional carbon source to oil. In spite of that, there was a good correlation between the total amounts of degraded alkanes, measured using GC–MS, and the amounts of cumulative produced CO_2 (**Table 2**) in the untreated and treated soils in the bottle experiment ($r = 0.75$, $P \leq 0.01$). Such positive correlation, where the produced CO_2 increases with increasing degradation of hydrocarbons, has been previously observed (Namkoong et al., 2002). The ratios of degraded alkanes to the amount of produced CO_2 in the SG and WS treated soils, after subtracting the produced CO_2 from soil-free SG and WS, were 0.66 and 0.81, respectively. Previous studies have reported values between 0.8 and 1.4 (Hwang et al., 2006). Our values were lower than those reported previously, mainly because our calculation was based on the amounts of degraded alkanes, which is a fraction of oil, and not on the total TPH. Moreover, it is known that a fraction of the carbon could be assimilated into biomass (Linton and Stephenson, 1978). Using GC–MS, the degradation of alkanes was slightly higher ($P < 0.05$) in the bottle experiment than in the microcosm experiment. However, when the incubation period is taken into account (i.e., 110 vs. 64 days), the degradation rates were still higher in the microcosm experiment (i.e., 0.172 and 0.166 vs. 0.29 and 0.28 mg of alkanes g^{-1} soil day^{-1}, for SG and WS amended soils, respectively). This is due to the clear differences in the incubation conditions between the bottle and the microcosm experiments, including water content, aeration, mixing, and scale. The continuous supply of oxygen in the microcosm experiment due to soil tilting apparently favored oil degradation.

Previous reports have used SG for bioremediation of oil-contaminated soils because of its richness in nutrients and the existence of high diversity and density of microorganisms therein (Chang et al., 2003; Gestel et al., 2003; Ros et al.,

2010; Ambrazaitiene et al., 2013). However, the low respiration activities of SG suggests a low activity of its microbial community. The remarkable increase in the produced CO_2 after the addition of SG to the contaminated soil indicates that SG was most likely a good source of nutrients. Previous reports showed that the addition of SG to diesel- and oil-contaminated soils resulted in a total degradation rate of 46–98% of TPHs (Namkoong et al., 2002; Hur and Park, 2003; Ros et al., 2010; Chorom and Hosseini, 2011). Although, the use of 5% (w/w) SG in our experiments was effective, the use of as high as 10 and as low as 1% was also shown to stimulate oil degradation rates (Hur and Park, 2003; Ros et al., 2010; Chorom and Hosseini, 2011). On the contrary, the excessive use of SG retarded the biodegradation of TPH in other cases (Namkoong et al., 2002).

The bioventing agent, WS, stimulated respiration activities and oil mineralization rates in the oil-contaminated desert soils. Since the studied desert soil had a silt loam texture with 50% silt, it is plausible that the addition of WS resulted in an increase in the soil porosity and oxygen availability to the oil-degrading bacteria. Previous biostimulation studies using WS reported degradation rates between 59 and 85% during a period of 1–3 months (Vasudevan and Rajaram, 2001; Marin et al., 2006; Rojas-Avelizapa et al., 2007). Other studies have used WS as a source of inorganic nutrients such as N and P and organic nutrients such as cellulose and hemicellulose (Zhang et al., 2008; Llado et al., 2015). It was postulated that the decomposition products of cellulose and hemicellulose promote the growth of bacteria including the oil-degrading types. WS was also used to enhance bioremediation in salty contaminated soils, where high salt levels may inhibit microbial growth, by cutting capillaries and decreasing salt accumulation at the soil surface (Zhang et al., 2008). This effect was shown to result in a remarkable increase in bacterial biomass and in an increase in TPH degradation from 29 to 48% (Zhang et al., 2008).

Bacterial Diversity and its Changes in the Untreated Desert Soil

MiSeq data showed shifts in the bacterial community structure of untreated as well as in biostimulated soils. The original desert soil was dominated by sequences belonging to the bacterial groups Alphaproteobacteria, Gammaproteobacteria and Firmicutes, and these groups have been previously encountered in oil-contaminated soils, including desert soils (Militon et al., 2010; dos Santos et al., 2011; Sutton et al., 2013). Several members of these bacterial groups are known for their ability to degrade aliphatic and aromatic hydrocarbons (Alonso-Gutierrez et al., 2008; Lafortune et al., 2009; Vila et al., 2010; Kostka et al., 2011). Among the detected genera in our desert soil that potentially contain hydrocarbon-degrading species were *Sphingomonas, Phenylobacterium, Sphingopyxis*, *Pseudomonas, Halomonas,* and *Alcanivorax*. The detection of these genera in the original soil suggests that oil-degradation could be naturally occurring in the soil even without any treatment. This process, which relies on the activity of indigenous oil-degrading microorganisms, is known as natural attenuation and has become an accepted low-risk and cost-effective bioremediation approach (Margesin and Schinner, 2001; Bento et al., 2005; Ruberto et al., 2009). The produced CO_2 levels and the measured natural degradation by GC–MS analysis in the untreated soil support the occurrence of natural biodegradation in the untreated soil.

Species belonging to the Alphaproteobacterial genera *Sphingomonas*, *Phenylobacterium,* and *Sphingopyxis* in the original soil have been previously detected in oil-contaminated environments and have been known for many years to contain degraders of PAHs (Tao et al., 2007; Lan et al., 2011; Yang et al., 2014; Zhang et al., 2014; Ronca et al., 2015). For instance, *Sphingomonas* sp. was shown to utilize phenanthrene as its exclusive source of carbon and energy (Tao et al., 2007) and to degrade 83% of applied diesel and 3–79% of mixed PAHs (Cui et al., 2008). *Phenylobacterium* and *Sphingopyxis* spp. were also shown to degrade several aromatic hydrocarbons as a carbon source (Eberspaecher and Lingens, 2006; Kertesz and Kawasaki, 2010; Lan et al., 2011; Kim et al., 2014; Yang et al., 2014). Similarly, strains of the genera *Pseudomonas, Alcanivorax* and *Halomonas*, which prevailed the class Gammaproteobacteria, are renowned for their global distribution in contaminated sites and for their ability to degrade hydrocarbons (Hara et al., 2003; Schneiker et al., 2006; Mnif et al., 2009; Linda and Bouziane, 2012; Oyetibo et al., 2013). The two dominant genera of the phylum Firmicutes; *Bacillus* and *Streptococcus*, have also been previously detected in contaminated soils with an active role in the degradation process (Makut and Ishaya, 2010; Mansur et al., 2014).

The incubation of the soil for 64 days in microcosms, even without any treatment, induced clear shifts in the microbial community composition. It is known that confined incubation of bacterial samples results in changes in the composition of bacterial community structure, an effect known as "bottle effect" (Hammes et al., 2010). While the abundance of Flavobacteria increased in S_{64}-A, the abundance of Actinobacteria increased in S_{64}-A and S_{64}-B and the abundance of Firmicutes increased in S_{64}-C. Genera affiliated to Firmicutes and Actinobacteria are known for their vast distribution in oil-contaminated soils and they contain strains that were able to degrade hydrocarbons. Actinobacteria play a central role in the decomposition of organic matter and recycling of nutrients (Prince et al., 2010). Among the detected Actinobacterial genera known to degrade hydrocarbons are *Corynebacterium and Micrococcus* (Santhini et al., 2009; Oyetibo et al., 2013; Hassanshahian et al., 2014).

Post-biostimulation Bacterial Community Shifts

The relative abundance of the bacterial groups exhibited high variability among the triplicate samples of each treatment, making it difficult to attribute specific shifts in the bacterial community to the applied treatment. Nevertheless, NMDS ordination based on the different treatments placed the microbial communities in separate clusters and this dissimilarity was supported by an ANOSIM *R* value of 0.66. This suggests that, in spite of the heterogeneity in the triplicate soil samples of each treatment, the species composition still varied between the different treatments. Moreover, the persistence of the same

bacterial groups after treatment indicates that these groups still favored the new conditions. In the SG-treated soils, the relative abundance of Alphaproteobacteria remained the same or increased in all replicates, whereas the abundance of Firmicutes, Gammaproteobacteria and Chloroflexi increased in some replicates but not in others. The alphaproteobacterial genera *Sphingomonas* and *Brevundimonas*, which constituted 35 and 20.4% of the total sequences, respectively are known to include species that degrade petroleum compounds (Ijah and Ukpe, 1992; Baraniecki et al., 2002; Vazquez et al., 2013). The persistence of the Firmicutes group after biostimulation with SG indicates that this group constitutes a stable and vital component of the bacterial community structure of the desert soil. Sequences in this group are prevailed by *Bacillus, Salirhabdus* and *Virgibacillus*, which are known to have oil-degrading species (Kothari et al., 2013; Borah and Yadav, 2014; Roy et al., 2014; Albokari et al., 2015). The detection of sequences belonging to the class Chloroflexi only in the treatment with SG and not in others, strongly suggests that these bacteria have been growing in the sludge and brought into the soil. The green non-sulfur Chloroflexi bacteria were previously found abundant in wastewater treatment plants (Bjornsson et al., 2002; Kragelund et al., 2007; An et al., 2013).

In the soil amended with SB, Firmicutes persisted while Gammaproteobacteria increased in abundance to reach 25–63% of total sequences, of which 97% were affiliated with the genus *Halomonas*. The detection of high abundance of *Halomonas* in this treatment suggests that these bacteria were introduced by the SB or favored the growth on the organics therein. This assumption is supported by the produced CO_2 of soybean alone, which indicated the presence of an active bacterial community. Although the genus *Halomonas* is known to include oil-degrading species (Mnif et al., 2009; D'Ippolito et al., 2011), most of the detected sequences were affiliated to the species *Halomonas xinjiangensis*, which was isolated from a pristine soil and is unable to degrade hydrocarbons (Guan et al., 2010). The amendment of the soil with WS induced the most remarkable shift in the bacterial community, indicating that WS created different conditions in this treatment that favored the growth of Firmicutes species. Although the phylum Firmicutes includes both aerobic and anaerobic microorganisms, all detected species in this treatment belonged to aerobic *Bacilli*-related species. This suggests that WS probably acted as a bioventing agent and increased oxygen penetration in the soil. Bacilli strains are known to include many oil-degrading species and were even detected in crude oils (Ijah and Ukpe, 1992; Khanna et al., 2012; Borah and Yadav, 2014). An interesting feature of *Bacilli* strains is their ability to produce biosurfactants, which increase the bioavailability of oil through emulsification and consequently facilitate the degradation process (Cubitto et al., 2004; Yamane et al., 2008; Perfumo et al., 2010; Najafi et al., 2011; Chandankere et al., 2013).

We conclude that SG, WS, and SB are suitable bioremediation agents that can be successfully used to enhance the activity of oil-degrading bacteria and facilitate the degradation of petroleum contaminants in desert soils. SG and WS had a stronger stimulatory effect on the soil's respiration activities and oil degradation than SB. Microbial community analysis revealed the dominance of sequences affiliated to Alphaproteobacteria, Gammaproteobacteria, and Firmicutes in the original soil, although there was a clear heterogeneity among the triplicate samples. The relative abundance of these groups showed variations after the addition of the biostimulating agents, with the most prominent shift in the case of WS-treated soils, where almost the whole community was composed of Bacilli.

AUTHOR CONTRIBUTIONS

SA-K and RA designed the experiments, SA-K performed the experiments and the chemical analysis using GC–MS, RA did the molecular work and the bioinformatics analysis. SA-K and RA wrote the manuscript.

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APPENDIX

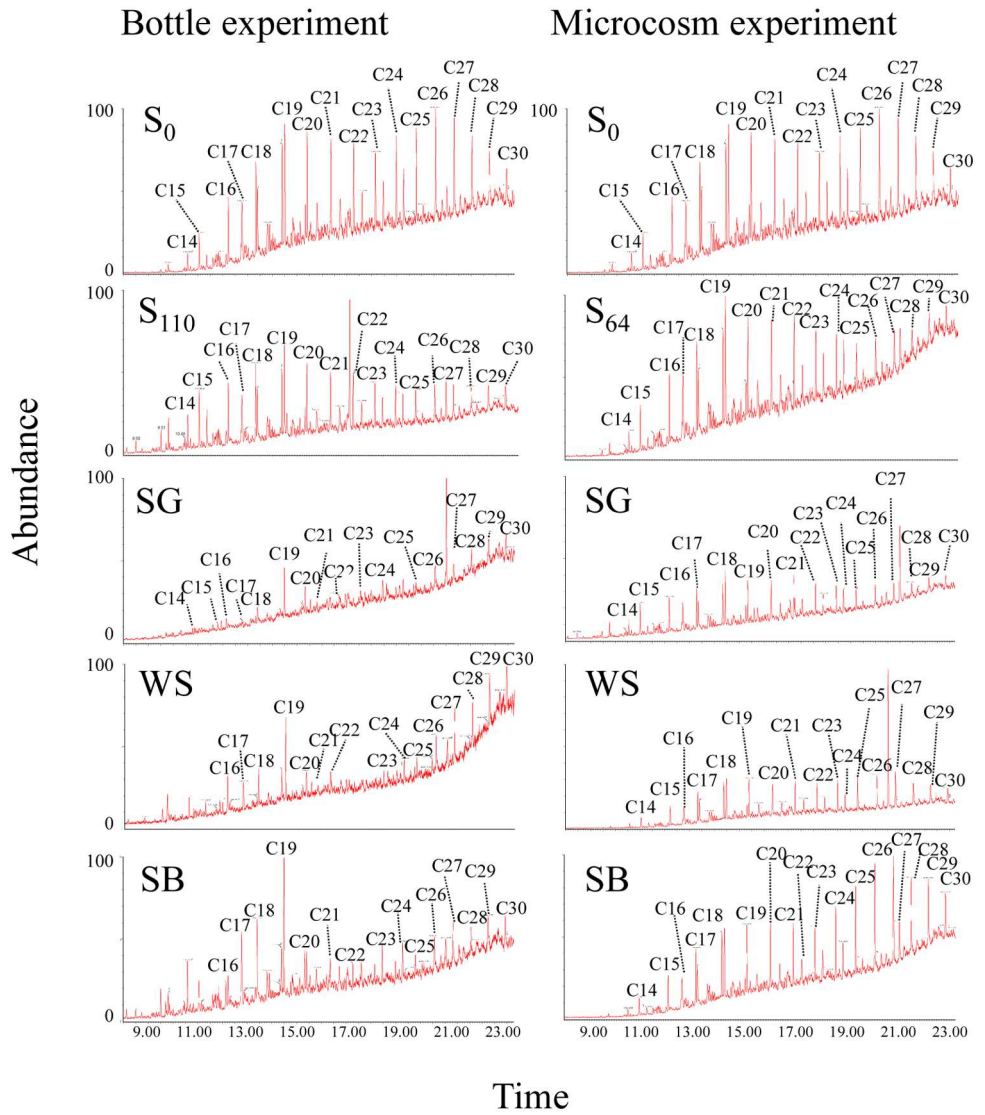

FIGURE A1 | Gas chromatography/mass spectrometry (GC/MS) chromatograms showing the concentrations of crude oil fractions before and after biostimulation with sewage sludge (SG), wheat straw (WS), and soybean meal (SB) in the bottle and in the microcosm experiments. S_0 denotes the original soil before incubation and S_{110} and S_{64} denote the same soil at the end of incubation without any treatment in the bottle and in the microcosm experiment, respectively.

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A Fluorescent Bioreporter for Acetophenone and 1-Phenylethanol derived from a Specifically Induced Catabolic Operon

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The β-proteobacterium *Aromatoleum aromaticum* degrades the aromatic ketone acetophenone, a key intermediate of anaerobic ethylbenzene metabolism, either aerobically or anaerobically via a complex ATP-dependent acetophenone carboxylase and a benzoylacetate-CoA ligase. The genes coding for these enzymes (*apcABCDE* and *bal*) are organized in an apparent operon and are expressed in the presence of the substrate acetophenone. To study the conditions under which this operon is expressed in more detail, we constructed a reporter strain by inserting a gene fusion of *apcA*, the first gene of the *apc-bal* operon, with the gene for the fluorescent protein mCherry into the chromosome of *A. aromaticum*. The fusion protein indeed accumulated consistently with the expression pattern of the acetophenone-metabolic enzymes under various growth conditions. After evaluating and quantifying the data by fluorescence microscopy, fluorescence-based flow cytometry and immunoblot analysis, mCherry production was found to be proportional to the applied acetophenone concentrations. The reporter strain allowed quantification of acetophenone within a concentration range of 50 μM (detection limit) to 250 μM after 12 and 24 h. Moreover, production of the Apc-mCherry fusion protein in the reporter strain was highly specific and responded to acetophenone and both enantiomers of 1-phenylethanol, which are easily converted to acetophenone. Other analogous substrates showed either a significantly weaker response or none at all. Therefore, the reporter strain provides a basis for the development of a specific bioreporter system for acetophenone with an application potential reaching from environmental monitoring to petroleum prospecting.

Keywords: ***Aromatoleum aromaticum*, acetophenone, bioreporter, fluorescence microscopy, flow cytometry, immunoblot, mCherry**

INTRODUCTION

Among many other aromatic substrates, the β-proteobacterium *Aromatoleum aromaticum* strain EbN1 degrades the aromatic ketone acetophenone (phenylmethylketone) as sole carbon and energy sources (Rabus and Widdel, 1995). When grown anaerobically in the presence of nitrate, it utilizes acetophenone either directly or as a metabolic intermediate in the degradation of

ethylbenzene (**Figure 1**), whereas under aerobic conditions, only acetophenone is metabolized, but not ethylbenzene (Rabus and Widdel, 1995; Rabus and Heider, 1998; Heider et al., 1999; Jobst et al., 2010). The industrial use of acetophenone includes applications as solvent and precursor for the production of resins, but also as a food-flavoring agent or fragrance for cosmetics. Acetophenone vapors may cause skin irritation and transient corneal injury in humans after short-term exposure, but no information is available on potential long-term effects (Hagan et al., 1967; US EPA, 1999). Compared to the relatively low toxicity of acetophenone, some of its halogenated derivatives (e.g., the tear gas compound 2′-chloroacetophenone) are highly toxic and cause environmental problems when spilled (Olajos and Salem, 2001). Toxicity of acetophenone toward growth of microorganisms has been reported at concentrations of 0.4–30 mM (Fedorov et al., 1993; Hage et al., 2001; Yang et al., 2008).

Microbial degradation of acetophenone and chloroacetophenones has been known for a long time and was initially believed to be restricted to aerobic microorganisms, which initiate the metabolic pathway by a Baeyer–Villiger oxidation to (chloro)phenyl-acetate esters (Cripps, 1975; Havel and Reineke, 1993). This reaction is catalyzed by a variety of oxygenases that usually accept many different substrate analogs (Jones et al., 1994; Kamerbeek et al., 2003). An oxygen-independent degradation pathway of acetophenone has been discovered in denitrifying bacteria capable of anaerobic ethylbenzene degradation, with *A. aromaticum* EbN1 as the best characterized strain (**Figure 1**; Rabus and Widdel, 1995; Ball et al., 1996; Rabus and Heider, 1998; Champion et al., 1999; Jobst et al., 2010). This pathway starts by oxidizing ethylbenzene to acetophenone in two enzymatic steps via (*S*)-1-phenylethanol, utilizing the molybdoenzyme ethylbenzene dehydrogenase (Johnson et al., 2001; Kniemeyer and Heider, 2001a) and an NAD-dependent (*S*)-1-phenylethanol dehydrogenase (Kniemeyer and Heider, 2001b; Höffken et al., 2006), while acetophenone is further degraded to benzoyl-CoA (Rabus and Heider, 1998; Jobst et al., 2010). *A. aromaticum* also degrades acetophenone as sole substrate, using the same pathway under anaerobic and aerobic growth conditions (Rabus and Widdel, 1995; Ball et al., 1996). The same strain is known to degrade the close chemical analog 4′-hydroxyacetophenone, albeit using a completely different pathway (Wöhlbrand et al., 2008).

Acetophenone degradation by *A. aromaticum* is initiated by a highly complex ATP-dependent acetophenone carboxylase (Apc) which produces benzoylacetate (Jobst et al., 2010). This enzyme has been purified and characterized from cells grown under denitrifying conditions on either ethylbenzene or acetophenone (Jobst et al., 2010). It consists of five subunits (ApcABCDE) that are encoded in an operon together with a gene coding for benzoylacetate CoA-ligase (*bal*; **Figure 1**; Rabus et al., 2002). Four subunits (ApcABCD) form a $(\alpha\beta\gamma\delta)_2$ core complex of 485 kDa, which contains tightly bound Zn atoms believed to be essential for carboxylase activity. The fifth subunit (ApcE) is separated from the complex during purification and needs to be added to restore activity *in vitro*. The reconstituted enzyme exhibits a stoichiometry of two ATP hydrolyzed to ADP per acetophenone carboxylated (Jobst et al., 2010). The generated benzoylacetate is subsequently activated to the CoA-thioester by a CoA-ligase (Rabus and Heider, 1998; Rabus et al., 2002; Muhr et al., 2015), and benzoylacetyl-CoA is cleaved by a so far unknown thiolase to acetyl-CoA and benzoyl-CoA, the common intermediate in

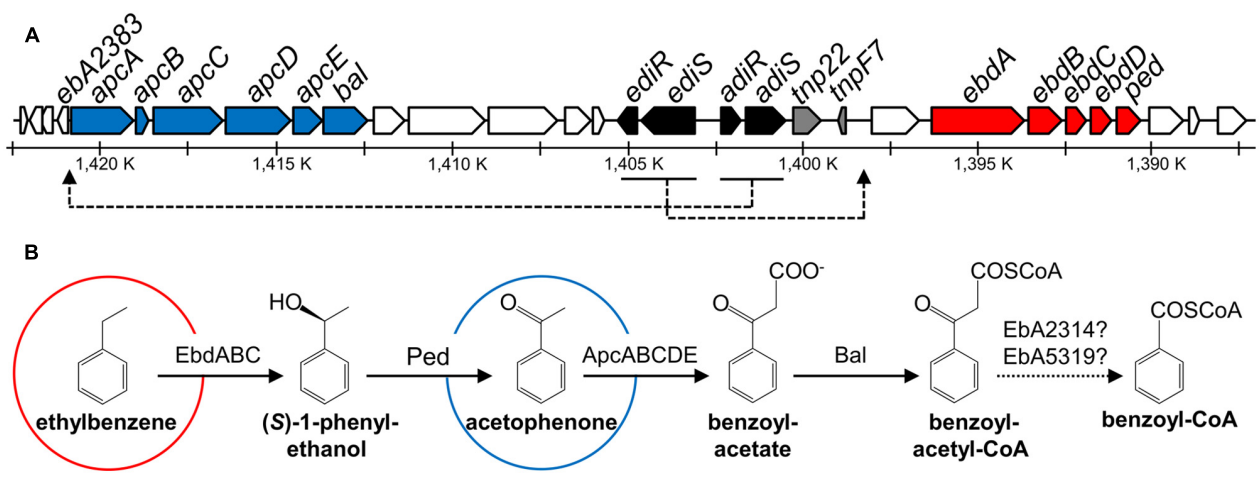

FIGURE 1 | Scheme of genes and enzymes involved in anaerobic degradation of ethylbenzene and acetophenone in *Aromatoleum aromaticum*. Modified from Rabus et al. (2002), Kühner et al. (2005), Muhr et al. (2015). **(A)** Predicted functions of gene products are indicated by different colors: blue: acetophenone and ethylbenzene degradation, red: ethylbenzene degradation, black: regulation, gray: transposase/transposase fragment, white: proteins of unknown function. **(B)** Enzyme names: EbdABC: ethylbenzene dehydrogenase, Ped: (*S*)-1-phenylethanol dehydrogenase, ApcABCDE: acetophenone carboxylase, Bal: benzoylacetate CoA-ligase and EbA2314 (accession number CAI07405) or EbA5319 (accession number CAI09148): potential β-ketothiolases. Shown are also genes encoding two predicted two-component regulatory systems, *ediSR* (*tcs2/tcr2*) for ethylbenzene degradation induction, and *adiRS* (*tcr1/tcs1*) for acetophenone degradation induction.

the anaerobic metabolism of aromatic compounds (Heider and Fuchs, 1997a,b; Carmona et al., 2009; Fuchs et al., 2011). The *apc-bal* operon is part of a larger gene cluster including the *ebd-ped* operon and genes coding for two two-component regulatory systems (**Figure 1**; Rabus et al., 2002).

The enzymes of acetophenone metabolism are apparently regulated in response to the availability of the substrate, but independently of those of ethylbenzene metabolism (Kühner et al., 2005; Rabus et al., 2014; Muhr et al., 2015) or those involved in degradation of the close chemical analogs 4′-ethylphenol and 4′-hydroxyacetophenone (Wöhlbrand et al., 2008; Rabus et al., 2014; Muhr et al., 2015). In order to analyze the mechanisms regulating the acetophenone-metabolic genes, we constructed an *A. aromaticum* strain carrying a chromosomally integrated fusion of the first gene of the *apc-bal* operon (*apcA*) with the gene for the fluorescent protein mCherry (Shaner et al., 2004) and tested for its expression in the presence of various potential inducers. This strain indeed responded to acetophenone and both enantiomers of 1-phenylethanol in a concentration- and time-dependent manner and showed high substrate specificity.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The streptomycin-resistant *A. aromaticum* strain EbN1-SR7 (Wöhlbrand and Rabus, 2009) was grown at 28°C under denitrifying conditions in mineral salt medium on a rotary shaker as described elsewhere (Rabus and Widdel, 1995; Rabus and Heider, 1998; Muhr et al., 2015). Bacterial growth was followed by measuring optical density at 578 nm and the consumption of nitrate by using semiquantitative test strips (Macherey-Nagel, Düren, Germany). For time- and concentration-dependent analysis we inoculated the reporter strain in carbonate-buffered mineral salt media containing 1.5 mM of benzoate in hungate tubes under aerobic conditions. These cultures were incubated for 24 h at 28°C, until they had reached an OD_{578} of 0.4–0.5 and most of the benzoate was consumed (typical residual benzoate concentrations were 0.2 mM). At this point ($t = 0$ h), different concentrations of acetophenone were added. For conjugational plasmid transfer, strain EbN1-SR7 was grown in a modified mineral salt medium described previously (Wöhlbrand and Rabus, 2009).

Escherichia coli strains were grown at 37°C in LB media (Sambrook and Russell, 2001). If required, the growth medium was solidified with 1.5% (w/v) agar. Antibiotics were added at the following final concentrations: kanamycin 30–50 μg ml^{-1} and streptomycin 30–50 μg ml^{-1}. The diaminopimelate (DAP) auxotrophic *E. coli* strain WM3064, a derivative of strain β2155 (Dehio and Meyer, 1997), was grown on media containing 0.3 mM DAP. *E. coli* strain DH5α was used for all cloning purposes (Taylor et al., 1993).

DNA Techniques and Plasmid Transfer

All enzymes and kits for the isolation of DNA or the purification of PCR products or restriction fragments were purchased from Thermo Scientific™ (Life Technologies GmbH, Darmstadt, Germany) or Analytik Jena (Jena, Germany). Oligonucleotide primers were ordered from biomers.net GmbH (Ulm, Germany). Sequences were analyzed using pDRAW32 (ACACLONE software) and DNAMAN (Lynnon Biosoft) software.

Plasmids were transferred into *E. coli* strains by transformation using chemically competent cells (Inoue et al., 1990) and into *A. aromaticum* by conjugation (Wöhlbrand and Rabus, 2009). Bacterial mating and conjugational plasmid transfer were performed as described before (Wöhlbrand and Rabus, 2009), with the exception that the DAP-auxotrophic *E. coli* strain WM3064 was used as donor strain.

Construction of a Chromosomal *apcA-mCherry* Insertion Mutant

To generate a strain with a chromosomal integration of an *apcA-mCherry* fusion, we used the suicide-vector pK19mobsacB (Schäfer et al., 1994), which had previously been used to create a knock-out mutant in *A. aromaticum* (Wöhlbrand and Rabus, 2009). The procedure was modified to promote only a single crossover event with the chromosomal DNA, leading to the stable insertion of the entire vector plus the cloned fragment (**Figure 2B**). A fragment of the *mCherry* gene lacking the start codon (starting from third codon) was amplified from plasmid pCHYC-2 (Thanbichler et al., 2007) using primers CHY_for_XbaI (5′-AATCTAGAAAGGGCGAGGAGGATAACATG-3′) and CHY_rev_EcoRI (5′-AAGAATTCTTACTTGTACAGCTCGTCCATG-3′), restricted with *Xba*I and *Eco*RI, and ligated into the equally treated vector pK19mobsacB to generate pK19CHY (6401 bp). In addition, a fragment including 607 bp of the 5′ upstream region and the first 96 bp of the first gene of the *apc*-operon (*apcA*) was amplified from genomic DNA of strain EbN1-SR7 using primers apcA_for_HindIII (5′-GCAAGCTTGGCGATTCACCCGTTTCG-3′) and apcA_rev_XbaI (5′-AATCTAGACGGGGTGGTGTCCACCTTG-3′), restricted with *Hind*III and *Xba*I, and ligated into the equally treated pK19CHY. The resulting plasmid, pK19*apc*-CHY (7086 bp), was sequenced and contained the first 32 codons of *apcA* fused to the *mCherry* gene. It was conjugated into EbN1-SR7, and the transconjugants were purified at least three times on DAP-free media to guarantee the purity of the cultures and absence of residual *E. coli* contamination. The colonies were screened for the presence of plasmid-mediated kanamycin resistance, which should indicate chromosomal integration by a crossover event in the homologous upstream sequence of *apcA*. The proper insertion of the plasmid at the predicted site was verified by colony PCR of the kanamycin-resistant *apcA-mCherry* mutant (data not shown), henceforth designated as strain APC-CHY.

Fluorescence Microscopy

Cultures of strain APC-CHY grown either aerobically or under denitrifying conditions were exposed to air for at least 1 h at room temperature before being immobilized on a 1% agarose-pad.

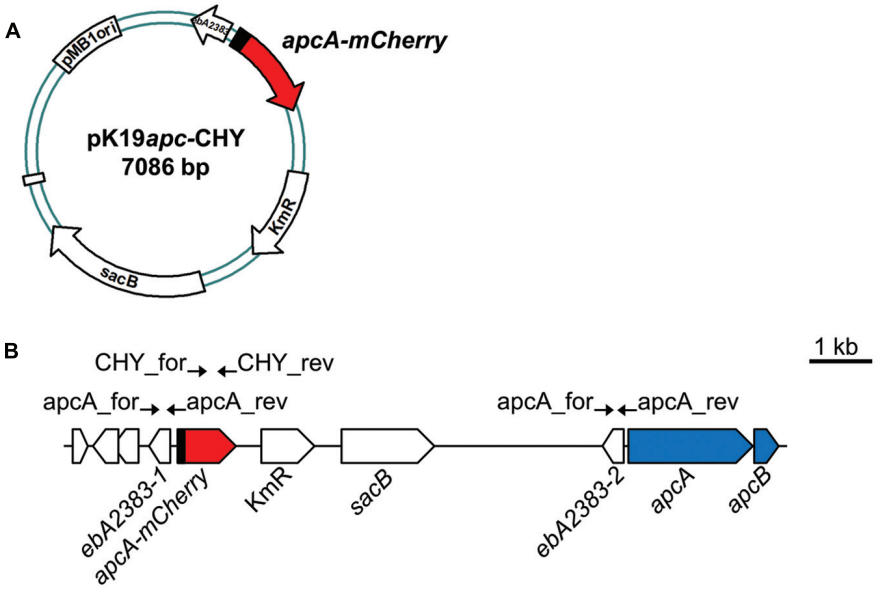

FIGURE 2 | Construction of *A. aromaticum* strain APC-CHY. (A) Schematic depiction of plasmid pK19*apc*-CHY. **(B)** Reporter strain APC-CHY containing the integrated plasmid pK19*apc*-CHY with a reporter fusion behind the unchanged *apc* upstream sequence resulting in a duplicated copy of the hypothetical gene ebA2383. Primers used for construction are indicated by arrows at their annealing positions.

Cells were analyzed using a Zeiss Axio Observer.Z1 microscope (Zeiss, Germany) equipped with a Zeiss Plan-Apochromat 100×/1.46 numeric-aperture oil differential interference contrast (DIC) M27 objective, a Chroma ET-mCherry filter set, and a pco.edge sCMOS camera (PCO, Germany). Images were analyzed and processed with ImageJ 1.48v (Wayne Rasband, National Institutes of Health, USA), Metamorph 7.7.5 (Universal Imaging Group, USA), and Illustrator CS5 (Adobe Systems, USA). All fluorescence images (500 ms exposure) shown in this study were scaled equally (monochrome, threshold setting 250/10,000) for comparison in brightness. For the quantitative analysis of relative mean and maximum fluorescence signal response (threshold set at 750 units), the fluorescence images were divided into 10–15 sectors each containing about 25 cells. Each sector was analyzed separately, and average values were generated.

Flow Cytometry

For fluorescence–based flow cytometry, bacterial cells were washed two times with fresh mineral salt medium in which the substrates were omitted and diluted 1:100 in tethering buffer (10 mM K_2HPO_4, 10 mM KH_2PO_4, 10 mM lactic acid, 0.1 mM EDTA, and 1 μM L-methionine, pH 7.0). Fluorescence-activated cell analysis was carried out on a BD LSRFortessa^{TM} SORP flow-cytometer (BD Biosciences, Franklin Lakes, NJ, USA; Piatkevich and Verkhusha, 2011; Telford et al., 2012). Fluorescence was detected using a 561-nm laser (yellow–green) at 100 mW for excitation and a 610/20 bandpass filter. The forward and side scatter values were monitored using a 488-nm laser (blue) at 50 mW. The acquired data were analyzed using BD FACSDiva^{TM} software version 8.0 (BD Biosciences, Franklin Lakes, NJ, USA) with data collected in FCS 3.0 file format.

Electrophoretic Methods and Immunoblotting

Defined amounts of protein (50 μg) from crude extracts were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% (w/v) polyacrylamide gels in the Mini-PROTEAN Tetra System (Bio-Rad, Munich, Germany) as described elsewhere (Laemmli, 1970). For extract preparation, cell suspensions were disrupted by sonication and cell debris and membranes were removed by ultracentrifugation at 100,000 × *g* and 4°C for 1 h. Protein concentrations were determined using a Coomassie dye binding assay with bovine serum albumin as a standard (Bradford, 1976). Immunodetection with anti-mCherry antiserum was performed as described before (Jung et al., 2014). For quantitation of the immunocomplexes, the signals were recorded by a ChemiDoc MP imaging system as volume (intensity) of the respective bands using Image Lab 5.0 software (Bio-Rad, Munich, Germany). All ApcA-mCherry fusion protein signals are presented in arbitrary units (a.u.), where one unit corresponds to an original volume (intensity) readout value of 10^6. Data were fitted using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). To control for equal loading, all membranes were stained with Ponceau S [0.1% (w/v) in 5% (v/v) acetic acid; Sigma-Aldrich, St. Louis, MO, USA].

Determination of Benzoate Concentrations

To determine the concentrations of benzoate in culture supernatants, 10% (v/v) of a 1 M $NaHSO_4$ solution was added to reach a final pH of ∼2.0. After centrifugation (15,000 × *g* for 5 min), the absorbance at 230 nm was detected using UV-cuvettes

(Robinson, 1991) and the concentration of benzoic acid was calculated from its extinction coefficient ($\varepsilon = 11{,}900\ M^{-1}\ cm^{-1}$).

RESULTS

Construction of a Chromosomal Fluorescent Reporter Fusion

Previous studies addressing enzyme activities in *A. aromaticum* cells grown on various substrates indicated that acetophenone-catabolic genes are exclusively induced in cells grown on acetophenone or ethylbenzene (Jobst et al., 2010; Rabus et al., 2014; Muhr et al., 2015). In order to further elucidate the induction pattern of these genes *in vivo*, we constructed a translational fusion of the first 32 codons of the *apcA* gene with the *mCherry* gene to generate a fluorescent reporter system (**Figure 2**). To avoid potential problems resulting from multiple copies of a plasmid-borne reporter gene, we integrated the *apcA-mCherry* reporter fusion into the chromosome of strain EbN1-SR7 (Wöhlbrand and Rabus, 2009) by inserting plasmid pK19*apc*-CHY upstream of the first gene of the *apc-bal* operon through a single cross-over event (**Figure 2**). The resulting mutant strain, APC-CHY, carried the reporter fusion behind the unchanged *apc* upstream sequence plus a duplicated copy of 607 bp of the *apc* upstream sequence, followed by the entire *apc-bal* operon. The identity of this strain was verified by its kanamycin resistance and by testing for proper integration of the plasmid using PCR analysis. Additionally, its growth properties with ethylbenzene, acetophenone, or benzoate did not differ significantly from those of the wild-type strain (data not shown).

Reporter Gene Response to Different Growth Conditions

The reporter strain derived from *A. aromaticum* was grown to mid-exponential phase (72 h) under different conditions in carbonate-buffered mineral salt media. The expression of the reporter gene fusion was observed in parallel by fluorescence microscopy and quantitative immunoblotting with antiserum against mCherry (**Figure 3**). We observed increased fluorescence intensities in all APC-CHY cells grown anaerobically on 2 mM acetophenone or ethylbenzene as well as in those grown aerobically on acetophenone, whereas none of the cultures grown on 4 mM benzoate, 1 mM toluene, or 1 mM 4′-ethylphenol showed significant fluorescence intensities (**Figure 3A**; cultures on toluene or 4′-ethylphenol not shown).

The presence of ApcA-mCherry was additionally confirmed by quantitative immunoblotting of extracts separated on an SDS gel (**Figure 3B**). The antiserum reacted strongly with ApcA-mCherry (30.1 kDa) and with a protein corresponding in size to free mCherry protein (∼27 kDa), which likely results from degradation of the fusion protein in cells grown on acetophenone (**Figures 3B,C**). The strongest signal intensity of ApcA-mCherry in cells grown on acetophenone was set as 100%. In comparison, cells grown anaerobically on benzoate plus acetophenone or aerobically on acetophenone showed signal intensities of 75 and 60%, respectively. Cultures of strain APC-CHY grown on ethylbenzene showed signal intensities of 20% under the same conditions. In contrast, controls grown anaerobically on benzoate did not show significant immunoblot signals (**Figures 3B,C**). The few cross-reacting bands in the control show the same pattern as an immunoblot of the parental strain without chromosomally integrated mCherry and are therefore unrelated to the mCherry protein (**Figure 3B**).

Development of a Quantitative Acetophenone Sensor System

We examined whether the newly constructed reporter system can be used to quantify the concentrations of acetophenone in growth media of *A. aromaticum*. To avoid problems with the oxygen demand for developing the mCherry fluorescence (Shaner et al., 2004), we decided to shift to aerobic growth conditions for setting up a sensory system. To this end, we inoculated the reporter strain in carbonate-buffered mineral salt media containing 1.5 mM of benzoate. After 24 h ($t = 0$ h), different concentrations of acetophenone were added and samples were taken for up to 96 h. The respective reporter output values were analyzed by quantitation of the relative fluorescence intensities from fluorescence microscopy images as well as fluorescence based flow cytometry and confirmed by quantitative immunoblot analysis of ApcA-mCherry signals.

Various tested cultures showed a strict dependence of their fluorescence output on the applied acetophenone concentrations. To illustrate this correlation, samples of two biological replicates were taken 24 h after different concentrations of acetophenone (0–1000 μM) had been added. From each replicate at least two fluorescence microscopy images were taken and scaled equally for comparison of differences in brightness (**Figure 4A**). The acetophenone-dependent relative fluorescence signals were quantified by measuring the mean and maximum fluorescence signals of individual cells (threshold set at 750 units) and averaging the data. In both cases, a linear correlation was observed between acetophenone concentration and fluorescence output up to an acetophenone concentration of 250 μM (**Figure 4B**, Supplementary Figure S1). For both evaluation methods, the fluorescence output was saturated at acetophenone concentrations over 250 μM. Therefore, the reporter strain allows quantification of acetophenone within a concentration range of 50 μM (detection limit) to 250 μM.

The correlation of acetophenone concentration with mCherry fluorescence was also independently proven by flow cytometric analysis of samples taken from the same cultures that had been used for fluorescence microscopic analysis. To achieve this, 20,000 cells per acetophenone concentration from each replicate were analyzed by measuring their relative mean fluorescence signals (**Figure 4C**). Again, a linear correlation was observed for concentrations up to 250 μM acetophenone, which turned over to a saturated reaction at higher substrate concentrations, exactly mimicking the results obtained from fluorescence measurements using microscopy images.

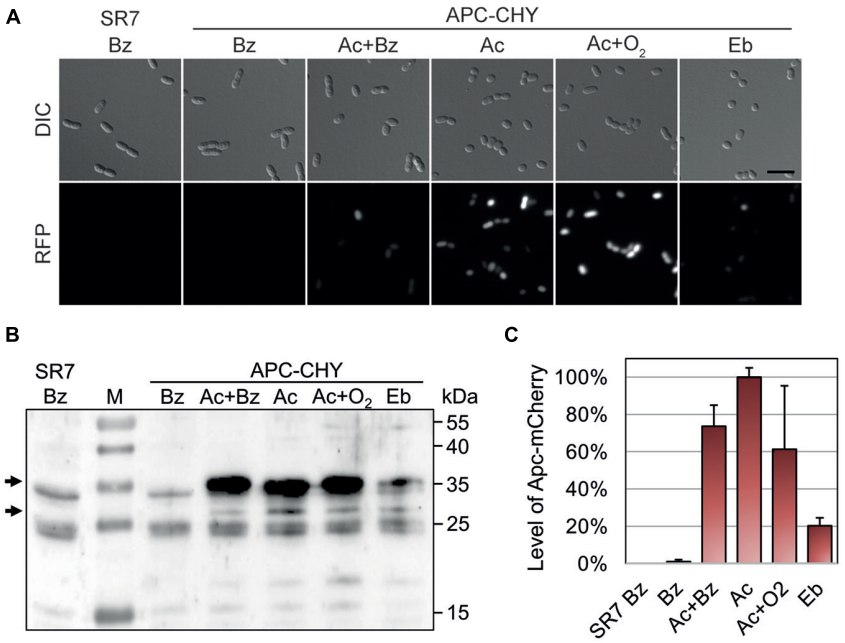

FIGURE 3 | Qualitative and quantitative analysis of ApcA-mCherry fusion protein production in strain APC-CHY. Cultures were grown to mid-exponential phase (72 h) under denitrifying conditions on 4 mM benzoate (Bz), 2 mM acetophenone plus 2 mM benzoate (Ac+Bz), 2 mM acetophenone (Ac), or 2 mM ethylbenzene (Eb). In addition, one culture was grown on 2 mM acetophenone under aerobic conditions (Ac+O_2). The parental strain EbN1-SR7 grown on benzoate served as control (SR7 Bz). **(A)** Differential interference contrast (DIC) and fluorescence microscopy images (RFP, 500 ms) of the cells. Scale bar, 5 μm. All fluorescence microscopy images were scaled equally (250/10,000) and shown in monochrome for comparison of differences in brightness. **(B)** Immunoblot analysis of different cell free extracts (50 μg) of APC-CHY using anti-mCherry antiserum. Molecular masses of the protein standard (M) are given along the right margin. Arrows indicate the positions of the ApcA-mCherry fusion product (30.1 kDa) and a degradation product corresponding in size to free mCherry (~27 kDa). **(C)** Quantification of signal intensities obtained for the ApcA-mCherry by immunoblot analysis in relative units (%). The values given are the average of at least three parallel measurements. Error bars indicate the standard deviation.

Time-Dependence of the Assay

In addition to the concentration dependence, the quantitation of various samples taken after different incubation times showed a time-dependency of the fluorescence output. Apart from using the two fluorescence-based methods mentioned above, we analyzed the same samples for presence of ApcA-mCherry fusion proteins directly, using quantitative immunoblot analysis of the respective cell free extracts with antiserum against mCherry. Samples were analyzed for up to 96 h after exposing the cultures to different concentrations of acetophenone (**Figure 5A**). In contrast to the linear correlation between substrate concentration and output observed for the fluorescence-based methods, the direct quantitation of fusion protein yielded obvious saturation kinetics curves, at least for the samples analyzed after 12 and 24 h (**Figures 5B,C**). The data fitted very well to the following equation, where P is the measured immunoblot signal, c is the acetophenone concentration, max is the saturation value, K is the concentration at half-maximum saturation, and B is the background value without added acetophenone.

$$P = \frac{max * c}{K + c} + B$$

As an additional control, a sample of strain APC-CHY grown under denitrifying conditions on 2 mM acetophenone to mid-exponential phase (72 h, see **Figure 3B**) was included in the immunoblots (**Figure 5A**, labeled with +). The concentration dependence of the Apc-mCherry levels after 12 and 24 h was very similar and again allowed a good resolution of acetophenone concentrations in the range of 50–250 μM. However, as seen in **Figure 5A**, the correlation between acetophenone concentration and Apc-mCherry content deteriorated with longer exposure times. Similar results were obtained by fluorescence microscopy from APC-CHY cells after 48 h exposure as well as by flow cytometry analysis after 48 and 72 h exposure (data not shown).

Specificity of Acetophenone Sensing

Activity of acetophenone carboxylase (ApcABCDE) has been shown to be highly specific toward its natural substrate acetophenone (Jobst et al., 2010; Muhr et al., 2015). Therefore, we investigated the fluorescence signal response to metabolic precursors as well as different chemical analogs of acetophenone using the same experimental procedure described above. All these substrates were added individually to a culture pre-grown on benzoate for 24 h under aerobic conditions at final concentrations of 0.5 mM. After 16 h of exposure, samples were analyzed by fluorescence microscopy (Supplementary Figure S2). The relative mean fluorescence signals (threshold set at 750 units) as well as the maximum fluorescence signals were quantified from fluorescence microscopy images as

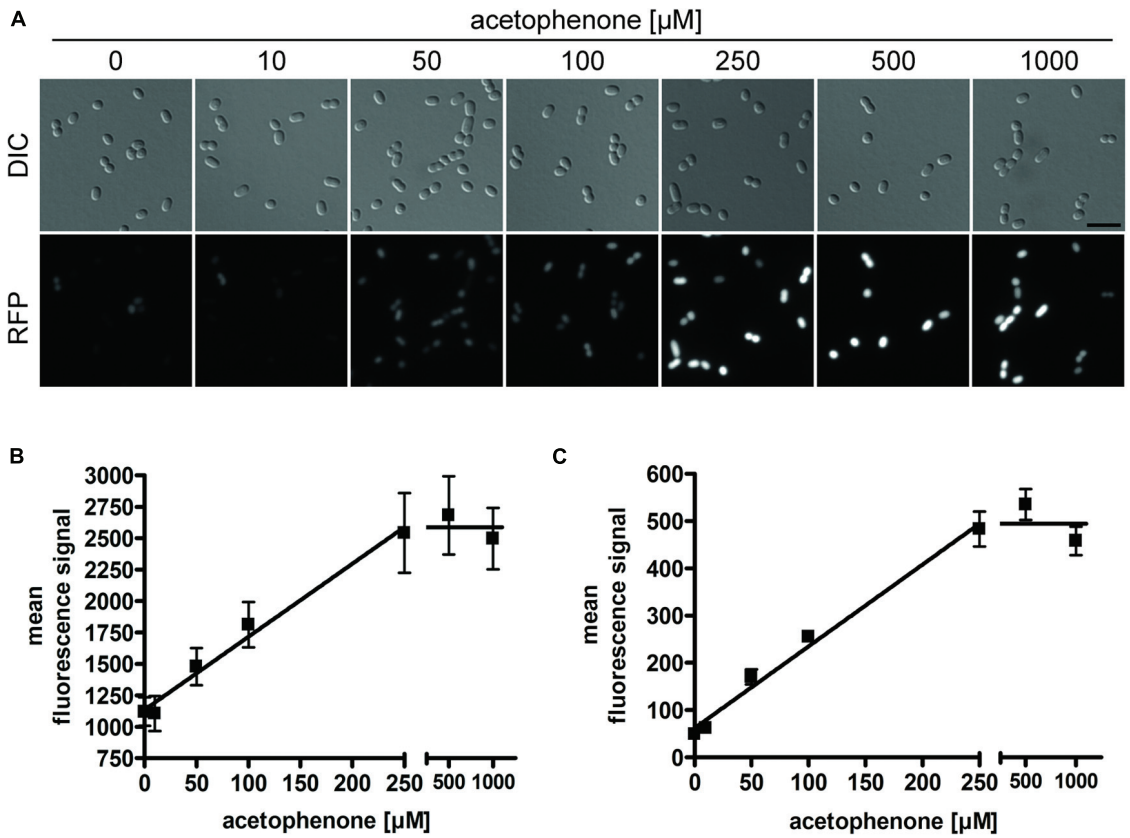

FIGURE 4 | Acetophenone-dependent fluorescence output. Cultures of strain APC-CHY were measured for mCherry fluorescence after 24 h of exposure to different concentrations of acetophenone. **(A)** DIC and fluorescence microscopy images for different acetophenone concentrations were shown in monochrome and scaled equally for comparison of differences in brightness. Scale bar, 5 µm. **(B)** Quantitation of relative mean fluorescence signals from fluorescence microscopy images. The values given are the average of the values obtained from at least 50 parallel measurements. **(C)** Relative mean fluorescence signals determined by flow cytometric analysis of 10,000 cells per culture from two biological replicates per acetophenone concentration. **(B,C)** Error bars indicate the standard deviations. Data were fitted by linear regression between 0 and 250 µM acetophenone, yielding R^2 values of 0.984 **(B)** and 0.988 **(C)**. The fitted lines correspond to slopes of 5.81 μM^{-1} **(B)** and 1.73 μM^{-1} **(C)** and background values of 1136 units **(B)** and 61.8 units **(C)**.

described above (**Figure 6A**, Supplementary Figure S3). The highest induction levels were observed if acetophenone or its immediate metabolic precursors, (*S*)- or (*R*)-1-phenylethanol were added (**Figure 6A**). The response obtained with acetophenone was taken as 100% reference. In both evaluation variants, the (*S*)-enantiomer of 1-phenylethanol showed a slightly higher fluorescence signal response than the (*R*)-enantiomer, reaching a mean value of 91% and a maximum value of 94% of the level obtained with acetophenone, compared to values of 82 and 80%, respectively (**Figure 6A**, Supplementary Figure S3). The inducing effect of the alcohols is most probably due to their conversion to acetophenone, as suggested by analyzing the concentration dependence for (*S*)-1-phenylethanol, which revealed virtually identical saturation kinetics as determined for acetophenone (Supplementary Figure S4).

Interestingly, the fluorescence signals observed under aerobic growth conditions with added ethylbenzene had approximately the same intensities as the ones obtaind for the close acetophenone analog propiophenone with mean values of 44 and 46% of the reference value, respectively (**Figure 6A**). Among all other substrate analogs tested, only the fluoroacetophenone isomers generated relatively high mean fluorescence signal values of 58, 43 and 58%, whereas cultures induced with the hydroxyacetophenone isomers showed very weak signals of 21–24% of the reference. A number of other tested compounds yielded only fluorescence values similar to the level of the negative control with benzoate (**Figure 6A**, Supplementary Figure S2). These substrates included further ring-substituted acetophenone derivatives such as 2′-chloroacetophenone, 2′-methylacetophenone, and 4′-methylacetophenone, the hydrocarbon analogs toluene and styrene, the phenolic analogs phenol, 4-cresol and 4′-ethylphenol, as well as the side chain analogs 2-phenylethanol, phenylacetaldehyde, and styrene oxide (for recorded data see Supplementary Figure S2).

The relative fluorescence intensities determined for the various substrates were confirmed by quantitative immunoblot analysis (**Figure 6B**, Supplementary Figure S5). The signal intensities of ApcA-mCherry proteins were quantified and compared to cells induced with 0.5 mM acetophenone (set as

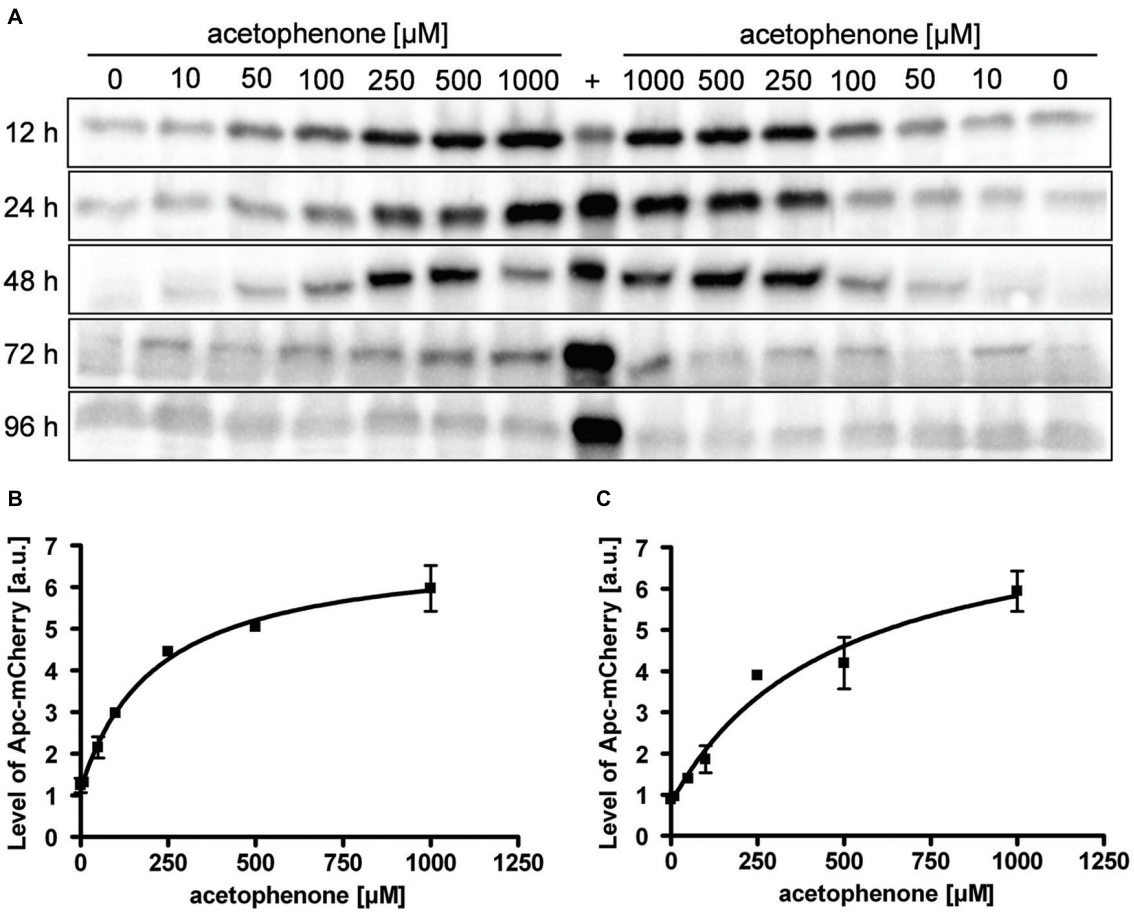

FIGURE 5 | Time- and concentration-dependency of reporter protein content. (A) Cultures of strain APC-CHY were tested for their content of ApcA-mCherry fusion protein by immunoblot analysis after various times of exposure to different concentrations of acetophenone. Samples were taken after the indicated time points given along the left margin. The lanes were loaded with cell free extracts (50 µg) from two biological replicates per acetophenone concentration and probed with anti-mCherry antiserum. A control culture of strain APC-CHY grown under denitrifying conditions on 2 mM acetophenone to mid-exponential phase (72 h, see **Figure 3B**) served as control (labeled with +). **(B,C)** Quantification of ApcA-mCherry signal intensities from the immunoblot analysis after 12 h **(B)** and **(C)** 24 h. The values given are the average of at least three parallel measurements including standard deviations in arbitrary units (a.u.). Data were fitted by the saturation kinetics equation given above, R^2 values were 0.996 **(B)** or 0.976 **(C)**. Curve parameters: max = 5.812 a.u. **(B)** or 7.395 a.u. **(C)**, K = 213.7 µM **(B)** or 467.4 µM **(C)**, and background values 1.133 a.u. **(B)** or 0.786 a.u. **(C)**. It should be noted that the units in **(B,C)** are not directly comparable because the experiments were performed independently.

100%). As for the data evaluated by fluorescence microscopy, the highest content of ApcA-mCherry fusion protein was observed if acetophenone or its immediate metabolic precursors (*S*)- or (*R*)-1-phenylethanol were present. The induction levels measured for the (*S*)- and (*R*)-enantiomers of the alcohol were even 1.2 and 1.3 times higher than with acetophenone, respectively (**Figure 6B**). In contrast, control cells grown on benzoate showed only a basal level of 7.9% of the value obtained with acetophenone. Moreover, as observed by fluorescence microscopy, cells exposed to the fluoroacetophenone isomers showed a medium content of ApcA-mCherry fusion protein (39, 28, and 41%, respectively, compared to acetophenone), whereas the hydroxyacetophenone isomers yielded very weak induction levels in the range of 16–18% of the reference value. All other substrates tested in this study gave rise to Apc-mCherry levels of less than 16%, which were very close to the level of the control.

DISCUSSION

In this study we present the first fluorescent reporter gene fusion constructed in the denitrifying betaproteobacterium *A. aromaticum*, which is a highly versatile degrader of many aromatic compounds (Rabus and Widdel, 1995; Rabus and Heider, 1998; Boll et al., 2002; Rabus et al., 2005, 2014). A strain containing a chromosomally integrated fusion of the first gene of the acetophenone-metabolic *apc-bal* operon with the gene for the fluorescent mCherry protein appears to serve as a reliable reporter system for the presence of certain aromatic compounds without changing the physiological properties of the host strain. Like most other available fluorescent protein reporter systems, mCherry is dependent on exposure to oxygen for the maturation of the protein-derived fluorophore, but it exhibits a relatively fast maturation rate (Shaner et al., 2004, 2005;

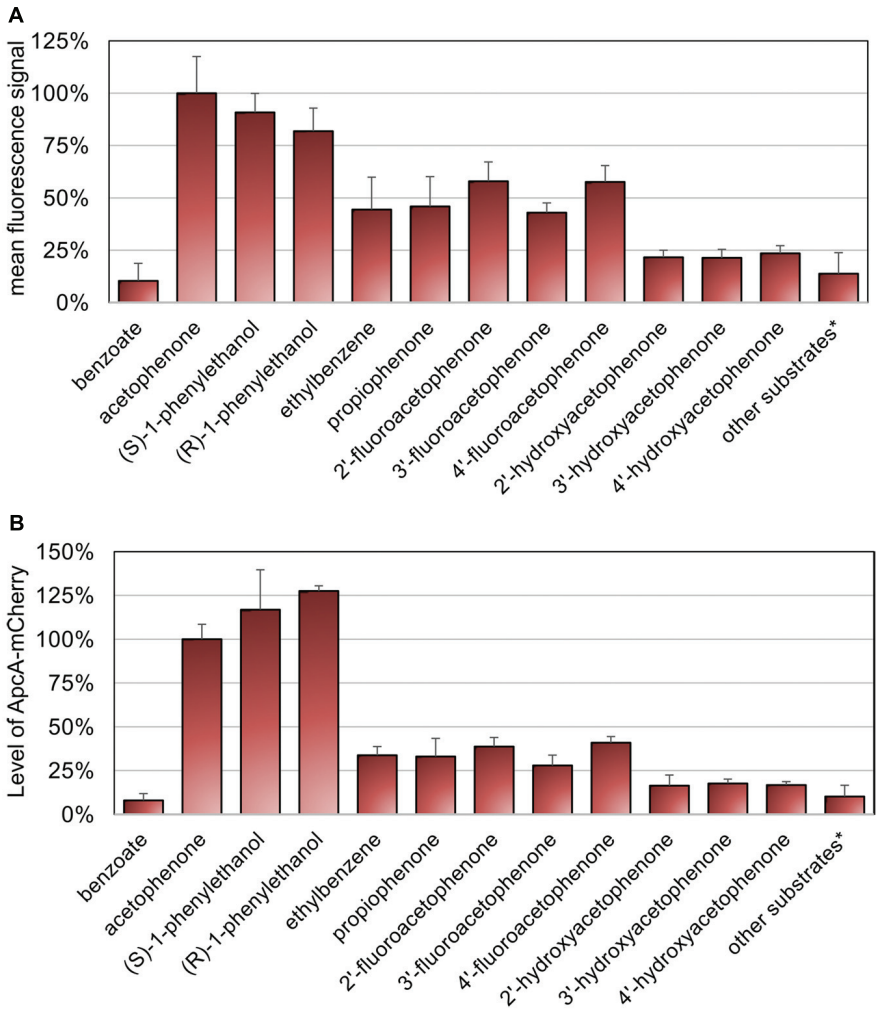

FIGURE 6 | Specificity of sensing. Cultures of strain APC-CHY were analyzed for mCherry fluorescence after 16 h of exposure to different substrates at final concentrations of 0.5 mM. Substrates yielding only background fluorescence, whose recorded values were treated summarily: 2-phenylethanol, phenylacetaldehyde, 2′-chloroacetophenone, 2′-methylacetophenone, 4′-methylacetophenone, styrene oxide, styrene, toluene, 4-cresol, phenol, and 4′-ethylphenol. **(A)** Quantitation of relative mean fluorescence signals from fluorescence microscopy images. The values given are the average of the data obtained for at least 30 parallel measurements. **(B)** Quantification of signal intensities of ApcA-mCherry fusion proteins from immunoblot analysis of cell free extracts (50 μg) using anti-mCherry antiserum. The values given represent the average of at least three parallel measurements. **(A,B)** Error bars indicate the standard deviations in relative units (%).

Merzlyak et al., 2007). We observed that even for cells from strictly anaerobic cultures, exposure of the samples to air for 60 min (including the required processing time for microscopy or other analytical procedures) was sufficient to ensure maturation of the mCherry fusion proteins. Cultures grown under aerobic conditions showed full fluorescence intensities already without preincubation. The presence and quantity of the mCherry fusion proteins was evaluated by three independent methods and showed an excellent correlation between reporter gene expression and the growth conditions under which the enzymes encoded by the induced operon are active (Rabus et al., 2002, 2014; Kühner et al., 2005; Muhr et al., 2015). It was also confirmed here that the *apc-bal* operon is highly induced during acetophenone metabolism under both anaerobic and aerobic growth conditions.

The reporter strain constructed in this study was used to establish a quantitative signal/response relation for the physiological substrate acetophenone based on three independent detection systems. Fluorescence intensities obtained from fluorescence microscopy images or flow cytometric analysis as well as mCherry fusion protein contents were proportional to the applied acetophenone concentrations, providing an effective means to determine unknown concentrations over a range spanning almost one order of magnitude. We observed a linear dependence of the fluorescence signals with acetophenone concentrations of up to 250 μM for the two fluorescence-based detection methods, which turned abruptly into saturation at higher substrate concentrations. In contrast, the concentration dependence of immunologically detected fusion protein was best described by saturation kinetics.

This apparent discrepancy might be due to self-absorbance phenomena at high concentrations of fluorescent proteins in the cytoplasm. The readout of the system was very similar after 12 and 24 h of incubation and only deteriorated after 48 h or longer incubation times, probably because of different extents of degradation of the added acetophenone. Another explanation could lie in toxic or growth limiting effects of acetophenone when added at high concentrations of 0.5 and 1 mM.

Notably, the sensing system seems to be rather specific for acetophenone and its immediate metabolic precursors, (*S*)- or (*R*)-1-phenylethanol. Its inability to discriminate between the 1-phenylethanol enantiomers and acetophenone was expected, since *A. aromaticum* is known to contain several alcohol dehydrogenases with significant activities capable of interconverting acetophenone and the 1-phenylethanol enantiomers (Kniemeyer and Heider, 2001b; Rabus et al., 2005; Höffken et al., 2006). This notion is corroborated by the identical response kinetics recorded for (*S*)-1-phenylethanol and acetophenone. Weaker, but still significant fluorescence signals were also recorded after exposure to ethylbenzene, propiophenone, and the isomers of fluoroacetophenone. This suggests that lack of the oxo-group of acetophenone, elongation of the side chain by one C, or presence of a fluoro-substituent at the aromatic ring still allow partial recognition by the regulator protein(s) responsible. Remarkably, propiophenone and the fluoroacetophenones are also among the few substrates efficiently carboxylated by acetophenone carboxylase (Jobst et al., 2010).

In contrast to either of these compounds, the hydroxyacetophenone isomers elicit almost no response, while other ring-substituted analogs like 2′-chloroacetophenone, 2′- or 4′-methylacetophenone only yield values in the range of the negative control. This correlates well with the respective bond lengths, which are shortest for fluoro- and subsequently longer for hydroxyl-, methyl-, and chloro-substituents at aromatic rings (Clugston and Flemming, 2000). Therefore, the fluoroacetophenones are the closest mimics of acetophenone, whereas larger substituents prevent the respective molecules from binding to either the catalytic enzyme or the respective regulator(s). The absence of any response with 2-phenylethanol, styrene oxide, or phenylacetaldehyde indicates the regiospecifity of recognition, which probably requires the correct placement of the oxo group. Moreover, the lack of induction by toluene or styrene compared to a weak inducing effect by ethylbenzene points to the importance of proper size and orientation of the side chain of an inducing compound.

Remarkably, some of the weakly inducing compounds (propiophenone or the fluoroacetophenone isomers) do not serve as growth substrates for *A. aromaticum*, whereas the (almost) non-inducing 4′-hydroxyacetophenone is degraded by a completely different and specifically induced pathway (Wöhlbrand et al., 2008; Rabus et al., 2014; Muhr et al., 2015). It seems that an efficient regulatory machinery has evolved in *A. aromaticum* to specifically discriminate between inducing the enzymes of acetophenone and 4′-hydroxyacetophenone degradation. Therefore, the reporter strain can principally be used as a rather specific bioreporter system for the presence of acetophenone (plus some closely related chemical analogs) in a concentration range of 50–250 μM. Because acetophenone as well as the 1-phenylethanol isomers are common intermediates of aerobic and anaerobic pathways of ethylbenzene degradation (Filipovic et al., 1992; Bernhardt, 2006; Carmona et al., 2009; Fuchs et al., 2011; Rabus et al., 2014), a specific detection system for these compounds may be useful for applications in environmental monitoring or even in prospecting for new petroleum reservoirs, based on indirectly screening for hydrocarbons via their degradation products. Alternative detection systems for acetophenone include GC/MS methods which reach detections limits of around 80 nM (EPA method index: EPA-OSW 8270D). However, we think that a fluorescence-based method which discriminates the target substrates in complex mixtures of other compounds may be preferable for a fast field survey of environmental samples. Typical acetophenone concentrations in pristine environments are far below the detection limit of the bioreporter system (<10 nM), but they are expected to rise above the detection limit in oil-contaminated sites, judging from ethylbenzene contents of crude oil of up to 40 mM (Kolpin et al., 2002).

It seems to be a general property of anaerobic aromatics-degrading bacteria that they use more substrate-specific catabolic pathways than their aerobic counterparts (Bernhardt, 2006; Fuchs et al., 2011), which may go along with higher substrate specificities of the corresponding regulatory systems. These regulatory systems of anaerobic aromatics degraders (compared to mostly unspecific systems in aerobic bacteria) may provide a basis for the development of many more bioreporter systems. The suicide-vector based system for constructing specific sensor strains for aromatic compounds presented in this study can easily be adapted more broadly to other specifically regulated catabolic genes of *A. aromaticum* or other bacteria for detecting and quantifying these compounds.

AUTHOR CONTRIBUTIONS

EM carried out the practical work, OL took and analyzed the fluorescence microscopy images, SGS performed the flow cytometry analysis, MT provided advice on fluorescence microscopy experiments and proofread the manuscript. EM and JH designed the experiments, analyzed the results, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01561

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Bacteria in the injection water differently impacts the bacterial communities of production wells in high-temperature petroleum reservoirs

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Water flooding is widely used for oil recovery. However, how the introduction of bacteria via water flooding affects the subsurface ecosystem remains unknown. In the present study, the distinct bacterial communities of an injection well and six adjacent production wells were revealed using denaturing gradient gel electrophoresis (DGGE) and pyrosequencing. All sequences of the variable region 3 of the 16S rRNA gene retrieved from pyrosequencing were divided into 543 operational taxonomic units (OTUs) based on 97% similarity. Approximately 13.5% of the total sequences could not be assigned to any recognized phylum. The Unifrac distance analysis showed significant differences in the bacterial community structures between the production well and injection water samples. However, highly similar bacterial structures were shown for samples obtained from the same oil-bearing strata. More than 69% of the OTUs detected in the injection water sample were absent or detected in low abundance in the production wells. However, the abundance of two OTUs reached as high as 17.5 and 26.9% in two samples of production water, although the OTUs greatly varied among all samples. Combined with the differentiated water flow rate measured through ion tracing, we speculated that the transportation of injected bacteria was impacted through the varied permeability from the injection well to each of the production wells. Whether the injected bacteria predominate the production well bacterial community might depend both on the permeability of the strata and the reservoir conditions.

Keywords: water flooding, petroleum reservoir, bacterial community, pyrosequencing, oil-bearing strata

Introduction

Microorganisms play various roles in petroleum reservoirs during oil exploration and post-operation processes (Magot et al., 2000). Recently, the microorganisms in petroleum reservoirs have received much attention, reflecting the effects of these microbes on oil recovery (Sen, 2008; Brown, 2010). Since the 1950's, water flooding has been a widely accepted method for increasing oil recovery from petroleum reservoirs. Flood water, obtained from the sea, river or groundwater, and

recycled production water contain not only nutrients, dissolved oxygen and inorganic ions but also microorganisms (Grassia et al., 1996). These microorganisms are continuously injected into the subsurface and likely affect the reservoir ecosystem (White, 1984; Liu et al., 2005). Struchtemeyer et al. (2011) indicated that the addition of a mud component might shift the bacterial community structure in the reservoir during drilling. However, to date, little is known about the effects of the microorganisms introduced into petroleum reservoirs during water flooding (Struchtemeyer et al., 2011). Benefitting from the development of molecular techniques, such as denaturing gradient gel electrophoresis (DGGE) (Yoshida et al., 2005; Wang et al., 2008), clone libraries (Li et al., 2006, 2007b; Pham et al., 2009) and sequencing technology, an increasing number of studies have addressed the microbial composition of oil reservoir ecosystems (Li et al., 2007a; Pham et al., 2009; Kotlar et al., 2011; Kryachko et al., 2012; Wang et al., 2012; Gao et al., 2013; Lenchi et al., 2013; Lewin et al., 2014). Most of these studies have been on the microbial communities of production well samples. Recently, Lewin et al. indicated that the microbial composition and relative abundance in two non-linked production wells from a same geographical area were extremely similar (Lewin et al., 2014). Lenchi et al. reported that there were no significant differences in the bacterial composition between flooded and non-flooded production wells (Lenchi et al., 2013). However, in a previous study, using PCR-DGGE and clone library technology, the comparison of community structures of one injection water and two production water samples collected from a long-flooded petroleum reservoir indicated that each production well had a specific bacterial structure, despite both wells being continuously flooded with the same injection water for over 30 years (Ren et al.). We also detected significant differences in the bacterial composition between injection and production water samples (Ren et al.). Subsequently, Tang et al. (2012) confirmed this conclusion using clone library technology to compare two production wells in a block and three production wells in another block. These authors observed significant differences among the samples from different wells. In contrast to previous studies, these production wells were connected to an injection well (Tang et al., 2012). Considering the contradictions in previous studies, whether and how water flooding affects the bacterial community structures of individual production wells in the same block remains unknown. Nevertheless, understanding the effects of injected microorganisms on the microbial communities of production wells and revealing the impact factors associated with these effects are crucial to the practice of Microbial Enhanced Oil Recovery.

The aim of the present study was to corroborate the influence of the injected microbes on the microbial community structure of the subsurface during water flooding and to identify the factors that impact the microbial community structures in production wells. To this end, we compared the bacterial structure of samples from one injection well and six adjacent production wells in the same working block of a long-term water-flooded thermophilic oil reservoir. In addition, we attempted to associate the microbial community structures with the various parameters of the sampled wells.

Materials and Methods

Site Description and Sample Collection

In 1961, the Shengli oil field was established in Shandong province in the Yellow River Delta of China. The sandy oil-bearing horizon in the oil field is approximately 1173∼1230 m, with an in situ temperature of 69°C and a pressure of 12 MPa. The block of Zhong Ng3 in the Shengli oil field has undergone water flooding for about 35 years. The water content in the formation water of this petroleum reservoir was over 96%.

The samples used in this study including one injection water sample (IW) and six production water samples (PWs) were collected from Zhong Ng3 working block. IW was collected from injection well (W-00) corresponding to injection well 6-313, and PWs were collected from six spatially independent production wells (W-01, W-02, W-03, W-04, W-05, and W-06, corresponding to production well 5-414, 6-13, 7N11, 8N11, 4-11, and 3-411, respectively). The relative positions of the injection well and the six production wells are shown in **Figure 1**. A tracer test was used to investigate the inter-correlation of the injection well and each production well. The tracer, thiocyanate (SCN^-) was injected at one well along with the injection water and detected at a producing well after some period of time. Injection water was collected from the water supply pipelines before injection, and production waters were collected directly from the well heads of production wells. The names of the samples collected from the above wells were the same as the name of their corresponding well. All of the samples were collected in May 2008. Each sample was full filled sterile 5-L plastic bottle to prevent the oxygen and stored at 4°C before pre-treatment.

Sample Pre-Treatment and DNA Extraction

The microbial biomass of each sample was concentrated by filtration using the Millipore vacuum/pressure pump (Millipore

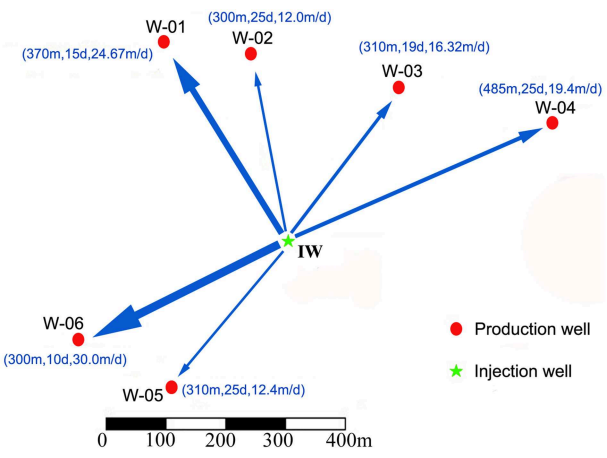

FIGURE 1 | Location maps and inter-correlations of the injection and production wells. The arrow indicates the direction of water flow; the thickness of the arrow shows the rate of flow; and the numbers in the parentheses represent distance, tracer breakthrough time and water flow rates from the injection well to the production wells. IW, injection water sample; W-01 to W-06, production water samples.

Corporation, Bedford, Mass.). Each 250-mL sample was mixed with 1/4 volume of saturated sodium chloride solution and incubated at 70°C for 2 min before filtration (Ren et al., 2011). A 0.22-μm filter (Millipore, USA) was used to collect microbial biomass following an 8-μm filter (Bandao, China). All filters of one sample were collected and stored in a sterilized 10-mL centrifuge tube at −20°C for DNA extraction. The genomic DNA was extracted from the filters using a bead-beating protocol as described previously (Zhang et al., 2007). The AxyPrep PCR cleanup kit was used to purify genomic DNA, which was stored at −20°C until 16S rRNA gene amplification.

Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

The bacterial V3 region of the 16S rRNA gene used for DGGE analysis was amplified using the primers described by Muyzer et al. (1993). The 25-μL reaction mixture contained 0.5 U Taq DNA polymerase, 2.5 μL of the corresponding 10x buffer, 2 μL of a 2.5 mM dNTP mixture (Promega, USA), 1.6 ng /μL of BSA, 6.25 pmol of each primer, and 10 ng of genomic DNA. PCR was performed on Thermal Cycler (Bio-Rad, USA) using a touchdown procedure described previously (Liu et al., 2006), and "Reconditioning PCR" was performed as described by Thompson (Thompson et al., 2002). The concentrations of the PCR products were determined using a DyNA Quant 200 fluorometer (Pharmacia, US) and were evaluated using 1.2% (wt/vol) agarose gel electrophoresis.

V3-PCR products were separated in 8% (wt/vol) denatured polyacrylamide gels by electrophoresis using a Dcode System (Bio-Rad, Hercules, CA): the linear denaturant gradient was 27–55% (100% denaturant corresponds to 7 M urea and 40% deionised formamide). Electrophoresis was performed at a constant voltage of 200 V and a temperature of 60°C for 240 min in 1× Tris-acetate-EDTA (TAE) buffer. A total of 200–250 ng of PCR products were loaded in each lane, and the DNA bands were stained using SYBR green I (Amresco, Solon, Ohio) and photographed using a UV gel documentation system (UVItec, Cambridge, UK). The UPGMA tree was constructed using Quantity One (Bio-Rad, Hercules, CA).

Pyrosequencing of the 16S rRNA Gene V3 Region

The V3 region of the 16S rRNA gene was amplified using the primers P1 (5′-CCTACGGGAGGCAGCAG-3′) and P2 (5′-ATTACCGCGGCTGCT-3′). A unique DNA barcode of eight nucleotides was added to the 5′ end of each primer and used to distinguish PCR products from different samples (Zhang et al., 2010). The 25-μL reaction mixture and PCR conditions have been described previously (Zhang et al., 2010). After the amplicon length and concentration were estimated, an equimolar mixture of all seven amplicon products was purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid, UKAS). Pyrosequencing was performed using the FLX Titanium system (Roche) (Margulies et al., 2005).

All the raw sequences were checked with the standards below: (i) matching primer; (ii) less than one for the edit distance of proximal and distal barcode; (iii) containing at least 100 bases; (iv) having no more than two ambiguous bases. Subsequently, V3 sequences were extracted and sorted to different samples according to barcodes. Sequences with 100% identity were considered as a unique sequence, and then was associated with the number of times observed in each sample (Zhang et al., 2010). Unique sequences were aligned using alignment function of web service in Greengenes (http://greengenes.lbl.gov). Aligned sequences were uploaded to ARB for calculating the distance matrix. Operational taxonomic units (OTUs) were divided using DOTUR based on 97% sequence identity (Schloss and Handelsman, 2005). Rarefaction curves and the Shannon index were also generated using DOTUR to estimate the diversity and richness of each sample. One representative sequence was randomly selected from each OTU on ARB, and its nearest phylogenetic neighbors were searched against database of RDP (http://rdp.cme.msu.edu). The relationship between various samples was illustrated by Principal coordinate analysis (PCoA) using UniFrac distance.

Nucleotide Sequence Accession Number

The sequences obtained in this study have been submitted to the GenBank databases under accession number SRP006479.

Results

Characteristics of the Injection Well and Production Wells

The location of the injection well and six production wells is shown in **Figure 1**. The distances from the injection well to the six production wells were between 300 and 480 m. According to the ion tracing results, the tracer breakthrough times were between 10 and 25 days, indicating that the water flow rates from the injection well to the six production wells ranged from 12 to 30 m/d (meters per day) (**Figure 1**). The working block contained three oil-bearing strata in the subsurface. W-01 and W-04 shared the same oil-bearing strata as W-02 and W-05, whereas W-03 and W-06 had distinct oil-bearing strata (**Table 1**). The physicochemical characteristics of these samples are also shown in **Table 1**.

TABLE 1 | Characteristics of sampling injection and production wells.

	IW	W-01	W-02	W-03	W-04	W-05	W-06
CHEMICAL SPECIES AND CONTENT (mg/L)							
Cl^-	4149.5	3933	3818	3683	3787	3578	3936
HCO_3^-	1073	988	931	1025	1061	1129	1042
Ca^{2+}	104.5	68	72	128	68	76	128
Mg^{2+}	36	55	41	17	43	17	19
K^++Na^+	2921	2790	2728	2624	2719	2627	2762
SO_4^{2-}	3.5	106	125	58	48	0	0
TDS*	8301	7938	7717	7535	7725	7525	7888
Oil bearing strata#		Ng33 Ng34 Ng35	Ng34 Ng35	Ng33 Ng35	Ng33 Ng34 Ng35	Ng34 Ng35	Ng35

*total dissolved solids; #the strata of well sampling from.

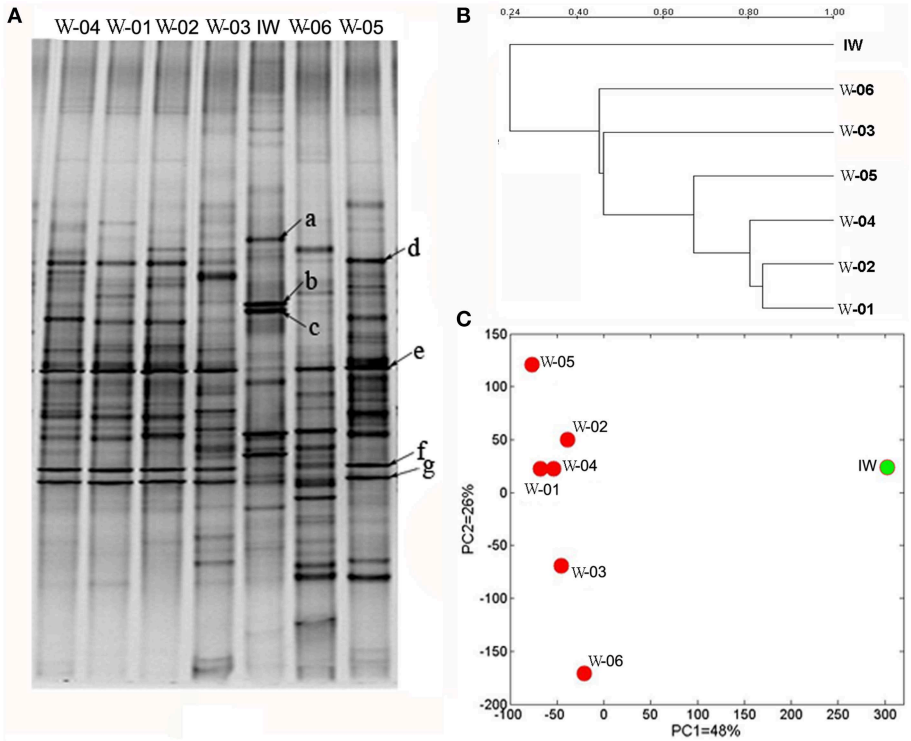

FIGURE 2 | DGGE profiles of PCR-amplified 16S rRNA gene fragments of bacterial communities from one injection water sample and six production water samples. (A) DGGE profiles of the bacterial communities, **(B)** clustering analysis of the DGGE digitalized data, and **(C)** scatter plot of the results from the principal component analysis (PCA) of the DGGE digitalized data. IW, injection water sample; W-01 to W-06, production water samples.

PCR-DGGE Analysis of the Injection and Production Water Samples

PCR-DGGE is a useful tool for comparing the microbial structures of different samples. As shown in **Figure 2A**, the PCR-DGGE profiles for the 16S rRNA gene V3 region revealed distinct bacterial structures between the IW and PW samples. The three predominant bands (a, b, and c) in the IW sample were weaker or absent in the PW samples. However, the four predominant bands (d, e, f, and g) in the PW samples were weaker or absent in the IW sample. The principal component analysis (PCA) of the DGGE fingerprints clearly differentiated the IW and PW samples (**Figure 2B**). Principal components (PCs) 1 and 2 accounted for 48 and 26% of the total variance, respectively. The bacterial structure of W-01 and W-04 was more similar among all samples in the PW samples. The clustering analysis of DGGE profile showed that the similarity between the IW and the PW samples ranged from 37 to 46%, which was lower than the 54 to 75% similarity among PW samples (**Figure 2C**). The results from the *t*-test of the distances among the wells showed a significant difference between the two types of wells.

Bacterial Structures in the Injection and Production Water Samples

The sequences of the V3 fragment of the 16S rRNA gene in the IW and PW samples were obtained using barcode pyrosequencing. A total of 5753 useable reads were obtained, with 1836 unique sequences. A total of 543 operational taxonomic units (OTUs) were defined based on 97% identity (Figure S1A). The coverage of each library was higher than 85%. The richness of the bacterial communities in each sample was estimated using a rarefaction analysis (Figure S1B). The rarefaction curves did not approach a plateau, despite the high number of reads. The curves of the Shannon diversity index of all samples reached saturation. No significant differences in the Shannon diversity index were detected among all samples, except for W-03, which showed slightly higher diversity than the other samples. These results suggest a higher complexity within the bacterial community in W-03 (Figure S1). The detailed taxonomic information for these OTUs is shown in Table S1.

The unweighted UniFrac principal coordinates analysis (PCoA) showed that the bacterial community in the IW sample was different from that in the PW samples (**Figure 3**). UniFrac significance tests for Unifrac distances indicated that the bacterial community of the IW sample was significantly different from that of each PW sample ($P < 0.05$). However, the bacterial communities of W-01 and W-04 showed no significant differences. This result is similar to the comparative findings of the bacterial communities of W-02 and W-05 ($P \geq 0.05$).

A total of 776 (13.5%) sequences shared less than 75% identity with the nearest reference and could not be assigned to any known phylum. The remaining sequences represented 17 phyla, of which Proteobacteria and Firmicutes were detected in

injection and production water samples, the remaining 15 phyla showed a remarkable transition from injection to production water samples (**Figure 4**). On the genus level, 40.4% of the sequences were classified into 59 genera (Table S1). Among these genera, *Pseudomonas* was the only predominant genus in the IW sample, whereas the predominant genera in the PW samples were *Pseudomonas*, *Acinetobacter*, *Halomonas, Thermodesulforhabdus, Thermacetogenium, Thermodesulfovibrio, Chryseobacterium* and *Thermodesulfobacterium*. Particularly, production wells W-01 and W-04 shared the same oil-bearing strata harboring the common genus, *Halomonas*, with the proportions of 18.5 and 4.7%, respectively. Similar results were also observed for production wells W-02 and W-05, which shared the common genus *Chryseobacterium*, with proportions of 18.3 and 26.2%, respectively. A total of 106 OTUs were detected in the IW sample, of which only 5.7 to 17.0% were detected in the PW samples, suggesting that most of the OTUs introduced were not observed in production wells. Among the OTUs detected in the IW sample and at least one PW sample, the abundance of the five OTUs, associated with Pseudomonadaceae, Alteromonadaceae, and Rhodocyclaceae, decreased in the PW samples compared with those in the IW sample (Figure S2A). Conversely, the abundance of 11 OTUs, associated with Pseudomonadaceae, Enterobacteriaceae, Hydrogenophilaceae, Moraxellaceae, Syntrophobacteraceae and Rhodocyclaceae, increased in the PW samples compared with those in the IW sample (Figure S2B). Among these OTUs, two OTUs (OTU5 and OTU6) associated with *Pseudomonas* accounted for 20.5 and 11.6%, respectively, of the total sequences in the IW sample. However, the abundance of OTU5 and OTU6 in the PW samples was significantly different. Particularly, OTU5 and OTU6 were weakly or undetectable in W-06, W-04, and W-03. However, either one or both of these OTUs showed high abundance in W-01, W-02, and W-05 (**Figure 5**). Significant differences in the abundance and ratio of these two OTUs between PW and IW samples suggested that these two bacteria had different fates in different production wells.

Several days passed between water injection and water recovery. To examine the changes in the microbial structure of the injection water over time, two injection water samples (IW/06 and IW/08) from the same injection well were collected at two different sampling times in December 2006 and May 2008, respectively, and the bacterial communities were analyzed using barcode pyrosequencing. A total of 991 and 760 sequences were compared to examine the community structure in these two samples (Figure S3A). The main bacteria phyla in the injection well were consistent at both sampling times, although there was a slight difference in terms of abundance (Figure S3B). One-way ANOVA based on the abundance of each OTU in the two samples from the same injection well indicated no significant difference between them, suggesting that the community structures in the water supply system barely changed after 18 months.

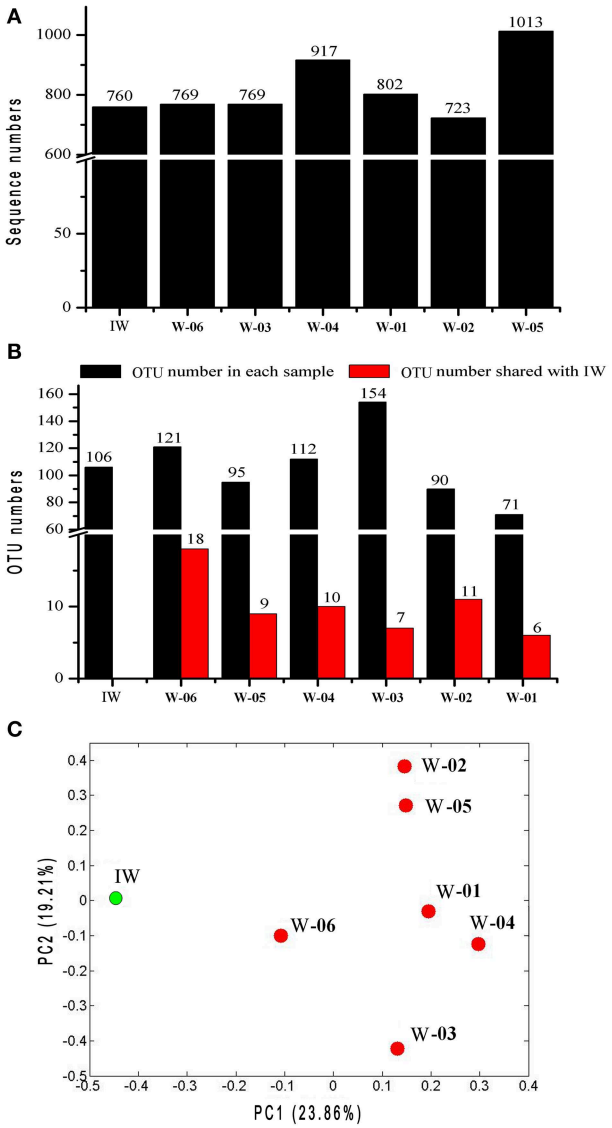

FIGURE 3 | Comparison of microbiota between the injection and production water samples based on pyrosequencing data and UniFrac metrics. (A) Number of sequences in each sample. **(B)** OTUs in each sample and OTUs shared between the injection water sample and each production water sample. **(C)** The PCoA plot was generated using weighted UniFrac analysis. IW, injection water sample; W-01 to W-06, production water samples.

Discussion

The water flooding of the Shengli petroleum reservoir has been continuous for more than 30 years. The production water was separated and subsequently recycled as injected water without sterilization. As bacteria proliferate within the pipeline and tanks of the water supply system, numerous microbial cells in the injection water are continuously introduced into the reservoir. However, the impact of this process has not been well characterized.

To compare the influence of injected bacteria on the microbial structure of production wells and associated reservoirs, we considered the stability of the bacterial community structure in injection water. Although it took 10 to 25 days for the injection water to arrive at each production well according to an ion tracer test, a relatively constant injection of bacterial communities

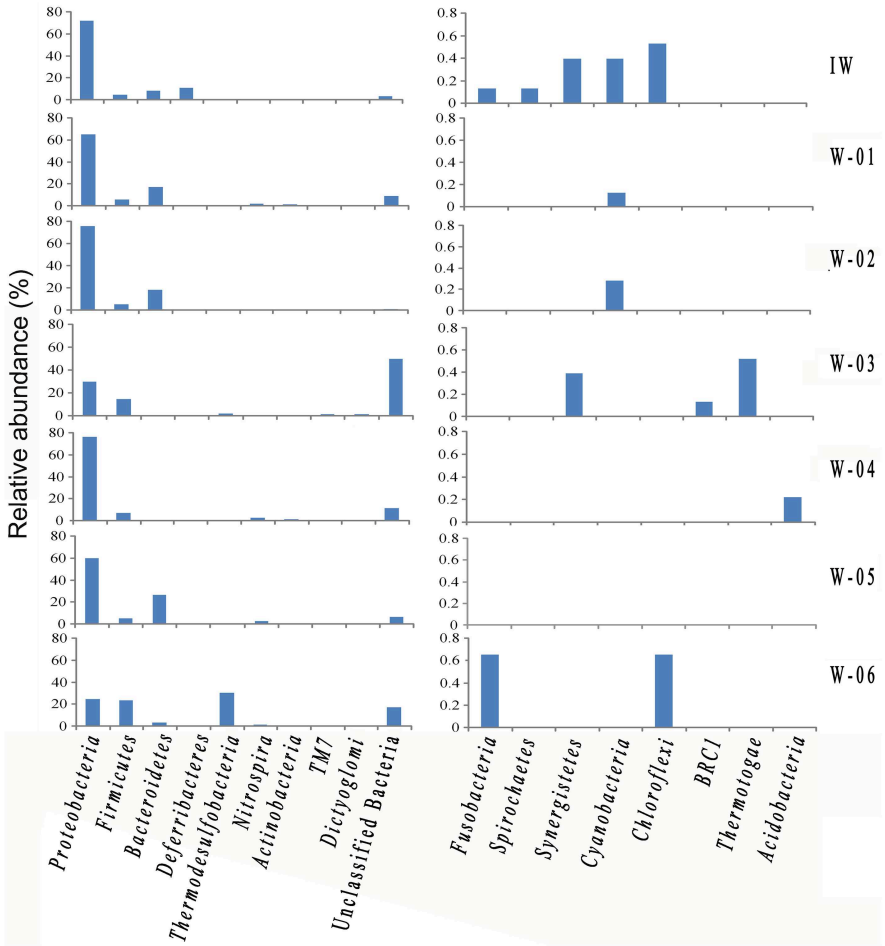

FIGURE 4 | The identity and abundance of representative phyla in the injection and production water samples. IW, injection water sample; W-01 to W-06, production water samples.

during this long period of time, as shown in the present study, warranted the reliability of a comparative study on injection water and production water, even when sampled at the same time. The influence of the sampling time on the effect of the injection water samples on the bacterial structure of the production wells was negligible, as previously reported (Lysnes et al., 2009).

Previous phylogenetic analyses identified a large number of unclassified bacteria in the production wells of petroleum reservoirs (Dahle et al., 2008; Pham et al., 2009). In the present study, 5753 sequences were obtained from seven Shengli petroleum reservoir samples using pyrosequencing technology. Among the total sequences, 63.2% of the sequences in the IW sample and 59.0% of the sequences in the PW samples might belong to new genera, indicating that further analysis is necessary to understand the microbial ecology in petroleum reservoirs due to their high diversity.

The results obtained in the present study showed that Proteobacteria, Firmicutes, and Bacteroidetes were the predominant phyla in Shengli petroleum reservoirs, consistent with previous reports on high-temperature petroleum reservoirs (Li et al., 2006, 2007b; Dahle et al., 2008). In addition, although detected in low abundance (<1.0%), some phyla in the Shengli petroleum reservoir, such as Saccharibacteria (formerly named TM7) and BRC1, have recently been reported in other high temperature oil reservoirs (Tang et al., 2012; Wang et al., 2012; Lenchi et al., 2013). Presenting more detail at the genus level, the present study showed many more genera than previous reports. The predominant genera, including *Pseudomonas, Halomonas, Acinetobacter,* and *Desulfothiovibrio,* have been previously described in other petroleum reservoirs (Orphan et al., 2000, 2003). Additionally, many other taxa, such as *Enhydrobacter, Pelomonas,* and *Weissella*, have rarely been detected in similar environments, indicating the complexity of petroleum microbial structures, and the strong power of the pyrosequencing method for elucidating the microbial community.

The results of both PCR-DGGE and massive parallel pyrosequencing suggested that the bacterial communities in the IW and PW samples were different. Similar conclusions have been reported using fingerprinting (She et al., 2005; Yuan et al., 2007) and a clone library approach (Ren et al., 2011; Tang et al., 2012). However, using pyrosequencing, we provided more detailed taxonomic information in the present study. We

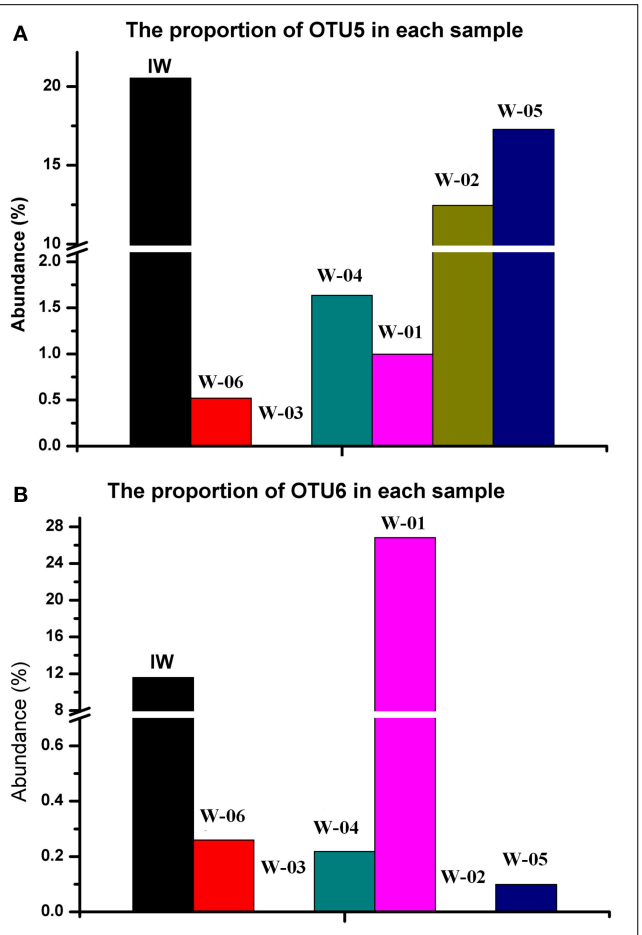

FIGURE 5 | Comparison of abundances of two OTUs from *Pseudomonas* in the injection and production water samples. (A) OTU5; **(B)** OTU6. IW, injection water sample; W-01 to W-06, production water samples.

observed that the abundance of 11 OTUs increased in the PW samples compared with the IW sample, indicating that some of the bacteria had adapted to the subsurface environment and might have proliferated there. However, 69.8% of the OTUs in the IW samples were different from those in the PW samples, indicating that many phylotypes of bacteria injected into the reservoir did not survive and were undetected in the production wells. In addition, the number of bacterial cells in the production water was at least one order of magnitude less than that in the injection water, although the reservoir had been continuously flooded for three decades, indicating a strong interception of microorganisms in the strata. Moreover, the two most predominant bacteria in the IW sample, namely OTU5 and OTU6, had different ratios in the PWs samples. OTU6 was almost undetectable in the W02 sample, whereas OTU5 was as high as 12% in the same sample. Considering the large difference in the number of bacterial cells between the injection and production water samples, we speculate that the bacterial biomasses of these two OTUs in the PW samples are not directly flushed from the injected biomass, further suggesting that only a small portion of the cells were transported to the production wells, even bacteria that were highly abundant in the injection water. Most of the cells likely were blocked during transit, reflecting the low permeability of the strata; therefore, only a few injected bacterial cells were transported to the far end in the production wells. The ion tracer test showed that the water flow rates from the injection well to the six production wells were different, suggesting the distinct porosity and water transferability of the formations along the pathway to the different production wells. Thus, in addition to their scarcity, the numbers of cells transported to these six production wells also varied. The high abundance of specific bacteria in the different production wells likely reflects the in situ reproduction of these microbes. The nanodarcy permeability and extremely small average pore throat size of the shale have prevented the pervasion of bacteria (Jack et al., 1991; Corinne Whitby, 2009; Brown, 2010). Consequently, the various bacterial communities formed within these wells, adjacent to the same injection well, undergo long-term water flooding. In the present study, the comparison of samples from multiple production wells and the different abundance of specific OTUs strongly supported this hypothesis. In the practice of Microbial Enhanced Oil Recovery (MEOR), selected bacteria are often injected into the oil reservoirs to enhance the recovery of crude oil. Based on the present study, we propose that the success of the injected bacteria in the oil reservoir depends on two factors: increased movement of the injected bacterial cells and proper environmental conditions for bacterial growth. Therefore, the previous unpredictable results of MEOR might reflect the low permeability of bacterial cells to the targeted reservoirs.

The results obtained in the present study also indicated similar bacterial structures in W-01 and W-04, which received water-oil fluid from the same oil-bearing strata. The same phenomenon occurred for W-02 and W-05 (**Table 1**). However, the other two production wells, W-03 and W-06, with distinct oil-bearing strata, contained unique bacterial communities. These results suggest that the bacteria in the production wells are closely associated with the oil-bearing strata that harbor them.

In conclusion, the differences in structure of the bacterial communities in the injection well and six associated production wells indicated that the bacterial composition in the production wells is strongly associated with the corresponding oil-bearing strata and the permeability from the injection well to the production wells. We emphasize that understanding bacteria cell flow mechanisms in situ might be key to the optimal design and evaluation of field applications of MEOR.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00505/abstract

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Microbial Communities in Sediments of Lagos Lagoon, Nigeria: Elucidation of Community Structure and Potential Impacts of Contamination by Municipal and Industrial Wastes

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Estuarine sediments are significant repositories of anthropogenic contaminants, and thus knowledge of the impacts of pollution upon microbial communities in these environments is important to understand potential effects on estuaries as a whole. The Lagos lagoon (Nigeria) is one of Africa's largest estuarine ecosystems, and is impacted by hydrocarbon pollutants and other industrial and municipal wastes. The goal of this study was to elucidate microbial community structure in Lagos lagoon sediments to identify groups that may be adversely affected by pollution, and those that may serve as degraders of environmental contaminants, especially polycyclic aromatic hydrocarbons (PAHs). Sediment samples were collected from sites that ranged in types and levels of anthropogenic impacts. The sediments were characterized for a range of physicochemical properties, and microbial community structure was determined by Illumina sequencing of the 16S rRNA genes. Microbial diversity (species richness and evenness) in the Apapa and Eledu sediments was reduced compared to that of the Ofin site, and communities of both of the former two were dominated by a single operational taxonomic unit (OTU) assigned to the family *Helicobacteraceae* (Epsilonproteobacteria). In the Ofin community, Epsilonproteobacteria were minor constituents, while the major groups were Cyanobacteria, Bacteroidetes, and Firmicutes, which were all minor in the Apapa and Eledu sediments. Sediment oxygen demand (SOD), a broad indicator of contamination, was identified by multivariate analyses as strongly correlated with variation in alpha diversity. Environmental variables that explained beta diversity patterns included SOD, as well as levels of naphthalene, acenaphthylene, cobalt, cadmium, total organic matter, or nitrate. Of 582 OTU identified, abundance of 167 was significantly correlated (false discovery rate $q \leq 0.05$) to environmental variables. The largest group of OTU correlated with PAH levels were PAH/hydrocarbon-degrading genera of the *Oceanospirillales* order (Gammaproteobacteria), which were most abundant in the hydrocarbon-contaminated Apapa sediment. Similar *Oceanospirillales* taxa

are responsive to marine oil spills and thus may present a unifying theme in marine microbiology as bacteria adapted for degradation of high hydrocarbon loads, and may represent a potential means for intrinsic remediation in the case of the Lagos lagoon sediments.

Keywords: sediment, PAH, hydrocarbons, microbial diversity, Illumina, Africa, estuary, Lagos lagoon

INTRODUCTION

Estuaries are important components of the global biosphere and play a variety of roles that range from providing habitat for a diversity of plants and animals, to the creation of unique biogeochemical zones that support key transformations in nutrient cycles. However, estuaries also often have significant exposure to anthropogenic activities and thus become polluted with a wide range of organic and inorganic compounds carried in agricultural, industrial, and municipal wastes (Chapman et al., 2013; Elliott and Elliott, 2013; Floehr et al., 2013; Chakraborty et al., 2014; Brady et al., 2015; Li and Duan, 2015; Tappin and Millward, 2015; Machado et al., 2016; Ribeiro et al., 2016). Estuarine sedimets are the ultimate repositories of these contaminants, and thus the in-depth knowledge of the impacts of contaminants upon sediment biology is an important prerequisite to understanding the broader effects on estuaries as a whole.

Sediments house a wide variety of benthic organisms, but prokaryotic microorganisms are particularly key in carrying out biogeochemical processes that are essential in natural nutrient cycling as well as in the fate and behavior of pollutant compounds. Sediments are unique microbial habitats in which a variety of aerobic and anaerobic processes can occur in different redox zones (Hunter et al., 2006; Crump et al., 2007; Zeng et al., 2011; Oni et al., 2015). The co-existence of these activities is important in natural biogeochemical cycling (e.g., linking methane production and consumption) as well as in affecting the fate of pollutants. For example, polycyclic aromatic hydrocarbons (PAHs) are widespread pollutants of sediments (Patel et al., 2013; Johnston and Leff, 2015; Louvado et al., 2015; Revathy et al., 2015; Waigi et al., 2015; Xia et al., 2015) and while anaerobic transformations of some PAH are known, aerobic processes tend to be more effective (Doyle et al., 2008; Fernandez-Luqueno et al., 2011). Conversely, halogenated organic compounds such as polychlorinated biphenyls (PCBs) and trichloroethylene are relatively recalcitrant to degradation by aerobic processes, but have significant potential for transformation by anaerobes (i.e., those of Chloroflexi class Dehalococcoidia) that dehalogenate these compounds as part of a respiratory process (Zanaroli et al., 2015; Matturro et al., 2016). Thus, an understanding about the impacts of pollutants upon sediment microbial communities should include consideration of effects on groups important in natural transformations, as well as those that may be active in biodegradation processes.

Lagos lagoon, located in Lagos State, southwestern Nigeria is one of Africa's largest estuarine ecosystems. It receives loads from four large rivers (Yewa, Ogun, Ona, and Osun), which collectively drain more than 103,626 km^2 of Nigeria. The estuary is urbanized, and borders the densely populated city of Lagos from which a large amount of wastewater is released into the lagoon daily. The lagoon is also industrialized, and is the location of Africa's largest port, as well as the location of extensive petroleum tank farms (Obafemi, 2008). Other areas of the lagoon boarder the forest belt, and are less intensively impact by urban and industrial activities (Alani et al., 2012; Nsikak et al., 2014; Adama et al., 2015; Amaeze et al., 2015). Thus, Lagos lagoon has areas that range from severely impacted by anthropogenic activities to those having comparatively low levels of direct impact. The potential effect that the varied types and intensities of anthropogenic impacts have had on the microbial communities in this ecosystem has not been explored. The goal of the present study was to fill that knowledge gap, and elucidate the structure of microbial communities in sediments of Lagos lagoon that varied in levels and types of anthropogenic impacts, with a view to identify microbes important in natural biogeochemical cycles that may be adversely affected by pollution, and those that may serve as degraders of environmental contaminants, especially PAHs.

MATERIALS AND METHODS

Study Sites and Sampling

Lagos lagoon is a tropical, coastal estuary that stretches from Cotonou in the Republic of Benin, and extends to the fringes of the Niger Delta in Nigeria along its 257 km course (located between Latitude 6°26′ 12.48 to 6°31′57″ and Longitude 3°19′ 48″ to 3°30′ 41″). Sediments from three sites were examined (**Figure 1**). The Apapa and Eledu sites were located adjacent to zones of dense population and municipal waste discharge. Additionally, the Apapa site was near Tin Can Island, which is the site of the largest port in West Africa and houses a high density of petroleum tank farms. The Ofin site lacked a major population on the adjacent shoreline and local industrial effluent was limited to a textile industry.

A 20 kg Ven Veen Grab (KC Research Equipment, Silkeborg, Denmark) sampler for collecting undisturbed bottom sediments was used in collecting sediments from six stations at each site. Samples were pooled in to a sterile aluminum foil pans and transported on ice to the lab and stored at −20°C prior to chemical analysis and nucleic acid extraction. The sampling strategy adopted was similar to other studies examining biogeography of microbial communities (Fierer and Jackson, 2006; Roesch et al., 2007; Bates et al., 2011, 2013) and employed compositing of sediment samples by site to focus on comparisons between sites rather than within sites.

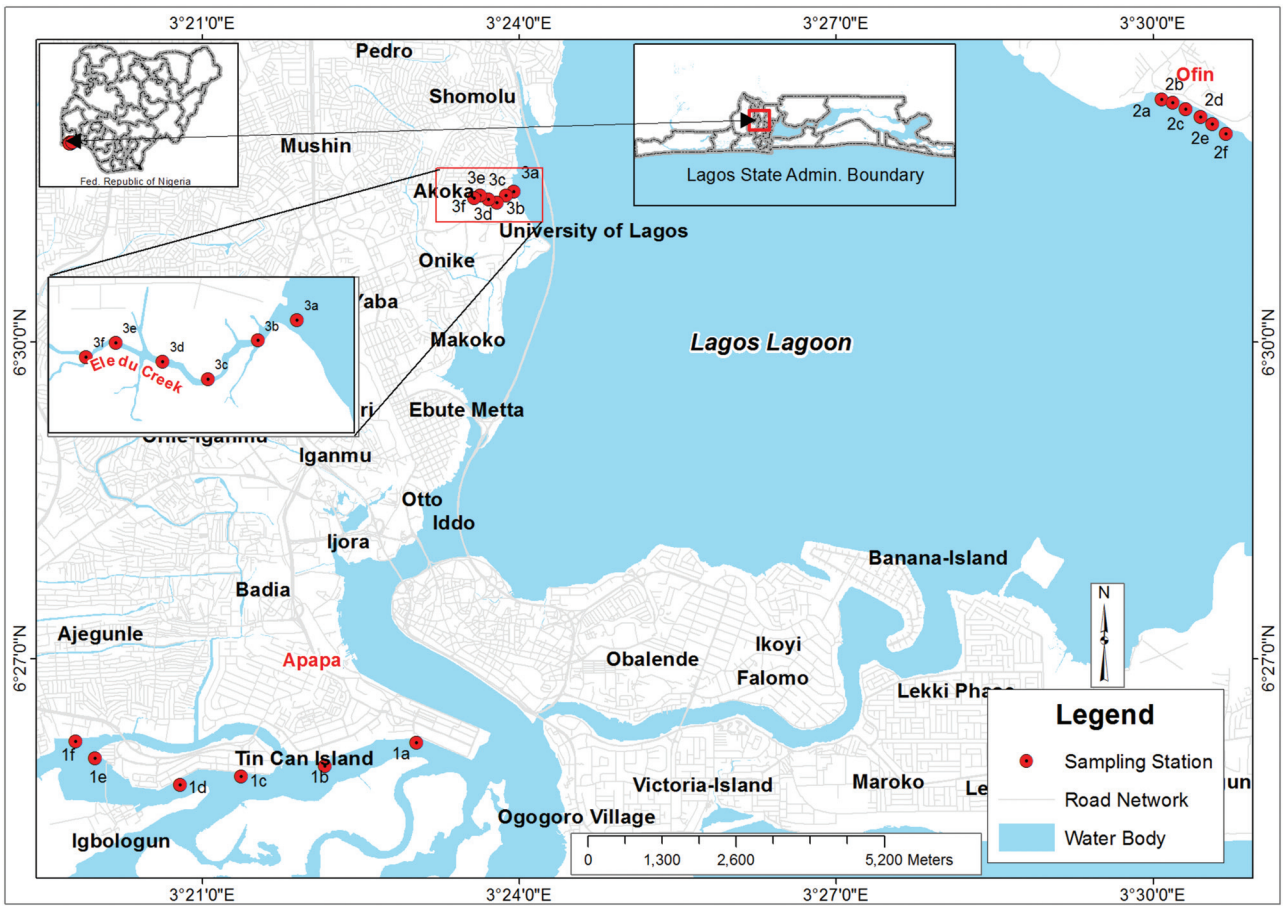

FIGURE 1 | Geographical map of the Lagos lagoon showing the sampling sites and stations. The sampling sites are given in red text: Apapa, Ofin, and Eledu. Expanded detail of the Eledu site is given in the inset.

Physicochemical parameters including pH determined with pH meter (model M501Rev Jenway CE350EU), total organic matter (TOM) and total phosphorus (TP) were determined as described previously (Froelich et al., 1979; Chopra and Kanwar, 1998) and sediment oxygen demand (SOD) was determined as described by Prince (Prince, 2015). Nitrate and chemical oxygen demand (COD) were measured according to Radojević and Bashkin (2006). For heavy metal analysis, sediment samples were dried at 80°C for 48 h, then gently ground with a rolling pin to disaggregate the sample then sieved to collect particles <63 μm. The size-fractionated samples (2.0 g) were digested with a solution of concentrated HNO_3 (0.3 ml) and HCl (6.0 ml) to near dryness and allowed to cool before 20 ml of 5.0 M HNO_3 was added (Tsai et al., 2003). The solutions were allowed to stand overnight and filtered. The filtrates were transferred into a 100 ml volumetric flask and made up to the mark with 0.5 M HNO_3 (Binning and Baird, 2001). Heavy metals present in the filtrate were determined by using flame atomic absorption spectrophotometry. PAH analysis was done by USEPA method 8270D. Sediment samples (10 g) were sonically extracted with methylene chloride. The extracts were then dried, concentrated, and exchanged into cyclohexane. Sample clean up was done by passage over silica gel. The purified extracts were then exchanged back to methylene chloride and analyzed by gas chromatography mass spectrometry. Extract composition was examined by total ion scans, and the USEPA 16 priority pollutant PAH were identified and quantified by using primary and secondary ions.

DNA Extraction, PCR Amplification and Sequencing

Methods for microbial community analysis were done by following protocols described previously (De Gannes et al., 2015). Microbial DNA from the sediment samples was isolated by using the UltraClean Soil DNA (MoBio Laboratories) extraction kit and quantified by fluorometry using a Qubit® 3.0 (ThermoFisher Scientific) with the dsDNA broad range protocol. To minimize variability from the DNA extraction process, 12 extractions were prepared from each sediment in batches of 0.25 g, which were pooled to generate three 1 g-equivalent samples. A 1 μl aliquot from each of the 1 g-equivalent extracts was used for generation of three amplicon libraries for each site. The libraries were created with universal prokaryotic primers 515F and 806R (barcoded)

that targeted the V4–V5 region of the 16S rRNA gene (Caporaso et al., 2010). The PCR conditions used were: denaturing for 3 min at 94°C, 35 cycles at 94°C for 45s, 50°C for 60s, and 72°C for 90 s, followed by 10 min of final primer extension at 72°C. PCR was done in 25 μl reaction mixtures; 13 μl of PCR grade H_2O (MoBio Laboratories), 5 Primer Hot master mix, 0.5 μl of 10 μM of each primer and 1.0 μl of DNA template. All amplification was done in three replicate 25 μl PCR reactions. Following initial amplification, library size was verified on an Agilent DNA 1000 chip, and cleaned using a 1X volume of Mag PCR clean-up beads (Axygen Biosciences, Union City, CA, USA). Following PCR, samples were cleaned and normalized by using a Sequal Prep Normalization Plate (Life Technologies, Carlsbad, CA, USA). Quantity and quality of the libraries were assessed using an Agilent DNA 1000 chip and Qubit® dsDNA High Sensitivity Assay Kit, respectively, and were standardized to 2 nM prior to pooling and sequencing. Sequencing was done with an Illumina Miseq system (Illumina, San Diego, CA, USA) by using Miseq reagent kit v.3 (Illumina) to generate 2 bp × 300 bp reads at the University of Wisconsin Biotechnology Center Madison, Wisconsin, USA. Images were analyzed using the standard Illumina Pipeline, version 1.8.2.

Sequence Data Processing and Analyses

Data analysis was done by the University of Wisconsin–Madison, Biotechnology Center. Illumina datasets were de-multiplexed by using MiSeq Reporter v. 2.2.31 (Illumina) with a Q20 minimum value as a quality filter, Reads were adapter- and quality-trimmed by using the Skewer trimming program (Jiang et al., 2014). Flash was used to merge paired end reads into amplicons (Magoč and Salzberg, 2011), which were then quality filtered. QIIME v. 1.9.1 (Caporaso et al., 2010) analysis used an open-reference OTU picking process: amplicon sequences were first clustered against a reference sequence collection (Greengenes v. 2013_08) using 97% similarity. Sequences that did not align with the reference sequence collection were clustered *de novo* at 97% similarity. Taxonomic assignment of each OTU was established by using the RDP Classifier (Wang et al., 2007). Sequence alignments were filtered to remove variable regions prior to phylogenetic tree creation. Singleton OTUs and OTUs that could not be aligned by using PyNAST were removed from the data set. For alpha diversity analysis (Chao1 and Shannon metrics), data from all three replicates for each sample was pooled and rarefaction applied using an upper limit that corresponded to the size of the smallest library (33,729 quality-filtered amplicons (**Supplementary Figure S1**)). Prism 6 (Graphpad, La Jolla, CA, USA) was used to display the composition of each library.

Statistical Analyses

Univariate linear regression of OTU abundance against environmental parameters was done by using the R programming environment[1]. False discovery rates were determined by the method of Benjamini and Hochberg (1995) and significance assessed by setting the resulting q values ≥ 0.05. The results of the linear regression were compiled in Excel to generate heat maps, wherein environmental parameters that were significantly correlated with the abundance of an OTU were cells highlighted in orange, and those lacking correlation were colored a background grey. The OTUs were organized by taxa (phyla), and thus the heat maps were used to display the patterns of environmental correlations across phyla. Multivariate analyses were done by using Primer-E v. 6 (PRIMER-E Ltd, Lutton, UK). For biological data (Illumina libraries) the numbers of quality-filtered amplicons assigned to a given OTU were normalized to the total quality-filtered amplicons assigned in a given library. The data set was square root transformed to down weight effects of highly abundant OTU and then a resemblance matrix constricted based on Bray-Curtis similarities (Clarke et al., 2008). Beta diversity patterns (among-sample similarities in microbial community structure) were examined by principal coordinate analysis (PCA) and results displayed by ordination. Similarity contours overlaid on PCA ordinations were based on groupings developed with the CLUSTER routine. Relationships between beta diversity patterns and environmental data were examined by using two non-parametric tests, the RELATE and the Bioenvironmental Step (BEST) routines. RELATE is a Mantel-type test that generates a Spearman Rho test statistic that measures congruence between matrices of biotic or abiotic data. BEST identified subsets of physicochemical variables that gave rank order similarities (Euclidean distance) between sediments that best matched the rank order Bray-Curtis similarities of microbial community composition (Clarke et al., 2008). Multivariate analysis was also done to examine the results of linear regression analysis to elucidate potential patterns in combinations of environmental variables that correlated with OTU abundance. For these tests, environmental variables were transformed to binary data (1 or 0) based on whether or not they showed a significant correlation to OTU abundance. Thus, for any given OTU, a variable that was significantly correlated with abundance was scored as "1", and any lacking a significant correlation were scored as "0". The data was then used to construct a resemblance matrix based on Euclidean distance, and CLUSTER analysis applied. The results were displayed as dendrograms, which illustrated groupings of environmental variables that tended to co-occur as significantly related to abundance of OTU. The output of the CLUSTER analysis was also applied to the heatmaps, and the environmental variables were listed in the tables in the same groups and order that were displayed in the dendrograms. The goal of this approach was to present the data in way that potentially conveyed information about the relationships between environmental variables and taxonomic identities of the OTU, rather than using an arbitrary listing of environmental parameters (e.g., alphabetical, by chemical type, *etc.*).

Accession Numbers

The Illumina sequence data reported here have been deposited in the NCBI Sequence Read Archive[2] under accession number SRP069095.

[1] www.R-project.org

[2] http://www.ncbi.nlm.nih.gov/sra

RESULTS

Sediment Characteristics

Out of the sixteen PAHs analyzed in the sediments, four composed the major fraction of these compounds: phenanthrene, fluoranthene, benzo(*b*)fluoranthene, and pyrene (**Table 1**). Total PAH levels decreased in the order Apapa > Ofin > Eledu. Eight heavy metals were determined, with the most abundant being (exclusive of iron) Zn, Cu, Ni, and Cr (**Table 1**). Total metal concentrations in the sediments decreased in the order Apapa > Ofin > Eledu. The three sites were highly dissimilar in levels of TOM, SOD, and COD, with the Apapa and Eledu sites having TOM and SOD levels at least twice that of the Ofin sediment (**Table 1**). A qualitative analysis of the sediments for halogenated organic compounds also revealed the presence of trichloroethylene in the Eledu sediment; halogenated organic compounds were not detected in the other two sites.

TABLE 1 | Sediment physicochemical characteristics [a].

	Sediment		
	Ofin	**Apapa**	**Eledu**
	PAH (ug/kg)		
Acenaphthene	20 ± 0.6	38 ± 0.8	5 ± 0.6
Acenaphthylene	115 ± 0.5	150 ± 0.3	60 ± 0.2
Anthracene	28 ± 0.1	64 ± 0.1	19 ± 2.1
Benzo(*a*)anthracene	261 ± 0.4	376 ± 7.7	308 ± 11.2
Benzo(*a*)pyrene	84 ± 6.8	334 ± 8.5	80 ± 0.3
Benzo(*b*)fluoranthene	492 ± 12.3	787 ± 6.6	426 ± 0.5
Benzo(*g,h,i*)perylene	231 ± 1.9	270 ± 1.5	68.6 ± 4.5
Benzo(*k*)fluoranthene	149 ± 7.9	208 ± 0.3.7	118 ± 10.1
Chrysene	196 ± 9.9	210 ± 16.1	176 ± 2.2
Dibenzo(*a,h*)anthracene	29 ± 7.9	43 ± 7.9	26 ± 7.9
Fluoranthene	531 ± 1.1	829 ± 8.4	411 ± 7.7
Fluorene	26 ± 3.5	75 ± 8.1	5 ± 1.01
Indeno(1,2,3-*cd*)pyrene	294 ± 1.2	517 ± 3.8	122 ± 5.2
Naphthalene	29 ± 3.1	35 ± 1.1	20 ± 1.1
Phenanthrene	802 ± 15.9	986 ± 12.3	710 ± 6.5
Pyrene	688 ± 12.1	719 ± 7.2	457 ± 15.1
Sum of all PAH	3,947	5,606	2,991
	Heavy metals (mg/kg)		
Cd	4.82 ± 0.02	4.06 ± 0.01	3.02 ± 0.03
Co	4.17 ± 0.1	4.14 ± 0.1	2.46 ± 0.4
Cr	10.43 ± 0.03	13.16 ± 0.02	8.36 ± 0.06
Cu	15.63 ± 0.57	16.96 ± 0.01	14.86 ± 0.04
Ni	15.11 ± 0.02	16.39 ± 0.21	12.16 ± 0.02
Pb	0.13 ± 0.01	0.14 ± 0.05	0.13 ± 0.01
Zn	16.73 ± 0.45	17.06 ± 0.20	13.63 ± 0.30
Sum of all metals	67.02	71.90	54.62
	Other parameters		
pH	8.4 ± 0.18	7.2 ± 0.2	7.8 ± 0.1
Total organic matter (g/kg)	149 ± 1.1	342 ± 0.5	428 ± 3.3
COD (mg/l)	480 ± 0.02	218 ± 0.01	1274 ± 0.1
SOD (mg/m^2/d)	4.02 ± 0.1	12.79 ± 0.1	20.48 ± 0.1
Nitrate (mg/l)	57.3 ± 0.2	63.3 ± 0.15	70 ± 0.06
Total phosphate (mg/kg)	1.0 ± 0.2	1.62 ± 0.06	0.4 ± 0.2
Trichloroethylene[b]	Negative	Negative	Positive
Fe (mg/kg)	104.74 ± 0.57	143.74 ± 0.03	659.31 ± 0.09

[a]*For all measures, n = 3. Abbreviations: COD, Chemical oxygen demand. SOD, Sediment oxygen demand.* [b]*Qualitative analysis.*

Alpha- and Beta-diversity Characteristics

Alpha diversity parameters (OTU richness and evenness) differed greatly between the sediments (**Table 2**). Eledu was lowest in both OTU richness and evenness while Ofin was the highest in both of these categories. Alpha diversity of the Apapa sediment was intermediate between the Ofin and Eledu.

Alpha diversity characteristics of the microbial communities were visualized with Whittaker plots, where line slopes reflect species (OTU) evenness and line lengths indicate OTU richness. Whittaker plots of the Apapa and Eledu communities were similar in displaying lines that were initially steeply sloped, reflecting a single dominant OTU that accounted for more than 50% of the reads in each of the libraries (**Figure 2**). The Apapa and Eledu samples were also similar in having a second highly abundant OTU. In contrast, dominant OTUs were absent from the Ofin sediment, and OTU abundance was comparatively evenly distributed (**Figure 2**). The Ofin sample also had the greatest OTU richness as evidenced by the longest sample line, followed by the Eledu and Apapa (**Figure 2**).

Identification of the environmental variables that were potential drivers of variation in microbial alpha diversity was explored by multivariate analyses. First, examination by a RELATE test yielded a Rho value of 0.395 and $p = 0.031$. The BEST routine identified the best fit between the among-sample patterns of the diversity measures with that of the environmental variables, and identified only variation in SOD levels as a strong predictor of microbial diversity ($\rho = 0.894$, $p = 0.003$), with both the Shannon and Chao1 metrics decreasing with increasing SOD.

Beta diversity patterns of the sediment communities (among-sample differences in OTU composition) were examined by PCA ordination (**Figures 3A,B**). The first axis (PCO1) accounted for the majority of the variance (83%) and separated the Ofin community from those of the Apapa and Eledu sites.

TABLE 2 | Characteristics of Illumina libraries and alpha diversity metrics of sediment communities.

	Sediment		
	Apapa	**Ofin**	**Eledu**
Number of reads	131,448	118,455	111,165
Number of quality filtered amplicons	64,403	33,729	65,868
Median amplicon length (bp)	440	443	440
Number of OTU	419	503	293
Chao1	2,791	3,069	2,173
Shannon	5.32	10.09	3.91

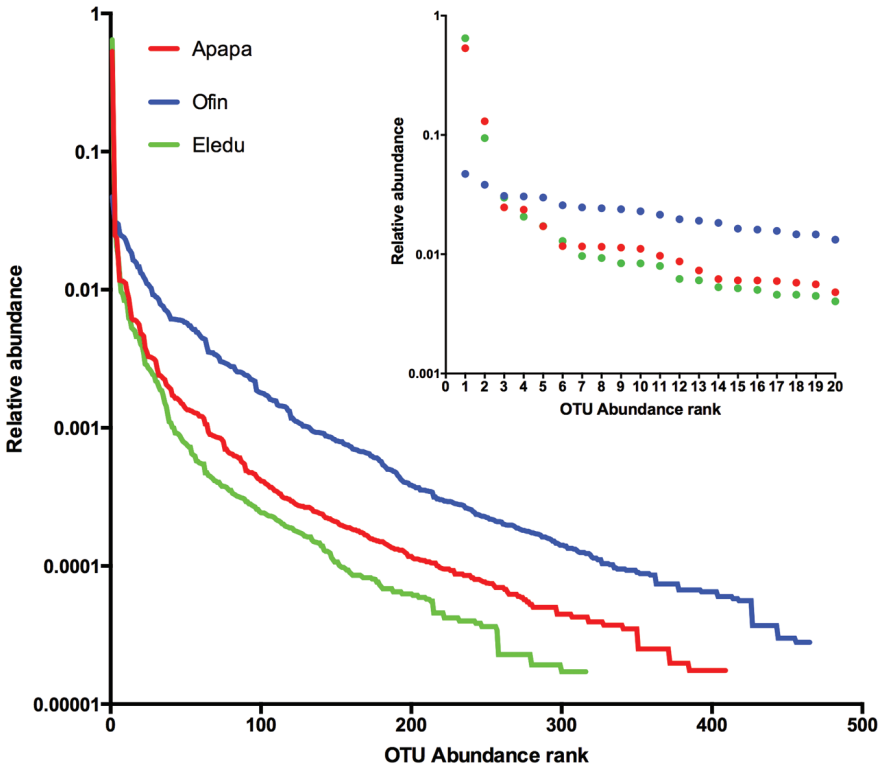

FIGURE 2 | Whitaker plots of average operational taxonomic unit (OTU) abundance in the sediment communities. For each sediment, OTU are plotted from most abundant (rank 1) to least. The large plot shows the entire data set for each sediment, while the inset shows a detailed view of the top 20 most abundant OTU in each sediment.

The Apapa and Eledu communities were separated by PCO2, which accounted for 11% of the variance. The Apapa and Eledu sediments were 80% similar to each other in microbial community composition but only 60% similar to the Ofin sediment community (**Figure 3A**). The sediment characteristics strongly associated with PCO1 were TOM, SOD, and nitrate while environmental vectors associated with PCO2 were PAH and metals (**Figure 3B**). The RELATE test yielded a relatively strong correlation between the sediment microbial community beta diversity and environmental parameters ($\rho = 0.651$, $p = 0.003$). The BEST routine was then employed to identify environmental variables that best explained variation in the beta diversity patterns. The ten correlations obtained identified either only one or two variables, but all yielded the same test statistic and significance ($\rho = 0.878$, $p = 0.001$). Factors that could individually explain beta diversity patterns were levels of Co, Cd or nitrate. The latter two variables also occurred in correlations combined with naphthalene or acenaphthylene. Two other variables, SOD and TOM, also occurred in correlations combined with naphthalene or acenaphthylene.

Microbial Community Composition

Across all libraries, a total of 565 bacterial OTU (97% of quality-filtered amplicons) and 17 archaeal OTUs (3% of quality-filtered amplicons) were identified (Supplementary Table S1). Archaea were divided between two phyla, the Crenarchaeota and Euryarchaeota. None of the archaeal OTUs was assigned to the phylum Thaumarchaeota. The relative abundance of Archaea at the Eledu site was greater than that in either the Apapa or Ofin sediments (**Figure 4A**). Each site had distinct archaeal community profiles (**Figure 4B**). Eledu was unique in being dominated by the Miscellaneous Crenarchaeotal Group [MCG; now reclassified to a new archaeal phylum, Bathyarchaeota (Lloyd, 2015)], which comprised 64% of archaeal OTUs in that sediment. Apapa and Eledu sediments were similar in that archaeal communities were primarily *Euryarchaeota*. However, the types of euryarchaeotal taxa present in the Apapa and Eledu sites differed markedly. In the Apapa community, the largest euryarchaeotal group was acetoclastic methanogens of the *Methanosarcinales*, while in the Ofin sediment, acetoclastic methanogens were a relatively minor constituent and the archaeal community was composed primarily of hydrogenotrophic or methylotrophic methanogens.

Proteobacteria predominated the microbial communities in the Apapa and Eledu sites representing 75–85% of the reads (**Figure 5**). In contrast, in the Ofin sediment, Proteobacteria comprised 30–35% of the libraries and three other phyla comprised large segments of the communities: Cyanobacteria (14–22%; excludes chloroplast sequences), Bacteroidetes (9–12%), and Firmicutes (8–17%). Other phyla that showed increased abundance in the Ofin sediment relative to the Apapa and Eledu sites were: Nitrospirae, Actinobacteria, Acidobacteria,

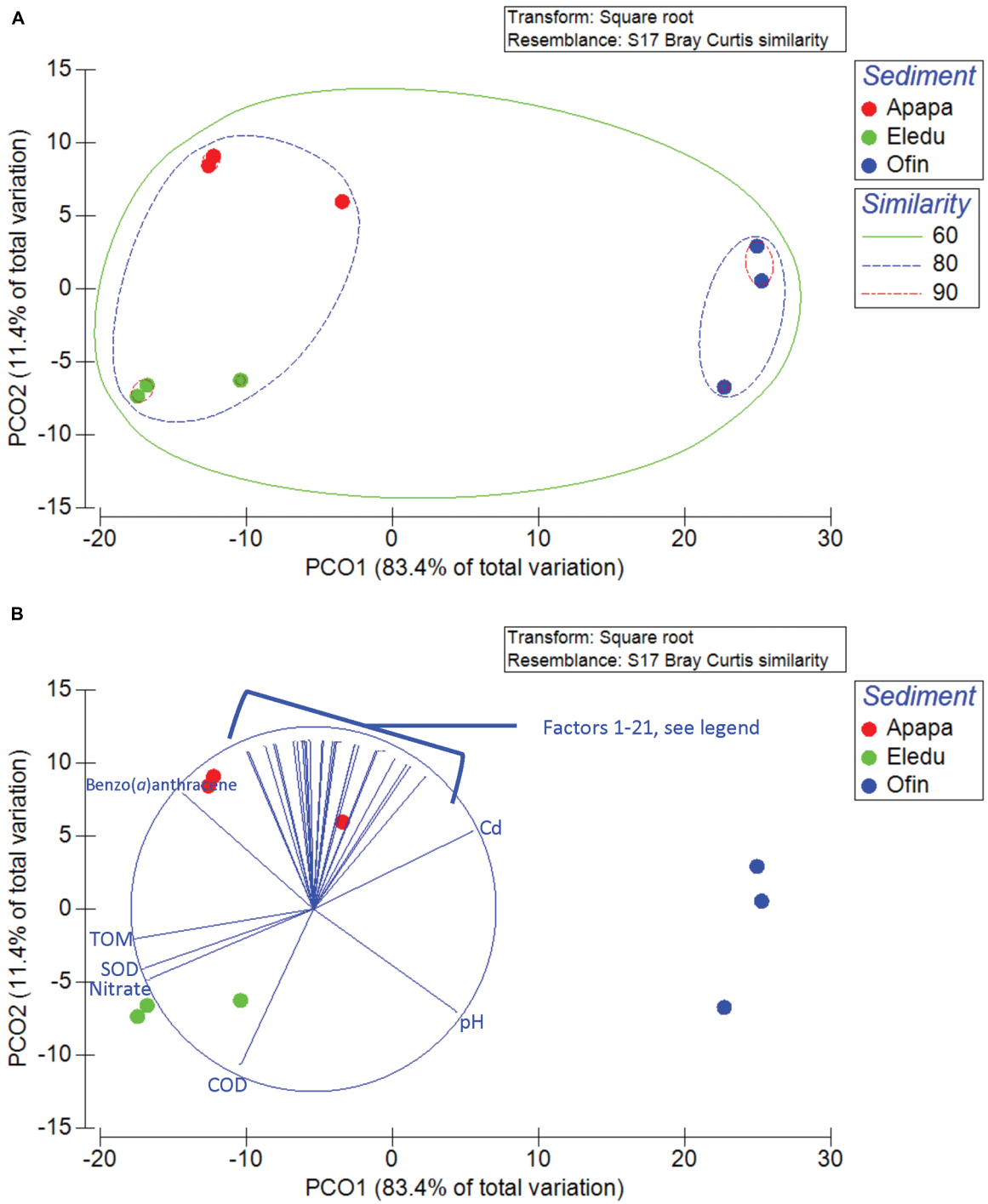

FIGURE 3 | Principal component ordinations of the sediment microbial communities showing levels of similarity among sampling sites (A) and environmental variables associated with the segregation of communities along the PCO axes (B). Environmental variables contained within the brackets are (left to right in the cluster): 1. Pb, 2. Benzo(*a*)pyrene, 3. Benzo(*b*)fluoranthene, 4. Benzo(*g,h,i*)perylene, 5. Cr, 6. Acenaphthene, 7. Cu, 8. Acenaphthylene, 9. Anthracene, 10. Benzo(*k*)fluoranthene, 11. Naphthalene, 12. Phenanthrene, 13. Indeno(1,2,3-*c,d*)pyrene, 14. Pyrene, 15. Chrysene, 16. Ni, 17. Dibenzo(*a,h*)anthracene, 18. Fluoranthene, 19. Fluorene, 20. Pyrene, 21. Co.

and Chlorobi. Chloroflexi was a significant phylum in all sediment communities (**Figure 5**), but comprised the largest share of library in the Eledu sediment (13%).

In the Apapa and Eledu sediments, the dominance of Proteobacteria was attributable to a single OTU of the family *Helicobacteraceae* in the Epsilonproteobacteria class (Supplementary

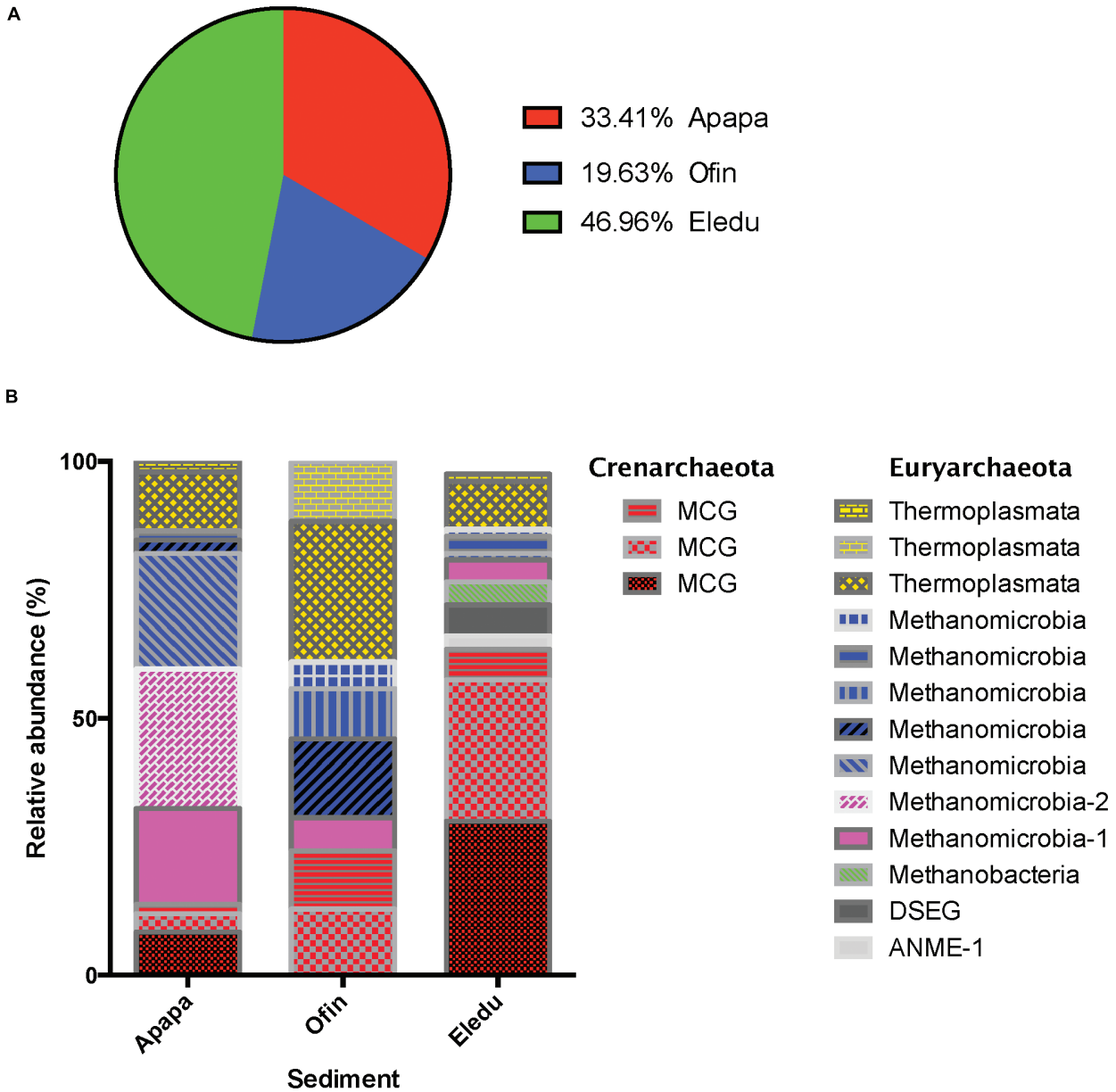

FIGURE 4 | Details of the archaeal community characteristics in the sediments. (A) Relative abundance of archaeal quality-filtered amplicons in each of the sediments as a percentage of the total archaeal quality-filtered amplicons across all libraries. **(B)** Relative abundance of major archaeal taxa in each of the sediment communities as a percentage of all archaeal quality-filtered amplicons in the indicated sediment. Abbreviations are: MCG, Miscellaneous Crenarchaeaotal Group; ANME-1, Anaerobic Methanotrophic-1; DSEG, Deep Sea Euryarchaeotal Group.

Table S1). The microbial community composition of the Ofin sediment was divergent from that of the other sites as the Epsilonproteobacteria were relatively minor (<1%). In the Eledu samples, the dominant *Helicobacteraceae* OTU accounted for an average of 65% of the reads, and was identified as *Sulfuricurvum*. In Apapa, the dominant *Helicobacteraceae* OTU (identified only to family level) accounted for an average of 53% of the reads, and a second *Helicobacteraceae* OTU (assigned to the genus *Sulfurimonas*) comprised an additional 13% of the sequences. In the Apapa and Eledu samples, all other OTU were of comparatively low abundance (**Figure 2**). For example, in the Apapa libraries, there were only eight OTUs comprising ≥1% of the libraries while Eledu had only four OTUs with ≥1% relative abundance. In contrast, the Ofin sediment was not dominated by any individual OTU (**Figure 2**), and the single most abundant of these was a Cyanobacteria sequence that on average comprised 4% of the libraries.

In the Ofin sediment, Dehalococcoidia accounted for 21% of all Chloroflexi reads. In comparison, 31% of Chloroflexi reads were assigned as Dehalococcoidia in the Apapa samples, while

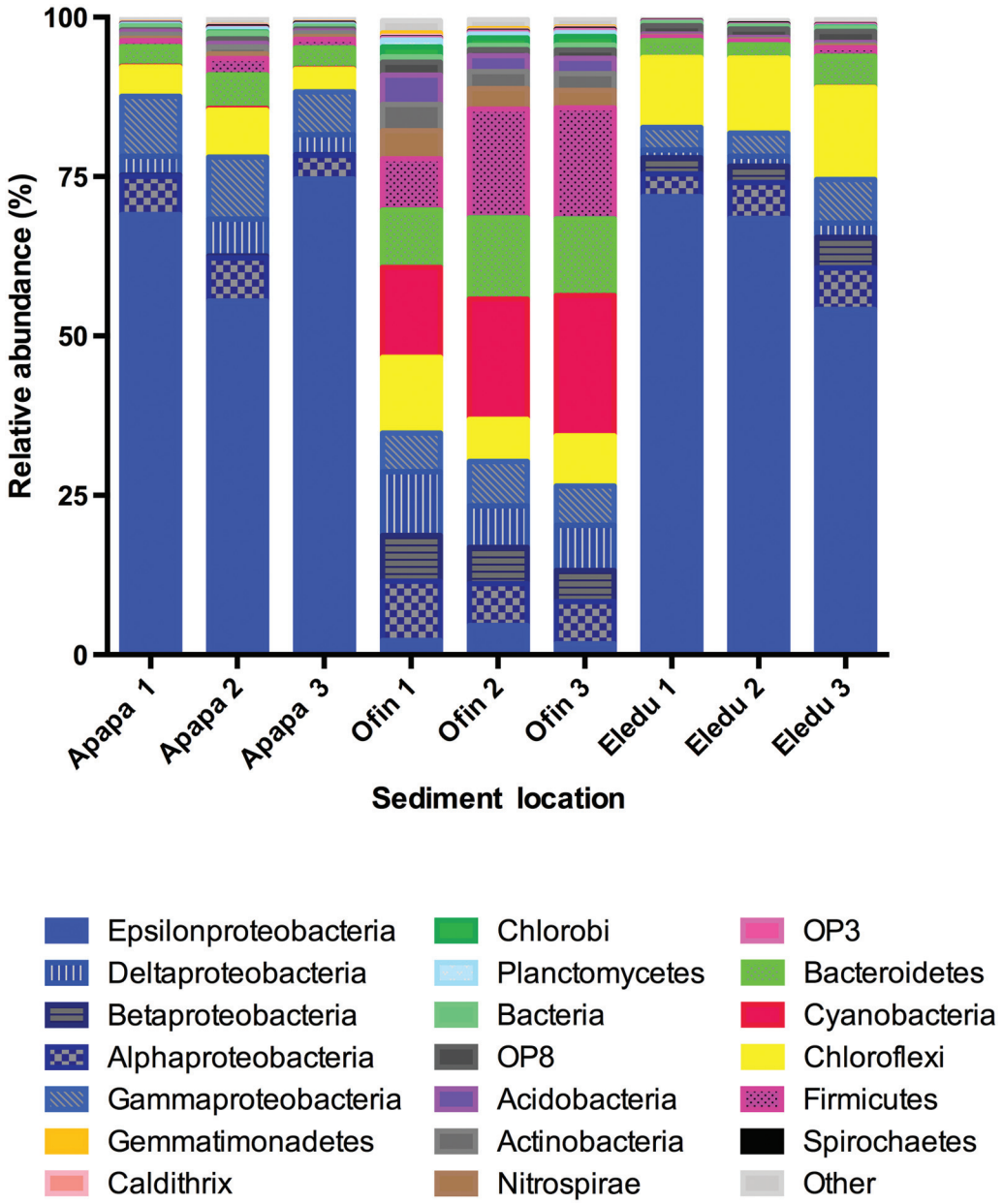

FIGURE 5 | Relative abundance of major bacterial phyla in Lagos Lagoon sediments. The phlyum Proteobacteria is subdivided by class (all color-coded blue).

Dehalococcoidia represented 84% of the Eledu Chloroflexi. Thus, there was an apparent enrichment of Dehalococcoidia in the Eledu sediment relative to the other two sites. Notably, the increased abundance of Dehalococcoidia in the Eledu sediment corresponded to the presence of trichloroethylene at that site, which was not detected in either of the other two sediments.

Six different *Oceanospirillales* genera accounted for 3% of all gammaproteobacterial sequences in the Apapa sediment, but the three main *Oceanospirillales* OTU were identified as the genera *Marinobacterium*, *Marinomonas,* and *Oleibacter,* all of which have been implicated in the biodegradation of PAH or other hydrocarbons (Teramoto et al., 2011; Dong et al., 2014). The *Vibrionales* OTU identified were assigned to the genus *Pseudoalteromonas* or to the family *Pseudoalteromonadaceae*, and were detected only in the Apapa sediment. Other known PAH/hydrocarbon degraders were *Alcanivorox* (Luan et al., 2014; Campos et al., 2015) and *Halomonas* (Gasperotti et al., 2015; Gutierrez et al., 2015). In the Eledu sediment, there was only one *Oceanospirillales* OTU (*Oleibacter*) and in the Ofin site there were no OTU identified as *Oceanospirillales*. While the Eledu and Ofin communities contained comparatively few of these key gamaproteobacterial OTU, other hydrocarbon genera were

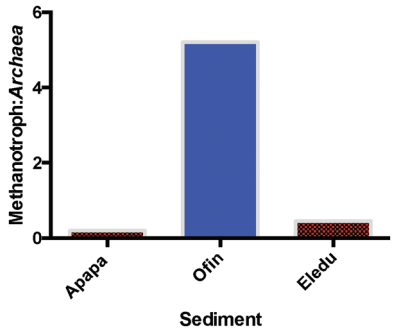

FIGURE 6 | Ratio of gammaproteobacterial methantroph quality-filtered amplicons to all archaeal quality-filtered amplicons in each sediment.

present especially from the Alphaproteobacteria (*Rhizobium, Rhodobacter*) and Betaproteobacteria (*Oxalobacteriaceae, Methylophilaceae*), which were either absent or of low abundance in the Apapa site.

In the Lagos lagoon a total of four OTUs were identified as alphaproteobacterial methanotrophs of the *Methylocystaceae* family, with three assigned to the genera *Methylopila*, *Methylosinus,* or *Pleomorphomonas*. Ten OTUs were assigned as gammaproteobacterial methanotrophs of the *Methylococcaceae* family identified to the genera *Methylocaldum, Methylomicrobium, Methylomonas,* and *Methylosarcina*. While both groups of methanotrophs were present at all sites, they were by far the most abundant in the Ofin sediment where they comprised 82% of all reads assigned as alphaproteobacterial methanotrophs, and 78% of all sequences identified as gammaproteobacterial methanotrophs. Furthermore, at the Ofin site, the ratio of all methanotroph reads to those of all archaeal sequences was at least ten times greater than that in communities of the Apapa or Eledu sites (**Figure 6**). Thus, there was an apparent enrichment of methanotrophs in the Ofin sediment, which may have reflected enhanced methanogenic capacity of the archaeal community in that site compared to that occurring in either the Apapa or Eledu sediments.

Relation of OTU Abundance to Sediment Physicochemical Characteristics

A total of 167 OTUs showed significant correlations (q·FDR $\leq$ 0.05) in abundance to environmental variables (Supplementary Table S2). There were 50 OTUs with a single correlation (mostly to TOM), and one OTU was correlated to 21 variables (Supplementary Table S3). Sediment TOM content was the environmental variable most frequently associated with OTUs abundance (90 OTUs) with SOD the second most frequently correlated factor (Supplementary Table S4). Other environmental factors that were frequently correlated with OTU abundance were dibenzo(*a*,*h*)anthracene, benzo(*a*)anthracene, benzo(*b*)fluoranthence, benzo(*a*)pyrene and Pb (Supplementary Table S4).

Multivariate analyses was applied to identify potential patterns in the frequency with which sediment physicochemical characteristics co-occurred as factors correlated with OTU abundance, and a dendrogram displaying results of that analysis had three main branches (**Figure 6**). Branch I contained the dominant environmental factor, TOM, alone. Branch II included SOD as well as nitrate and Cu. Branch III was represented predominantly by PAHs and metals. Within branch III, there were four subclusters (branches IIIA-D) of PAH and/or metals that frequently co-occurred as factors correlated with OTU abundance (**Figure 7**).

The OTUs that were correlated to factors in those branches showed in some cases distinct distribution patterns in terms of both taxonomy and site. For example, the abundance of Gammaproteobacteria OTU was correlated primarily to factors in Branch IIIC (six PAHs and Pb) and to lesser extent those of branch IIID (**Figure 8A**). Furthermore, the majority of Gammaproteobacteria OTU correlated to the branch IIIC factors were most abundant in the Apapa sediment (**Figures 8B** and **9**), and many of those OTUs were identified as genera in the order *Oceanospirillales* associated with PAH/hydrocarbon degradation (discussed above). The Alphaproteobacteria and Betaproteobacteria also showed a relatively high frequency of correlations to PAH and metals, but primarily to those of branch IIIA (**Figure 8A**). In contrast to the Gammaproteobacteria, most of these Alphaproteobacteria and Betaproteobacteria OTUs were from the Ofin or Eledu sites and comparatively few were derived from the Apapa sediment (**Figures 8B** and **9**). The Epsilonproteobacteria, including the dominant *Helicobacteraceae* OTUs from the Apapa site, were also correlated with PAH and metals (branches IIIA, C, D). Compared to other proteobacterial classes, the Deltaproteobacteria had relatively few correlations to metals and PAHs, but instead were most frequently correlated with TOM (**Figure 8A**). Other taxa, such as the Acidobacteria, Chlorobi, Cyanobacteria, and Firmicutes, displayed a pattern similar to that of the Deltaproteobacteria, and had extensive correlations to TOM, but few or no correlations to metals or PAH (**Figure 8A**). These taxa were in general most abundant in the Ofin sediment (**Figure 8B**).

DISCUSSION

Estuarine sediments are significant repositories of pollutants worldwide (Chapman et al., 2013; Elliott and Elliott, 2013; Floehr et al., 2013; Chakraborty et al., 2014; Brady et al., 2015; Li and Duan, 2015; Tappin and Millward, 2015; Machado et al., 2016; Ribeiro et al., 2016), an in-depth knowledge of the impacts of pollutants on microbial community structure is essential to gain insights into processes that may affect the fate of pollutants specifically and biogeochemical cycling more broadly. In the Lagos Lagoon, PAHs and heavy metal pollutants were spread throughout the sediments, but concentrations of both were highest in proximity to heavily industrialized port area of Apapa. However, the Eledu sediment was also contaminated by trichloroethylene, which was not present at either of the other sites. While SOD reflects the sum of biological and biochemical processes consuming oxygen, biological decomposition of organic matter is generally the major

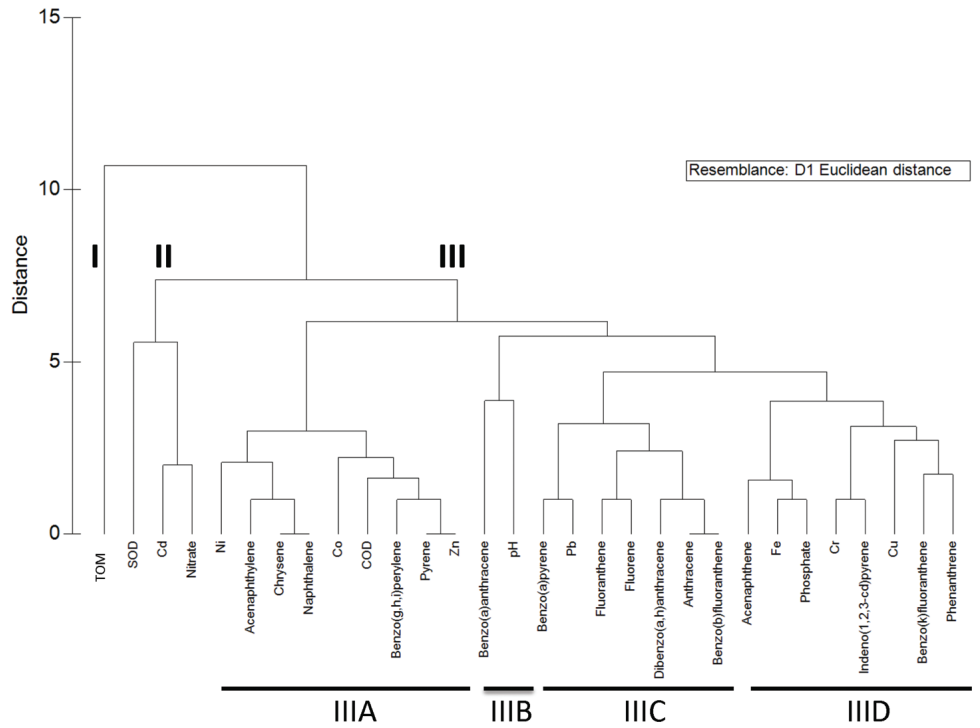

FIGURE 7 | Dendrogram illustrating groupings of environmental characteristics that frequently co-occurred as variables significantly correlated with OTU abundance. Three main branches are indicated by I-III, and four sub-branches within branch III are marked IIIA-D.

contributor and elevated SOD values are commonly measured in sediments impacted by organic wastes (Miskewitz and Uchrin, 2013; Fields et al., 2014; Rucinski et al., 2014; Azzoni et al., 2015). The elevated SOD of the Apapa and Eledu sites was thus a strong indictor that these sediments were impacted by organic materials to a level far greater than that of the Ofin site. An impact of organic wastes on the Apapa and Eledu sediments would also be consistent with the much higher levels of TOM at these two sites compared to the Ofin site. A key environmental effect of elevated SOD is a heightened drive to hypoxia in the water column and anaerobic conditions in the sediment, the latter of which would be consistent with dominance of anaerobic Epsilonproteobacteria in the Apapa and Eledu microbial communities, bacteria that were comparatively minor constituents of the Ofin sediment.

Potential impacts of anthropogenic pollutants upon microbial communities in the lagoon sediments were reflected in variations in alpha diversity. Compared to the Ofin site, the Apapa and Eledu communities had significantly decreased species (OTU) richness as well as decreased species evenness. Furthermore, the results of the RELATE and BEST tests collectively demonstrated that differences in alpha diversity were correlated with variations in environmental parameters, with differences in SOD the most significant. Other surveys of microbial communities in marine sediments have also detected decreases in species richness (Quero et al., 2015; Fuentes et al., 2016; Wang et al., 2016) which may be linked to reduction in either the variety of ecosystem services supported by these communities or in the redundancy of microbial processes, rendering the ecosystems less resilient to perturbations.

In the present study, the impact of sediment contamination on reduction of species evenness was perhaps even more striking, with both the Apapa and Eledu communities dominated by a single OTU, *Helicobacteraceae*. Quero and coworkers also reported a single *Helicobacteraceae* OTU dominated sediments contaminated by complex mixtures of PAHs, PCBs and heavy metals (Quero et al., 2015). The apparent enrichment of *Helicobacteraceae* was consistent with a trend observed for the Epsilonproteobacteria more broadly, which are now proving to be highly abundant in wide range of anoxic environments impacted by hydrocarbons including aquifers, soil, sludge and oil reservoirs (Kasai et al., 2005; Zhang et al., 2005; Hubert et al., 2012; Keller et al., 2015). However, hydrocarbon metabolism has not been demonstrated for *Helicobacteraceae* or other Epsilonproteobacteria, thus the physiological characteristics that enable their dominance of environments such as the highly polluted areas of Lagos lagoon (as indicated by SOD) remain to be determined. One possibility is that the Epsilonproteobacteria grow as syntrophs with bacteria that metabolize hydrocarbons (Hubert et al., 2012), a role that is supported by a recent study using stable isotope probing with $[^{13}C_2]$-acetate of an anerobic community consuming benzene (Starke et al., 2016).

Potential effects of sediment contamination were also apparent in microbial community structure. The RELATE and BEST tests demonstrated that differences among sites in composition were correlated with variations in environmental

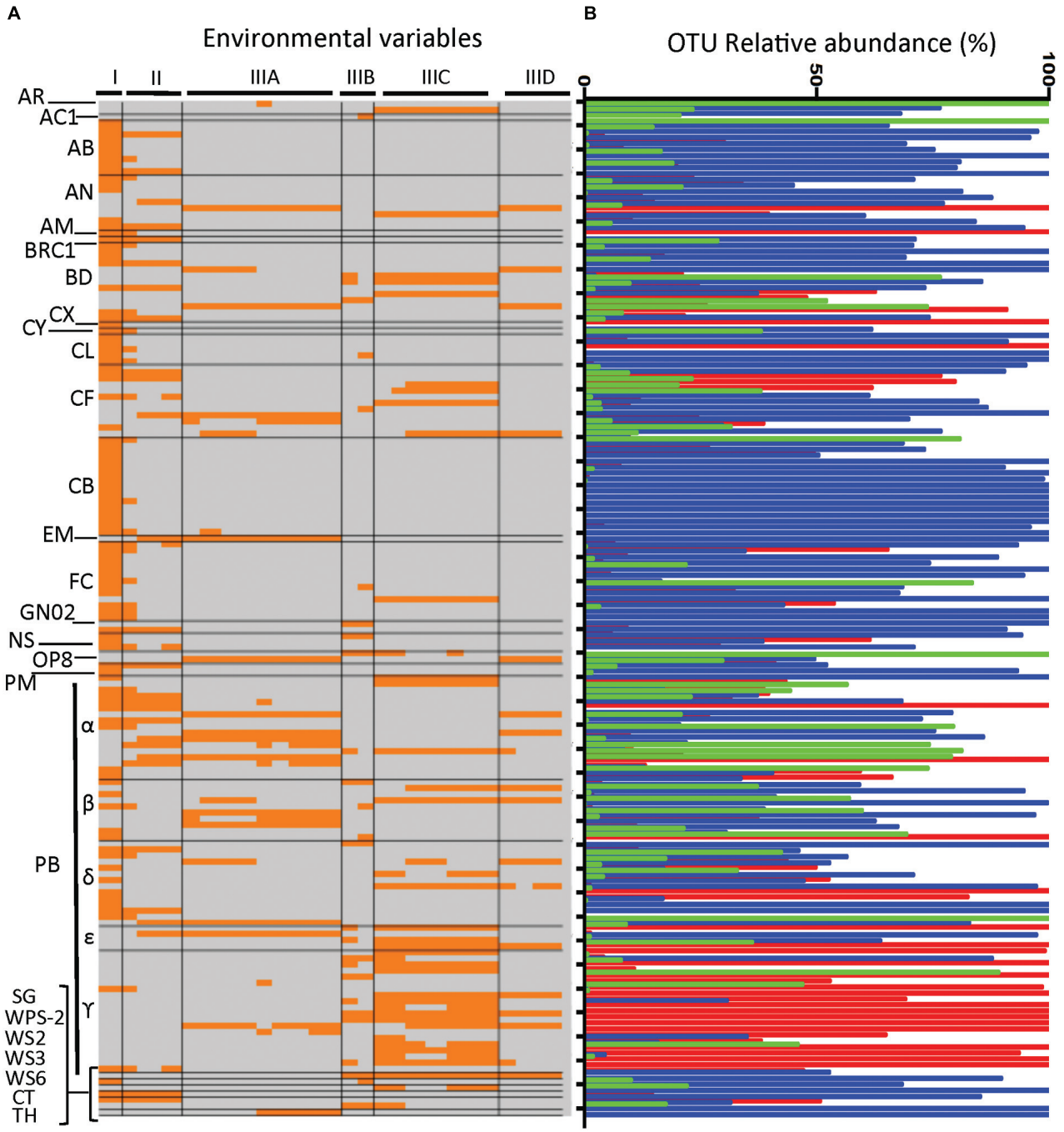

FIGURE 8 | (A) Heat map comparing environmental variables correlated with OTU abundance as a function of OTU taxonomic identity. **(B)** Comparison of OTUs taxonomic identity with OTU abundance across sites. **(A)** Environmental factors are given across the top as the groupings determined by CLUSTER analysis (see groupings in dendrogram, **Figure 7**). The OTUs are listed along the left and are grouped alphabetically by taxa. Each line is an OTU, and sections of the line that are colored orange identify environmental variables that were significantly correlated with abundance of the OTU; line sections colored gray correspond to variables that were not significantly correlated with OTU abundance. Taxa identifications are abbreviated as: AR, Archaea; AB, Acidobacteria; AC1, candidate phylum, AN, Actinobacteria; AM, Armatimonadetes; BD, Bacteroidetes; CX, Calithrix; CY, Chlamydiae; CB, Cyanonbacteria; CF, Chloroflexi; EM, Elusimicrobia; FC, Firmicutes; GN02, candidate phylum; NS, Nitrospira; OP8, candidate phylum; PM, Planktomycetes; PB, Protoebacteria; CL, Chlorobi; BRC1, candidate phylum; SG, Synergistetes; WPS-2, WS2, candidate phylum; WS3, candidate phylum; WS6, candidate phylum; CT, Caldithrix; TH, Thermi. Greek letters denote proteobacterial classes: α, alpha; β, beta; γ, gamma; δ, delta, and ε, epsilon. **(B)** Relative abundance of each OTU (each line from **A**) in each of the sediments is indicated by the bars in **(B)**, which are colored by site: Apapa, red; Ofin, blue; Eledu, green. Abundance of a given OTU at each site is expressed as a percentage of the total number of quality-filtered amplicons assigned to that OTU across all sites.

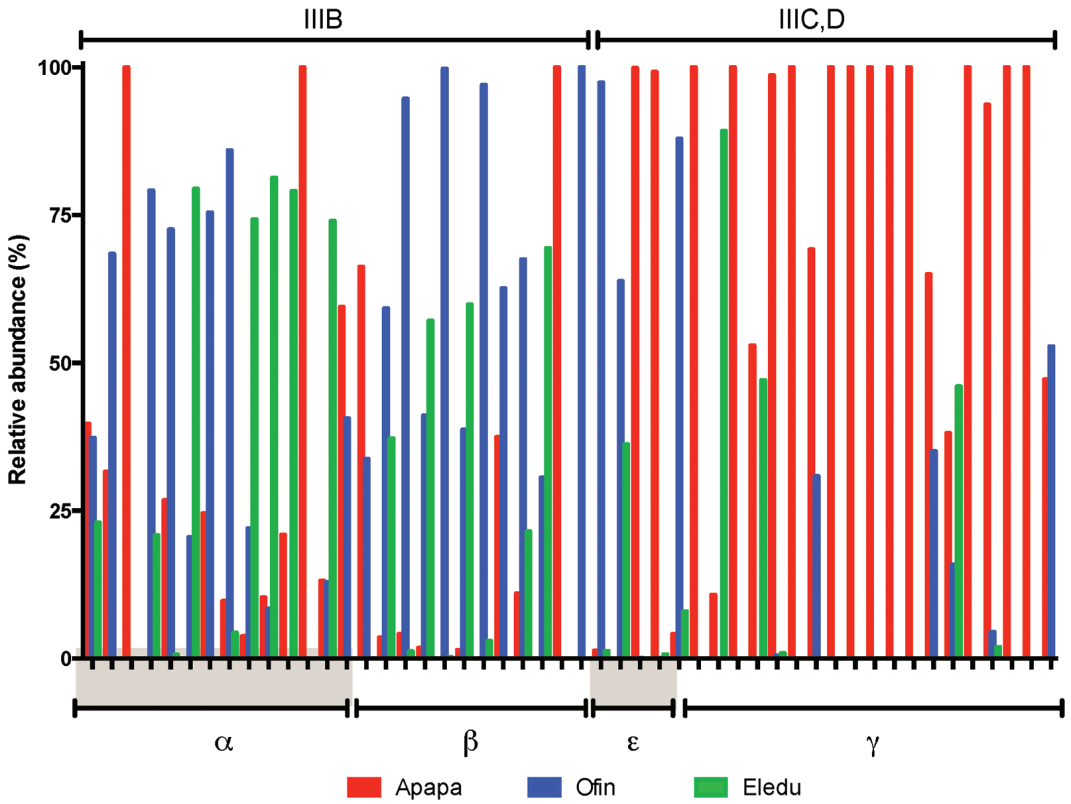

FIGURE 9 | Expanded view of proteobacterial OTU showing significant correlations in abundance to PAH/metals of subcluster III (see Figures 7 and 8A,B). Bars show the relative abundance of each OTU in the indicated sediment, expressed as percentage of the total abundance of the OTU across all samples. Proteobacterial subclasses illustrated are α, alpha; β, beta; γ, gamma, and ε, epsilon. Abundance of each OTU in each sediment is in each of the sediments indicated by bar color (Apapa, red; Ofin, blue; Eledu, green) and expressed as a percentage of the total number of quality-filtered amplicons assigned to that OTU across all sediments.

parameters, which were best explained by organic and inorganic contaminants. The composition of the Apapa and Eledu communities was similar in the dominance of the Epsilonproteobacteria, which resulted largely from a single *Helicobacteraceae* OTU (as discussed above). In contrast, in the Ofin sediment, Epsilonproteobacteria were minor constituents, and the community was composed primarily of other proteobacterial classes, as well as Cyanobacteria, Bacteroidetes, Firmicutes, which were all comparatively minor constituents of the Apapa and Eledu communities. Thus, there were significant alterations in microbial community structure associated with sediment contamination that could lead to shifts in pathways of fundamental biogeochemical processes. For example, the Cyanobacteria represented a potentially significant pathway for nitrogen uptake *via* biological nitrogen fixation (Howarth et al., 1988; Karlson et al., 2015; Adam et al., 2016), which was effectively eliminated in the contaminated sites. Also, the classes of Bacteroidetes and Firmicutes identified in the Ofin sediment have well established activities such as organic matter decomposition and fermentation that are keystone processes in anaerobic food webs (Hunter et al., 2006; Crump et al., 2007; Zeng et al., 2011; Oni et al., 2015). The absence or diminution of these groups in the Apapa and Eledu communities could thus have consequent effects on the mechanisms and pathways of carbon and H_2 flow.

Alterations in carbon cycling resulting from sediment contamination could also have been reflected in the archaeal communities that impact methane flux. Archaea were least abundant in the Ofin site, but the community was composed predominantly of hydrogenotrophic and methylotrophic methanogens, representing a significant potential for methane production. Notably, the Ofin site displayed evidence of a strong enrichment in methanotrophic bacteria, which would be consistent with enhanced methane formation. The Eledu site harbored the largest archaeal community, which was dominated by the crenarchaeotal MCG class that may, or may not, be involved in either production or consumption of methane (Kubo et al., 2012; Evans et al., 2015; Chen and He, 2016). The Apapa sediment harbored a relatively large archaeal community that was distinguished by a dominance of acetoclastic methanogens, possibly reflecting limiting H_2 levels (Beckmann et al., 2011; Beckmann and Manefield, 2014; Lv et al., 2015). While the exact impact that these variations in archaeal community structure may have on the levels of methane production is unknown, fundamental physiological differences between these groups would probably result in differences in methane flux.

A goal of this study was to gain insights into the microbial communities that may mediate PAH biodegradation *in situ* in sediments of Lagos lagoon, and there were strong parallels in the types of potential hydrocarbon degraders apparently enriched in the lagoon with those identified in other marine ecosystems impacted by point source hydrocarbon spills (Hazen et al., 2010; Dubinsky et al., 2013). The massive oil release from the *Deepwater Horizon* has been the subject of field studies utilizing deep sequencing analyses (Illumina or pyrosequencing) and provided a database of marine bacteria that are responsive to hydrocarbon exposure, at the heart of which are Gammaproteobacteria of the *Oceanospirillales* and *Vibrionales* orders (Dubinsky et al., 2013; Gutierrez et al., 2013; King et al., 2015). Moreover, hydrocarbon metabolism has been demonstrated for these taxa in laboratory experiments by *in situ* labeling with stable isotopes and/or physiological characterization of pure cultures (Gutierrez et al., 2013). In the present study, the same gammaproteobacterial genera implicated as key PAH/hydrocarbon degraders responsive to large oil spills formed an important core of potential hydrocarbon-degraders that were enriched in the Apapa sediment, which had the highest levels of PAHs contamination. In contrast, in the sediments less heavily impacted by PAHs, the genera of potential hydrocarbon degraders shifted to the Alphaproteobacteria and Betaproteobacteria. Thus, Proteobacteria were a significant group potentially affiliated with PAH, but the classes varied depending upon levels of PAHs and perhaps other factors. In future studies, approaches such as stable isotope probing (SIP) combined with meta-omics (genome, transcriptome, and/or proteome) could be applied to investigate the functions of organisms that have been hypothesized from the present study. For example, as noted above, SIP *via* [$^{13}C_2$]-acetate pulsing has recently provided evidence of Epsilonproteobacteria as the predominant acetate consumers in a community of anaerobes that consumed benzene (Starke et al., 2016).

CONCLUSION

This study provided insights into the potential impacts that PAHs and complex pollutants may have upon the sediment community in the Lagos lagoon, Nigeria. The communities of two sites strongly impacted by organic wastes (as reflected in elevated SOD) were significantly different from that of a comparatively non-impacted site, and exhibited diminution of OTUs involved in key biogeochemical processes such as nitrogen fixation and methane metabolism. The contaminated sites were also similar in that, although waste streams of differing composition impacted each site, communities of both sediments were dominated by one or two species of *Helicobacteraceae,* which were insignificant in the non-impacted site. The apparent enrichment of *Helicobacteraceae* in heavily polluted sites was consistent with findings of prior investigators, and highlighted a need to understand the physiological characteristics of *Helicobacteraceae* that enable effective colonization of such ecosystems. Lastly, data from this project indicated that hydrocarbon/PAH degrading genera of the order *Oceanospirillales* where potentially important in the sediment most heavily impacted by hydrocarbon pollution, which was consistent with findings from studies of marine oil spills. Thus, the *Oceanospirillales* appear to present a unifying theme in marine microbiology as adapted for degradation of high hydrocarbon loads in general, and present a potential means for intrinsic remediation in the case of the Lagos lagoon sediments in particular.

AUTHOR CONTRIBUTIONS

Experimental design and sample collection (CO, SA, EU, MI, and OA), Sample analysis (CO, SA, EU, MI, OA, and WH), Data analysis (CO, SA, EU, MI, OA, and WH), manuscript preparation (CO, SA, OA, and WH).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01213

FIGURE S1 | Rarefaction analyses of entire libraries for the indicated parameters: observed species (A), Chao1 metric (B), and Shannon metric (C). Note: for comparisons between sites of Chao1 and Shannon metrics, rarefaction was done to a common maximum number of sequences, which corresponded to the size of the smallest library (Ofin, 33,729 quality-filtered amplicons).

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Species Divergence vs. Functional Convergence Characterizes Crude Oil Microbial Community Assembly

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Oil reservoirs exhibit extreme environmental conditions such as high salinity and high temperature. Insights into microbial community assemblages in oil reservoirs and their functional potentials are important for understanding biogeochemical cycles in the subterranean biosphere. In this study, we performed shotgun metagenomic sequencing of crude oil samples from two geographically distant oil reservoirs in China, and compared them with all the 948 available environmental metagenomes deposited in IMG database (until October 2013). Although the dominant bacteria and the proportion of hydrogenotrophic and acetoclastic methanogens were different among oil metagenomes, compared with the metagenomes from other environments, all the oil metagenomes contained higher abundances of genes involved in methanogenic hydrocarbon degradation and stress response systems. In addition, a "shape-sorting" manner was proposed for the assembly of microbial communities in oil reservoirs, with the oil reservoir acting as a function sorter to select microbes with special functions from its endemic pool of microorganisms. At the functional level, we found that environmental metagenomes were generally clustered according to their isolation environments but not their geographical locations, suggesting selective processes to be involved in the assembly of microbial communities based on functional gene composition.

Keywords: metagenome, microbial community, oil reservoirs, petroleum, methanogenesis, hydrocarbon degradation

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INTRODUCTION

Oil reservoirs, which are located deep within the earth, exhibit extreme environmental conditions such as high pressure, high osmosis (due to high salinity or high hydrophobicity), and high temperature (Head et al., 2003). Culture-dependent and culture-independent analyses based on DNA/RNA fingerprints and high throughput 16S rRNA gene sequencing (Li et al., 2006, 2007, 2014; Dahle et al., 2008; Kaster et al., 2009; Pham et al., 2009; Zhang et al., 2012; Gao et al., 2016) have shown that oil reservoirs harbor various microbes. Because of the extreme environmental conditions in oil reservoirs, it is important to understand the functions and assemblies of microial communities in this environment and differences between these microbial communities and those in other environments such as freshwater, seawater, and surface soils. To date, knowledge about functional genes of microbial communities in oil reservoirs is limited

despite recent studies on functional genes associated with sulfate reduction, ammonia oxidation, and methanogenesis by analyzing clone libraries (Li et al., 2011; Guan et al., 2013; Liu et al., 2015) and on metagenomes of offshore oil reservoirs of the Norwegian Sea by using shotgun sequencing (Kotlar et al., 2011; Lewin et al., 2014).

Despite the identification of various microbes in oil reservoirs, mechanisms underlying microbial assemblages in these environments remain unclear. Variation in environmental factors such as temperature and pH and biological interactions may influence the microbial communities. The structures of natural microbial communities can be explained by niche-based mechanisms, and differences in the abundance of taxa reflect the differences in the environmental factors. It is supported by numerous evidences that microbial communities change rapidly in response to changes in environmental factors. In contrast, microbial compositions are extremely different in the abundance of rare taxa, sometimes in the high abundant taxa despite similar geochemical conditions in some oil reservoirs (Ren et al., 2011; Kryachko et al., 2012; Tang et al., 2012). These variations in compositions of microbial communities among different oil reservoirs with similar geochemical conditions cannot be explained by environmental factors. It is possible that stochastic processes such as dispersal and colonization play significant roles in structuring microbial communities in these oil reservoirs with similar geochemical conditions. However, this stochastic process cannot explain how oil reservoirs with different geochemical properties show microbial communities with similar taxonomic and functional gene compositions.

Here, we analyzed the crude oil metagenomes from two oil reservoirs in Daqing and Qinghai oil fields in China. These metagenomes were compared with other two crude oil metagenomes from offshore oil reservoirs of Norwegian Sea (Kotlar et al., 2011; Lewin et al., 2014), and 948 environmental metagenomes which were all environmental metagenomes available in IMG database until October 2013, to examine how they differed within and among other environments, and how taxonomic and functional gene compositions were linked to oil reservoirs characteristic conditions.

MATERIALS AND METHODS

Sample Collection

Oil samples were collected from the wellhead of the high temperature Qinghai (QH) oilfield located in the Tibetan Plateau situated in the northwest of China and from the wellhead of the mesophilic Daqing oilfield (DQ) located in the northeast of China (Supplementary Figure S1). The distance between the two sampling sites is more than 2,500 km. Blocks of Qinghai and Daqing oilfield from where the oil samples were obtained have been subjected to water flooding for 15 and >30 years, respectively. Samples were collected directly from sampling valves located on the wellheads before the oil entered production facilities such as treatment station for separating water, oil, gas, and sand (Supplementary Figure S2). To avoid contamination, sampling cans were washed, sealed, and sterilized by heating at 121°C for 20 min. The sampling valves were also washed and sterilized with 70% alcohol immediately before sampling. During sampling, an initial 20-L sample was discarded before collecting the oil samples in the sterilized cans. Once the cans were completely filled, they were sealed using screw caps, stored at <5°C, and sent to our laboratory within 2 days. Further analyses were performed immediately. Since the wellheads were directly connected to the working layers in reservoirs, the microbial community in the crude oil samples should reflect the microbial compositions in the reservoirs.

DNA Extraction, Sequencing, and Analysis

DNA was extracted from the samples as described previously (Kryachko et al., 2012) and the genomic libraries for Illumina sequencing were generated according to the Illumina paired-end library preparation protocol. The average size of the library fragments was 200 bp for both QH and DQ, while the average of the insert size was 190 bp for QH and DQ. The libraries were sequenced using HiSeq 2000 (Illumina, San Diego, CA, USA) with 2 × 100 bp paired-end sequencing.

For taxonomic analysis, low quality paired-end reads were removed using Illumina CASAVA pipeline with default parameters, and the remaining reads were compared against the NR database by using BLASTX to estimate functional and taxonomic composition and abundance as described before (Wang J. et al., 2013). MEGAN software (Huson et al., 2011) was used to parse the results of BLASTX and to estimate microbial composition using the lowest common ancestor (LCA) algorithm. Briefly, each read matching the sequence of any gene was placed onto the LCA node of those species in the NCBI taxonomy that are known to have that gene, and the taxonomic information of the LCA node was retrieved. To confirm the results, the 16S rRNA genes were also extracted from the reads based on hidden Markov Models (HMMs; Huang et al., 2009), and classified using the RDP classifier (Cole et al., 2009).

For metagenome assembly and gene prediction, after quality filtering of the raw reads, the remaining paired-end reads were used to build a *de novo* assembly by using SOAPdenovo (Luo et al., 2012) with default settings. Open reading frames (ORFs) of assembled contigs were predicted using MetaGeneMark program (Zhu et al., 2010). The predicted ORFs were compared against the NCBI NR database (downloaded from NCBI in September, 2012) of non-redundant protein sequences, the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologous (KOs) database (Kanehisa et al., 2008), and Clusters of Orthologous Groups (COG) database (Tatusov et al., 2003) to assess the functional classification. For searching genes encoding alkane 1-monooxygenase (AlkB), cytochrome P450 CYP153 family of alkane hydroxylases (CYP153), long-chain alkane monooxygenase (LadA), flavin-binding monooxygenase (AlmA) involved in aerobic *n*-alkane metabolism, and hydrocarbon succinate synthases (AssA/BssA) involved in anaerobic hydrocarbon degradation in the two metagenomes, reference sequences were downloaded from GenBank (Supplementary Table S1). Genes encoding AlkB and CYP153 were annotated as described previously (Nie et al., 2014).

Two crude oil metagenomes NorOil1 (Kotlar et al., 2011) and NorOil2 (Lewin et al., 2014) from offshore oil reservoirs of Norwegian Sea were also analyzed using the same methods.

To identify special genes and pathways in the metagenomes of the oil reservoirs, all the published 1,233 metagenomic datasets from the IMG database (until October, 2013) were downloaded and compared. After removing metagenomes that were not environmental samples, or were without detailed information about the sampling sites, 948 metagenome datasets were selected for analysis. Both the oil metagenomes and all the reference metagenomes were clustered using Cluster 3.0 program (de Hoon et al., 2004) with average linkage method based on the relative abundance of KEGG genes after normalization according to *z*-score. To identify specific functions of adaptive importance in different samples, genes in different regions were affiliated to KEGG pathways. Next, abundance of genes in different pathways was normalized using the *z*-score and was clustered. Bray–Curtis dissimilarity was calculated using Vegan: community ecology package (version 1.8–6) in R statistical program 2.9.1 (R Development Core Team). Based on the KO abundance, principal component analysis (PCA) was performed to evaluate similarity among various metagenomes by using the Vegan package in the R statistical program.

The metagenomics data have been submitted to NCBI SRA and are accessible under the BioProject identifier PRJNA251580.

RESULTS

Microbial Community Structure in Oil Samples

After filtering low-quality reads, 68,937,779 and 68,362,793 read pairs were generated from the metagenomes of Qinghai (QH) and Daqing (DQ) oilfields, respectively. Approximately 72.2% reads obtained from the QH metagenome were assigned to bacteria and archaea (Supplementary Figures S3 and S4); of these, approximately 93% reads were assigned to 34 known phyla (**Figure 1**; Supplementary Figure S4; Supplementary Table S2). Approximately 73.6% reads obtained from the DQ metagenome were assigned to bacteria and archaea (Supplementary Figure S4); of these, approximately 93% reads were assigned to 35 known phyla (**Figure 1**; Supplementary Figure S4; Supplementary Table S2). However, approximately 36.2% and 45.4% reads obtained from the QH and DQ metagenomes, respectively, that were assigned to known phyla could not be assigned to known genera (Supplementary Figure S4). The rarefaction analysis of reads based on the taxonomic information showed that a reasonable number of individual genomes were sampled, with the coverage over 99% both in QH and DQ at the genus level (Supplementary Figure S5).

The QH metagenome predominantly included bacteria belonging to *Proteobacteria*, which accounted for 80.8% of the total reads assigned to known phyla (**Figure 1**). Of the *Proteobacteria*, *Gammaproteobacteria* was the most dominant bacterial class, with genera such as *Pseudomonas* (54.4%), *Acinetobacter* (1%), and *Marinobacter* (1.3%) (Supplementary Figure S6). The remaining microbes mainly belonged to *Euryarchaeota* (11.1%), *Firmicutes* (3.8%), and *Deferribacteres* (1.2%). The QH metagenome included methanogenic archaea (47 genera) such as hydrogenotrophic *Methanococcus*, *Methanothermococcus*, *Methanobacterium*, *Methanoculleus*, and *Methanosarcina* that could produce methane from H_2, acetate or methyl compounds (Supplementary Figure S7; Supplementary Table S3). The DQ metagenome predominantly included bacteria belonging to *Firmicutes* (64.2%) and *Proteobacteria* (26.7%), namely, *Anoxybacillus* (37.2%), *Geobacillus* (2.8%), *Bacillus* (1.1%), *Pseudomonas* (6.4%), and *Brevundimonas* (0.9%) (Supplementary Figures S6 and S8). Microbes belonging to

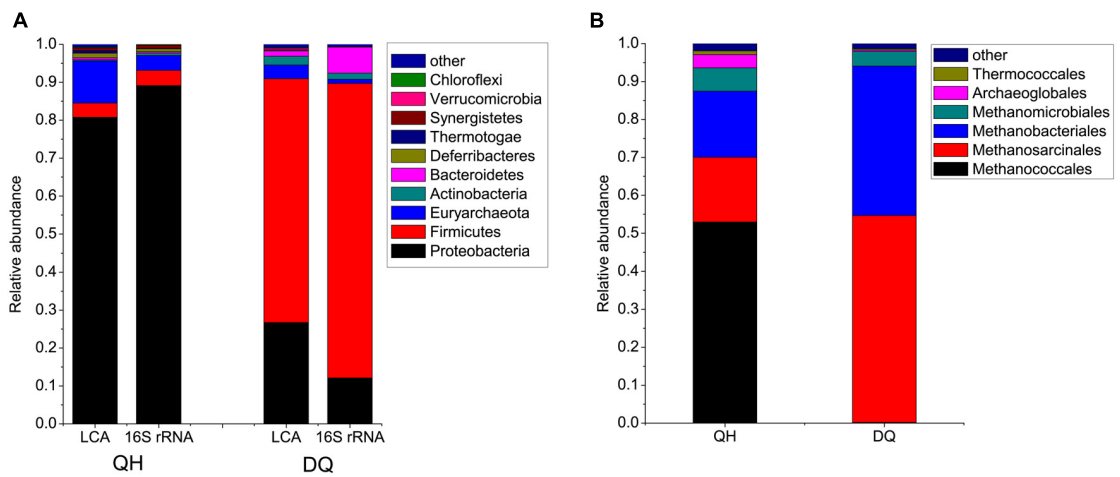

FIGURE 1 | Microbial communities in crude oil samples. (A) Microbial compositions at the phylum level. The phyla with average abundances in Qinghai (QH) and Daqing oilfield (D) Q more than 0.2% were shown, and the remaining phyla were combined as "other" in the figure. LCA, the reads were compared against the NR database by using BLASTX. The MEGAN software was used to parse the results of BLASTX and to estimate microbial composition and abundance. 16S rRNA, microbial compositions at the phylum level based 16S rRNA genes extracted from the reads and classified using RDP classifier. **(B)** Archaeal compositions at the order level.

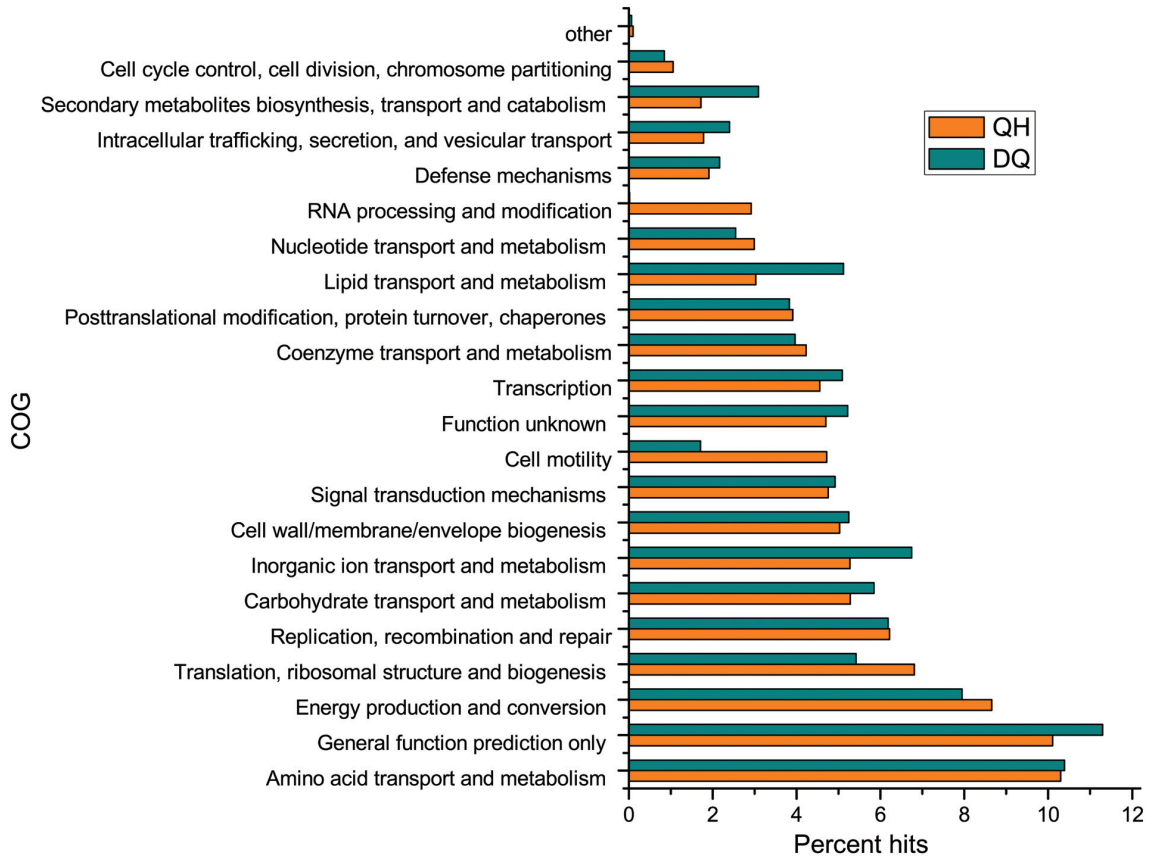

FIGURE 2 | Functional composition of the metagenomes from crude oil samples according to the clusters of orthologous groups (COG) database. Open reading frames (ORFs) of the assembled contigs were predicted using MetaGeneMark program and were compared against the COG database to assess the functional classification.

Euryarchaeota (3.6%), *Actinobacteria* (2.2%), and *Bacteroidetes* (1.2%) were also detected in QH metagenome. The DQ metagenome included methanogenic archaea such as acetoclastic *Methanosaeta* (Supplementary Figure S7; Supplementary Table S3). *Poribacteria* and *Fibrobacteres* were only detected in the DQ metagenome, and *Nanoarchaeota* were only detected in the QH metagenome; however, abundances of these bacteria were very low in both the metagenomes (Supplementary Table S2).

To confirm the results, the 16S rRNA genes were also identified from metagenome reads and classified using the RDP classifier. A total of 3,210 and 1,792 16S rRNA gene fragments were extracted from the reads from QH and DQ metagenomes, respectively. The results revealed that the taxonomic distributions were similar to those from BLASTX-based classification (**Figure 1**).

Genes Related to Petroleum Degradation Pathways in Oil Reservoirs

In all, 184,871 and 305,582 ORFs were found in the QH and DQ metagenomes, respectively. Of these, 54.9 and 60.0% ORFs, respectively, were assigned to COG database (**Figure 2**) and 34.3 and 34.8% ORFs, respectively, were assigned to KOs database (Supplementary Figure S9). Functional community profiles of the QH, DQ, NorOil1, and NorOil2 metagenomes were created based on KEGG annotations. KEGG annotations determined genes involved in complete hydrocarbon metabolism in both QH and DQ metagenomes; however, these genes showed remarkably different taxonomic affiliations. Both QH and DQ metagenomes contained high abundance of genes in lipid metabolism. For example, 129 and 395 genes for 3-oxoacyl-(acyl-carrier-protein) reductase (KO: K00059) were detected in the QH and DQ metagenomes, respectively. These genes were similar to sequences from *Pseudomonas* and *Marinobacter* in the QH metagenome and *Anoxybacillus* and *Pseudomonas* in the DQ metagenome. These data were consistent with the microbial taxonomic compositions of the two metagenomes. Both QH and DQ metagenomes contained glycosyltransferase genes essential for glycolipid and lipopeptide biosynthesis (Supplementary Tables S4 and S5). Moreover, both QH and DQ metagenomes contained high abundance of genes in lipid metabolism. For example, 129 and 395 genes for 3-oxoacyl-(acyl-carrier-protein) reductase (KO: K00059) were detected in the QH and DQ metagenomes, respectively. These genes were similar to sequences from *Pseudomonas* and *Marinobacter* in the QH metagenome and *Anoxybacillus* and *Pseudomonas* in the

DQ metagenome. These data were consistent with the microbial taxonomic compositions of the two metagenomes.

Alkane hydroxylases act in the first step of aerobic hydrocarbon degradation. Genes encoding alkane hydroxylase (EC: 1.14.15.3), alcohol dehydrogenase (EC: 1.1.1.1), and aldehyde dehydrogenase (EC: 1.2.1.3) were detected in both the metagenomes, indicating complete alkane degradation through β-oxidation pathway for fatty acid degradation. The QH and DQ metagenomes included ten and 46 genes encoding alkane 1-monooxygenase (AlkB; EC: 1.14.15.3), three and 66 genes encoding cytochrome P450 CYP153 family of alkane hydroxylases (EC: 1.14.15.3), 10 and 67 genes encoding long-chain alkane monooxygenase (LadA; Feng et al., 2007), and 36 and 149 genes encoding protein *n*-alkane metabolism A (AlmA; Throne-Holst et al., 2007), respectively (Supplementary Figures S10–S13). These results indicated that the DQ metagenome included more diverse genes encoding alkane hydroxylases than the QH metagenome. Genes encoding aerobic alkane hydroxylases were not detected in the NorOil metagenomes. Besides genes involved in aerobic alkane degradation, 16, 14, 18, and 18 genes encoding hydrocarbon succinate synthases (AssA/BssA) were present in the QH, DQ, NorOil1, and NorOil2 metagenomes, respectively. Proteins encoded by these genes activate anaerobic hydrocarbon degradation followed by benzoyl-CoA or fatty acid metabolism (Widdel and Rabus, 2001). Further, *assA*/*bssA* genes were detected with similarities to sequences from *Desulfotomaculum*, *Anaerofustis*, *Desulfatibacillum*, *Pelobacter*, *Bacteroides*, *Pseudomonas*, *Anaerobaculum*, *Spirochaeta*, and *Thermococcus* in the QH metagenome; *Desulfatibacillum*, *Desulfoglaeba*, *Desulfosporosinus*, *Desulfotomaculum*, *Anoxybacillus*, *Pseudomonas*, and *Sanguibacter* in the DQ metagenome; *Desulfovibrio*, *Pelobacter*, *Clostridium*, and *Thermovirga* in the NorOil1 metagenome; and *Desulfoglaeba*, *Desulfobacula*, *Geobacter*, *Azoarcus*, *Aromatoleum*, *Thauera*, and *Clostridia* in the NorOil2 metagenome (Supplementary Figure S14).

Bifunctional carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS, EC: 2.3.1-, 1.2.99.2, 1.2.7.4) is known as one of the key enzymes in Wood-Ljungdahl (W-L) pathway (Ragsdale, 2007). Here, we detected the genes encoding CODH/ACS in the QH and DQ metagenomes, to estimate the distribution of bacteria with W-L pathway and syntrophic acetate oxidation (SAO) potentials. In all, 79 and 60 reads matching the genes encoding CODH/ACS were detected in the QH and DQ metagenomes, respectively. Only 44 reads in the QH metagenome were assigned to classes *Clostridia*, *Deltaproteobacteria*, and *Synergistia* and 20 reads in the DQ metagenome were assigned to classes *Alphaproteobacteria*, *Deltaproteobacteria*, and unclassified *Poribacteria* (Supplementary Figure S15).

Clustering of Metagenomes of Oil Reservoirs

Taxonomic and functional compositions of QH, DQ, NorOil1, and NorOil2 metagenomes of oil reservoirs were compared. Bray–Curtis dissimilarity analysis indicated that the metagenomes of the four oil reservoirs (QH, DQ, NorOil1, and NorOil2) showed 58.4% average dissimilarity in taxonomic compositions and 32.2% average dissimilarity in functional compositions (KEGG). These results showed that despite large differences in taxonomic compositions across metagenomes of oil reservoirs, they shared many genes, especially enriched or reduced genes with the same or similar functions, from different species that constituted the core functions of these environments.

Hierarchical clustering of KO abundances was performed for metagenomes from the four oil samples and the 948 environmental metagenomes deposited in the IMG database (until October 2013). PCA analysis was performed using the KO abundances to analyze the relationship among the functional compositions of metagenomes from different environments, which also classified all the metagenomes into four groups according to their isolation environments (**Figure 3**). Notably, all the environmental metagenomes did not cluster according to their geographical locations but clustered according to their isolation environments (Supplementary Figure S16A), which formed four major clusters (cluster I to cluster IV). Generally, metagenomes from soil, freshwater, and marine samples were clustered, respectively, and were separated from the metagenomes from oil reservoirs and other extreme

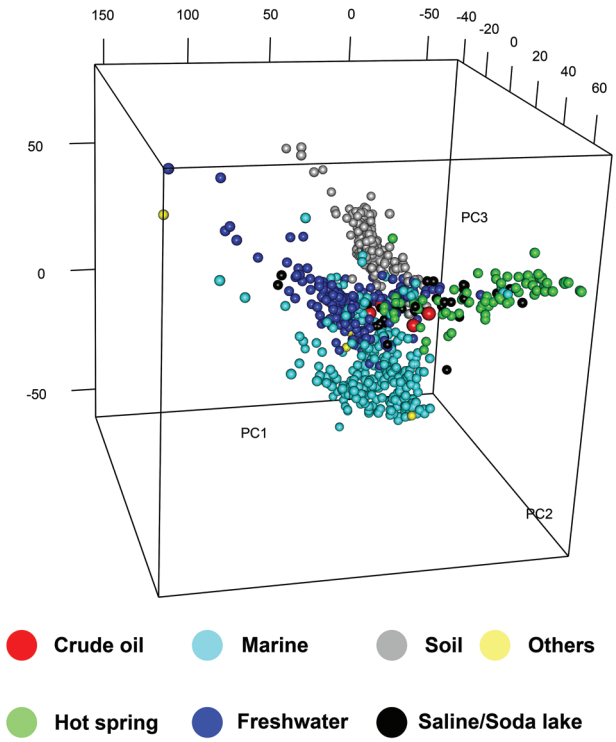

FIGURE 3 | Principal component analysis (PCA) of all the metagenomes based on the KO abundance. Four metagenomes from oil samples and the 948 environmental metagenomes deposited in the IMG database were used for PCA analysis using the KO abundances (For representative environmental samples, see Supplementary Tables S6–S9). Different colors denote different isolation environments.

environments (**Figure 3**; Supplementary Figure S16A). In cluster I, the metagenomes from the QH, DQ, NorOil1, and NorOil2 reservoirs were clustered with metagenomes from other extreme environments (Supplementary Table S6). Cluster II included metagenomes mainly from terrestrial soil environments (Supplementary Table S7). Cluster III included metagenomes mainly from marine environments (Supplementary Table S8) and cluster IV included metagenomes from freshwater environments (Supplementary Table S9). These different metagenome clusters showed different enriched functional genes and were viewed as four "hot" gene pools, denoted as groups A–D (Supplementary Figure S16B). In these gene groups, Groups A, B, C, and D corresponded to Clusters I, II, III, and IV, respectively, in the heat map (Supplementary Figure S16A). Of these enriched genes, genes in group A were associated with 43 pathways, including pathways of carbon metabolism, methane metabolism, amino acid biosynthesis (Supplementary Figure S16B; Supplementary Tables S10 and S11). For example, 146 KOs in group A (5.1% of the total KOs in group A) were assigned to the carbon metabolism pathway, including genes encoding CODH/ACS involved in W-L pathway. In contrast, other genes in carbon fixation, such as RuBisCO in Calvin cycle and ATP-citrate lyase in the reductive tricarboxylic acid cycle (Atomi, 2002), were less abundant in group A and in the metagenomes of oil reservoirs. We also identified pathways in which genes in groups A and B were both highly distributed. These genes were mainly related to nitrogen metabolism, two-component systems (TCSs), ABC transporters, and bacterial chemotaxis (Supplementary Figure S16B; Supplementary Table S11).

Specific Functional Profiles of Metagenomes of Crude Oil Reservoirs

We closely examined the enriched gene pool in group A. Methanogenesis-related functions were among the enriched pathways. In all, 93 KOs of methane metabolism were detected, constituting both complete hydrogenotrophic (KEGG module M00567) and acetoclastic (M00357) methanogenic pathways. The QH and DQ metagenomes included 78 and 27 reads, respectively, matching methyl-coenzyme M reductase alpha subunit (McrA), a key enzyme for methanogenesis and specific marker of methanogens (Luton et al., 2002; Supplementary Figure S17). *mcrA* genes similar to those from *Methanococcales* were dominant in the QH metagenome, accounting for 16.7% of the total *mcrA* genes. *Methanobacteriales* and *Methanosarcinales* similar *mcrA* genes were dominant in the DQ metagenome, accounting for 37.0 and 33.3% of the total *mcrA* genes, respectively. These results also showed that the DQ metagenome had more genes with similarities to sequences from *Methanosarcinales* and *Methanobacteriales*. Further, nitrogen fixation-related genes *nifDKH* (K02586, K02591, and K02588) were similar to the *nif* genes from methanogens such as *Methanococcales* (144/289 reads), *Methanobacteriales* (22/289 reads), and *Methanosarcinales* (19/289 reads) in the QH metagenome and *Methanobacteriales* (13/90 reads), *Methanomicrobia* (5/90 reads) in the DQ metagenome (Supplementary Figure S18).

In all, 15 complete TCSs such as PhoR-PhoB, KinB-AlgB, YesM-YesN, and VicK-VicR (Supplementary Table S12); 19 complete ABC transporter systems (Supplementary Table S13) were detected in the enriched KOs obtained from the QH and DQ metagenomes. TCSs are important for microbes to adapt to the extreme environment in oil reservoirs, which is anaerobic and hydrophobic, lacks phosphates and amino acids, and contains hydrocarbons as carbon sources and solvents. Genes of TCSs were similar to sequences from *Methanococcaceae*, *Methanosarcinaceae*, *Clostridiales*, *Proteobacteria*, and *Deferribacteres* in the QH metagenome and *Anoxybacillus* in the DQ metagenome. The complete ABC transporter systems in the metagenomes of the oil reservoirs included membrane transport for glutamine and cysteine. However, transporters for L-arabinose, lactose, and oligogalacturonide and for multiple sugars were absent.

Although not enriched, many genes related to bacterial osmotic stress responses, including those encoding transcriptional regulators and proteins involved in compatible solute transport and synthesis, were present in the metagenomes of the oil reservoirs (Supplementary Table S14), which was similar to that observed in other extreme environments.

DISCUSSION

Although oil reservoirs exhibit extreme conditions to support life, accumulating evidence has indicated that oil reservoirs are complex ecosystems comprising numerous microbes (Li et al., 2007; Dahle et al., 2008; Kaster et al., 2009). To adapt to these ecosystems, microbes have developed compatible functions such as enriched systems for carbon metabolism, methane metabolism, stress response, sensing, and transport. Enrichment of genes involved in carbon metabolism pathways might be because of the special requirements of carbon metabolism in these harsh environments. For example, although petroleum constituents are hydrophobic and toxic, they are the only potential organic carbon source for microbes in oil reservoirs and contribute to the enrichment of genes involved in both aerobic and anaerobic alkane degradation pathways, methane metabolism, TCSs, and ABC transporter systems. Both QH and DQ metagenomes contained genes essential for glycolipid and lipopeptide biosynthesis (Supplementary Tables S4 and S5), which could help bacteria to access hydrophobic oil constitutes (Wang X.B. et al., 2013). Enriched genes encoding AlkB, CYP153, LadA, and AlmA for aerobic alkane degradation were detected in the QH and DQ metagenomes but were absent in the NorOil1 or NorOil2 metagenomes. This might be because the DQ and QH reservoirs have been subjected to water flooding for more than 10 years. This may have provided small amount of oxygen through the injected water into the oil reservoirs and that may have stimulated aerobic alkane metabolism. Oxygen can also be provided by oxygen-bearing meteoric water, which results in the aerobic degradation of crude oil in shallow subsurface oil reservoirs

(Aitken et al., 2004). However, anaerobiosis is dominant in oil reservoirs (Head et al., 2003; Aitken et al., 2004; Dolfing et al., 2007; Jones et al., 2008). The metabolites such as organic acids generated during aerobic hydrocarbon degradation at the shallow subsurface could be further utilized in the deep surface of the oil reservoirs. Besides, anaerobic degradation of hydrocarbons in deep subsurface oil reservoirs are linked to methanogenesis via acetate or H_2/CO_2 (**Figure 4**; Dolfing et al., 2007). The acetate and H_2/CO_2 could be linked to several reactions and they might form a complex regulating network (**Figure 4**) which is influenced by factors such as H_2 and CO_2 concentration and temperature. SAO bacteria were thought using the reversal of W-L pathway to convert acetate to CO_2/H_2 that could be subsequently utilized by hydrogenotrophic methanogens (Hattori, 2008). Moreover, the enzyme activities in W-L pathway such as CO dehydrogenase, were detected in SAO bacteria (Hattori et al., 2005). The high abundance of hydrogenotrophic methanogens such as *Methanococcales*, *Methanobacteriales*, and *Methanomicrobiales* in the metagenomes of the oil reservoirs (**Figure 1**; Supplementary Figure S17), and the low concentration of detectable H_2 in oil reservoirs and microcosms of methanogenic hydrocarbon degradation (Mayumi et al., 2011) suggest a quick consumption of H_2 by hydrogenotrophic methanogens and low possibility of homoacetogenesis in oil reservoirs. The low concentration of H_2 could also benefit SAO bacteria (Dolfing et al., 2007; Kotlar et al., 2011), which convert acetate to H_2 and CO_2. Moreover, at high temperatures, acetoclastic methanogenesis becomes thermodynamically less favorable (Dolfing et al., 2007). Thus, at high temperatures, hydrogenotrophic methanogens is dominant. Therefore, hydrogenotrophic methanogens such as *Methanothermococcus* were dominant in the NorOil1 (at 85°C) and NorOil2 (at 83°C) metagenomes (Kotlar et al., 2011; Lewin et al., 2014), and the proportion of *Methanococcus* was higher in the QH metagenome while that of *Methanosaeta* was higher in the DQ metagenome. These results were consistent with those reported by Pham et al (Pham et al., 2009) who showed that acetoclastic *Methanosaeta* were dominant in some mesophilic oil reservoirs.

In contrast to the abundance of hydrocarbons, absence of saccharides in oil reservoirs should contribute to the low abundance of genes associated with sugar metabolism in the metagenomes of oil reservoirs. Although genes such as RuBisCO in Calvin cycle, and ATP-citrate lyase in the reductive tricarboxylic acid cycle were detected in low abundances, the detection of genes in WL pathway suggested the existing of chemoautotrophic microbes in oil reservoirs. Besides metabolism of hydrocarbons, this pathway could also provide organic carbon sources for other microbes. Furthermore, due to the absence of amino acids in oil reservoirs, microbes in these environments

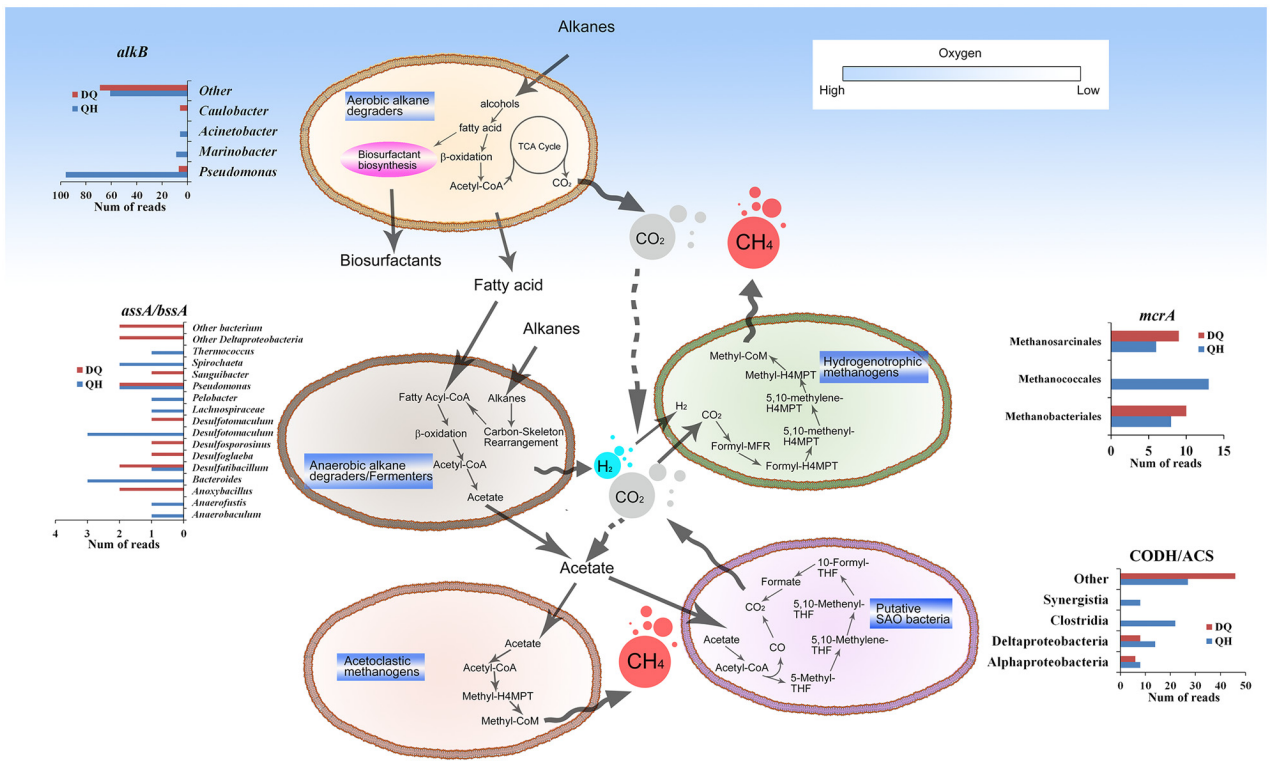

FIGURE 4 | General microbial metabolic pathways in the oil reservoirs. The pathways in the oil reservoirs were reconstructed by functional genes annotation. Briefly, hydrocarbons were oxidized aerobically in the shallow subsurface oil reservoirs or anaerobically in the deep subsurface oil reservoirs. The metabolites such as acetate and H_2 and were linked to methanogenesis from CO_2 reduction, acetoclastic methanogenesis, or syntrophic acetate oxidation (SAO). The genes involved in the pathways were similar with sequences from different taxa, the abundances of which were listed aside the pathway.

evolved effective systems to synthesize and transport amino acids for protein translation, as observed in group A (cluster I, Supplementary Figure S16A). Interestingly, *nif* was mainly enriched in methanogens in group A. It was not unusual because such genes have also been detected in some methane seep and consuming environments (Dekas et al., 2009; Miyazaki et al., 2009). Nitrogen-fixing archaea in these environments might use nitrogen fixation to overcome the scarcity of bioavailable nitrogen and may serve as the source of nitrogen for microbial communities (Dekas et al., 2009). Due to the low concentration of ammonia in oil reservoirs, it was suggested the important role of methanogens in the nitrogen cycle in oil reservoirs.

Besides hydrocarbon metabolism-related genes, microbes in oil reservoirs have developed special systems to adapt to crude oil habitats, to survive, to obtain nutrients and metabolize it, and to escape toxic compounds and high osmosis. For example, enriched VicR/VicK TCS, which mediates exopolysaccharide biosynthesis and biofilm formation (Fabret and Hoch, 1998; Winkler and Hoch, 2008), and LiaS/LiaR TCS, which provides resistance against organic solvents (Mascher et al., 2004), are beneficial for microbes to mitigate the impact of osmosis from petroleum hydrophobicity and to promote the survival of microbial communities. AtoS/AtoC can induce the bacterial growth in short-chain fatty acids, and positively regulate the levels of poly-(R)-3-hydroxybutyrate (PHB; Kyriakidis and Tiligada, 2009). PHB is a member of the polyhydroxyalkanoate (PHA) which serves as the intracellular carbon and energy storage compounds and enhances the survival of bacteria, usually when an essential nutrient such as nitrogen or phosphorus is limited in the presence of excess carbon source (Anderson and Dawes, 1990). These functions are important for microbes to survive in extreme environments of oil reservoirs, where the organic carbon sources, i.e., hydrocarbons are abundant, but the bioavailable nitrogen and phosphorus are limited.

High functional similarity observed among the metagenomes of the oil reservoirs was also observed among those of other environments. Functional profiles of all the 948 environmental metagenomes deposited in the IMG database (until October 2013) showed that metagenomes from soil, freshwater, marine, and extreme environments had their own special functions, irrespective of their geographical location (**Figure 3**; Supplementary Figure S16). This was consistent with the results of recent studies, which showed that microbes in similar environments selected similar functions to adapt to their surroundings (Nemergut et al., 2013). For example, higher availability of aromatic compounds such as aromatic carboxylic humic acids in soil (Van Trump et al., 2011) plays an important role in enriching genes involved in aerobic degradation of aromatic compounds (group D; Supplementary Figure S16B). In marine environments, high concentrations of nitrate and nitrite in euphotic and mesopelagic zones below the nitracline (Shi et al., 2010) and extreme oligotrophy in deep sea (Smedile et al., 2014) are important for enriching genes involved in nitrogen metabolism, bacterial chemotaxis, and flagellar assembly (group B; Supplementary Table S11). Therefore, it is not surprising that many marine bacteria are motile, with the proportion of motile cells being as high as 20–60% (Stocker, 2012). Consequently, specific environmental factors to the four environments resulted in the selection of characteristic microbial functions and clustering of metagenomes from these environmental (**Figure 3**). Similarly, genes related to methane metabolism were enriched in the metagenomes of the oil reservoirs. This was consistent with the finding that methanogenic anaerobic degradation of crude oil is an important process in subsurface petroleum reservoirs, which distinguishes oil reservoirs from other environments (Jones et al., 2008).

Microbial compositions at the taxonomic level varied remarkably among oilfields. Microbial compositions of the metagenomes from offshore oil reservoirs of Norwegian Sea (NorOil1 and NorOil2; Kotlar et al., 2011; Lewin et al., 2014) which were located in the same geographical region but were physically separated, were remarkably different from the metagenomes of the QH and DQ reservoirs. *Delta/Epsilon-proteobacteria* and methanogens were abundant in the NorOil1 metagenome (Kotlar et al., 2011). However, archaea were the most dominant microbes in the NorOil2 metagenome (Lewin et al., 2014). In contrast, *Pseudomonas* and *Anoxybacillus* were dominant in the QH and DQ, respectively (**Figure 1**). Microbial compositions at the taxonomic level were remarkably different even between these oil reservoirs. However, functional compositions, i.e., metabolic pathways and functional genes, were common among geographically distant oil reservoirs and extreme environments such as hot spring and soda lakes. Bray–Curtis dissimilarity analysis also confirmed the results that despite large differences in taxonomic compositions across metagenomes of oil reservoirs, they shared many genes, especially enriched or reduced genes with the same or similar functions, from different species. These phenomena may be attributed to the mechanisms underlying the assembly of microbial communities. The popular niche theory suggests that the structure of a microbial community is shaped mainly by factors such as environmental conditions, competition, and niche differentiation. Environment and microbial interactions dominate this process (Ofiteru et al., 2010). In contrast, neutral theory proposes that species in environments are ecologically equivalent and that the structures of microbial communities are determined randomly (Woodcock et al., 2007). However, these two theories can only explain a part of our results. The niche theory explained the selection of similar microbial functions by similar environments (**Figure 3**) but could not explain the remarkable difference in taxonomic microbial compositions. In contrast, the neutral theory explained the variation in the distribution of microbes at the taxonomic level among different oil reservoirs, which was consistent with the stochastic model of community assembly. However, this theory could not explain the clustering of samples from four typical environments. Divergence of species and convergence of functional compositions observed in the metagenomes of the oil reservoirs suggested that the assembly and structure of bacterial communities may not be based on species but may be based on functional genes determined by the respective environments, which was consistent with the findings of a previous study (Burke et al., 2011).

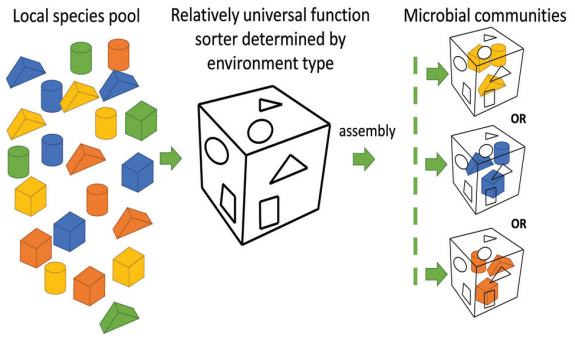

FIGURE 5 | Microbial community assembly in a shape sorter manner. Right-shaped blocks (genes with similar functions), despite their color (different taxa), have equal possibility of passing the shape filter (the characteristic of an environment) and getting into the sorter, microbes with genes that match the environmental functional filters, irrespective of their taxa, can be selected stochastically.

In this aspect, the assembly of microbial communities in oil reservoirs was affected by the effects of the deterministic assembly processes in a "shape-sorting" manner based on the functional compositions. Like a shape sorter that contains different shape filters and that only allows blocks with the correct shape to pass (**Figure 5**), an environment with characteristic functional filters acts as a sorter of functions. This sorter, which is determined by the environment (niche theory) and which is a characteristic of that environment type, is used to select microbes from nearby pools. Similar to the game in which right-shaped blocks, despite their color, have equal possibility of passing the shape filter and getting into the sorter, microbes with genes that match the environmental functional filters, irrespective of their taxa, can be selected stochastically (neutral theory). Next, these randomly selected successful microbes outcompete other microbes, colonize the environment, and occupy the niche. Therefore, different microbial species with similar properties or functions can randomly occupy the same niche in an ecosystem. Like oil reservoirs, soil, freshwater, marine, and extreme environments have their specific environmental factors and form four types of sorters with environment-specific functional filters for selecting genes.

Although the shape sorter equally allows the right-shaped blocks with different colors to pass through, colors with more number of blocks have higher possibilities to get into the shape sorter. Similarly, although the functional sorter is environment determined and is relatively universal within an environment type, microbes close to the sorter may be endemic because of their biogeographic patterns, especially at a larger scale (Martiny et al., 2006). Therefore, endemic differences in microbial composition should play a role in the assembly of microbial communities by offering endemic pools of diverse microbial candidates for selection. Thus, the shape-sorting system is comprehensively determined by the niche and neutral theories, local legacies of historical microbial events on a large scale, and differences among different microbial taxa. For example, crude oil has its own functional sorting system that selects specific functions from different taxa. However, further research is needed to test this hypothesis.

In summary, we characterized microbial functional gene compositions in crude oil from two geographically distant oil reservoirs (QH and DQ) in China and found that these genes clustered with genes from extreme environments but not with genes from soil, freshwater, and marine environments. Although microbial compositions were taxonomically different between oil reservoirs with different geochemical conditions and between different wells from the same oilfield with similar geochemical conditions, functional gene compositions in the metagenomes of these oil reservoirs showed high similarities. The preserved common complete methanogenic hydrocarbon degradation pathways and enriched stress response genes were the key factors for distinguishing the metagenomes of oil reservoirs and extreme environments from those of soil, freshwater, and marine environments. These results suggested that microbial communities in oil reservoirs with consistent functional profiles are functionally selected by the niche theory but taxonomically selected by the neutral theory. Moreover, all the environmental metagenomes were clustered according to their isolation environments based on the hierarchical clustering of functional genes, suggesting that both selective and neutral processes were involved in the assembly of microbial communities in oil reservoirs.

AUTHOR CONTRIBUTIONS

YN and J-YZ contributed equally to this work. YN, X-LW, and FZ designed the research. YN, J-YZ, and Y-QT performed research. J-YZ re-analyzed the datasets, and revised the manuscript. PG and YY contributed analytic tools. YN, J-YZ, YY, X-LW, and FZ analyzed data. YN, J-YZ, YY, and X-LW wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01254

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Metagenomic Analysis Indicates *Epsilonproteobacteria* as a Potential Cause of Microbial Corrosion in Pipelines Injected with Bisulfite

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Sodium bisulfite (SBS) is used as an oxygen scavenger to decrease corrosion in pipelines transporting brackish subsurface water used in the production of bitumen by steam-assisted gravity drainage. Sequencing 16S rRNA gene amplicons has indicated that SBS addition increased the fraction of the sulfate-reducing bacteria (SRB) *Desulfomicrobium*, as well as of *Desulfocapsa*, which can also grow by disproportionating sulfite into sulfide, sulfur, and sulfate. SRB use cathodic H_2, formed by reduction of aqueous protons at the iron surface, or use low potential electrons from iron and aqueous protons directly for sulfate reduction. In order to reveal the effects of SBS treatment in more detail, metagenomic analysis was performed with pipe-associated solids (PAS) scraped from a pipe section upstream (PAS-616P) and downstream (PAS-821TP) of the SBS injection point. A major SBS-induced change in microbial community composition and in affiliated *hynL* genes for the large subunit of [NiFe] hydrogenase was the appearance of sulfur-metabolizing *Epsilonproteobacteria* of the genera *Sulfuricurvum* and *Sulfurovum*. These are chemolithotrophs, which oxidize sulfide or sulfur with O_2 or reduce sulfur with H_2. Because O_2 was absent, this class likely catalyzed reduction of sulfur (S^0) originating from the metabolism of bisulfite with cathodic H_2 (or low potential electrons and aqueous protons) originating from the corrosion of steel (Fe^0). Overall this accelerates reaction of of S^0 and Fe^0 to form FeS, making this class a potentially powerful contributor to microbial corrosion. The PAS-821TP metagenome also had increased fractions of *Deltaproteobacteria* including the SRB *Desulfomicrobium* and *Desulfocapsa*. Altogether, SBS increased the fraction of hydrogen-utilizing *Delta-* and *Epsilonproteobacteria* in brackish-water-transporting pipelines, potentially stimulating anaerobic pipeline corrosion if dosed in excess of the intended oxygen scavenger function.

Keywords: corrosion, pipeline, microbiologically influenced corrosion (MIC), microbial community analysis, metagenomics, sulfate reducing bacteria (SRB), *Epsilonproteobacteria*, hydrogenase

INTRODUCTION

Pipeline failure caused by corrosion can have serious consequences for the oil and gas industry (Ossai et al., 2015) and understanding the causes of corrosion is therefore important. While chemical reaction between oxygen and iron is the main cause of external pipeline corrosion, microbiologically influenced corrosion (MIC) under mostly anoxic conditions can account for up to 40% of internal pipeline corrosion in the oil and gas industry (Zhu et al., 2003). The application of high-throughput sequencing technologies has indicated that diverse microbes are involved in internal pipeline corrosion, such as sulfate reducing bacteria (SRB), acid-producing fermentative bacteria, including acetogens, as well as methanogens (Dinh et al., 2004; Park et al., 2011; Mand et al., 2014; Okoro et al., 2014; Yang et al., 2014). Among these SRB are often considered to be the major MIC causative agents.

Fundamentally all SRB can corrode iron indirectly by producing the corrosive chemical agent hydrogen sulfide (H_2S). This has been referred to as "chemical microbially-influenced corrosion" (CMIC) by Enning and Garrelfs (2014). H_2S is produced during sulfate reduction by SRB with electrons usually derived from organic acids, alcohols, or hydrogen (H_2), which is formed by fermentation of organic compounds in anoxic settings (Muyzer and Stams, 2008). In the absence of organic electron donors and in the presence of metallic iron, SRB may obtain energy from oxidation of cathodic H_2 formed by chemical reaction between protons from water and electrons from anodic dissolution of iron, accelerating corrosion (Mand et al., 2014). Whether SRB are capable of accelerating corrosion by scavenging cathodic H_2, which was proposed long ago (Von Wolzogen Kühr and Van der Vlugt, 1934), is still controversial (Enning and Garrelfs, 2014). Instead, some SRB are thought to corrode iron through direct uptake of the anodic electrons with protons from water for sulfate reduction ("electrical microbially-influenced corrosion"; EMIC; Enning and Garrelfs, 2014).

The microbial consumption or production of H_2 is catalyzed by hydrogenases that can be divided into two main phylogenetically unrelated groups, the [NiFe]- and [FeFe]-hydrogenases (Vignais and Billoud, 2007). [NiFe]-hydrogenases are common in *Archaea* and *Bacteria*. Many are external to the cytoplasm and are primarily associated with H_2 oxidation in oxic and anoxic metabolism. [FeFe]-hydrogenases are found in *Bacteria* and *Eukarya*. These enzymes are especially prevalent in the cytoplasm of anaerobic fermentative organisms (e.g., the *Firmicutes*), where they form hydrogen in metabolic reactions coupling the oxidation of reduced electron carriers (NADH, NADPH or reduced ferredoxin, Fd_{red}) to the reduction of protons. SRB of the genus *Desulfovibrio* are exceptional in having a periplasmic [FeFe]-hydrogenase, which functions in hydrogen oxidation at high hydrogen concentration (Caffrey et al., 2007). Hence, assuming that consumption of cathodic hydrogen is important in MIC, both types of hydrogenases can contribute. However, [NiFe]-hydrogenases may be more important than [FeFe] hydrogenases because these enzymes are more widespread and act at lower hydrogen concentrations. Likewise, if metabolism of anodic electrons and aqueous protons in EMIC involves formation of H_2 both types of enzymes may contribute. In addition to SRB, hydrogenotrophic methanogens and acetogens have been found to contribute to MIC by using cathodic H_2 or anodic electrons for reduction of CO_2 to methane and acetate, respectively (Dinh et al., 2004; Mand et al., 2014).

As indicated previously (Park et al., 2011), the presence of MIC-causing SRB can be promoted by injection of sodium bisulfite (SBS), which is used as an oxygen scavenger to decrease oxygen-mediated corrosion in pipelines and other steel infrastructure. Injection of SBS into pipelines transporting brackish subsurface water to a plant generating steam for production of bitumen by steam-assisted gravity drainage caused a drastic change in microbial community composition of pipe-associated solids (PAS). Relative to solids from a pipe section upstream of the SBS injection point (PAS-616P), solids from a downstream pipe section (PAS-821TP) had a smaller fraction of methanogens of the family *Methanobacteriaceae* and larger fractions of SRB of the genera *Desulfomicrobium* and *Desulfocapsa* (Park et al., 2011). *Desulfocapsa* can also grow by disproportionating bisulfite into sulfide and sulfate (Finster, 2008). Here we evaluate the genetic potential of the microbial communities in these two PAS samples in more detail by an in depth metagenomic analysis with a focus on hydrogenase genes.

MATERIALS AND METHODS

Sample Collection

Two cutouts from a brackish water-transporting pipeline system were collected upstream (616P) and downstream (821TP) from the SBS injection point. These were the same as described elsewhere (Park et al., 2011). The pipeline cutouts were immersed in pipe-associated water (PAW) from the site, were shipped in sealed, airtight buckets and received in the lab within 24 h. The cutouts and the associated waters were then immediately transferred to a Coy anaerobic hood with an atmosphere of 90% (v/v) N_2 and 10% CO_2. PAS-616P and PAS-821TP were obtained by scraping the drained surface of the cutouts with a sterile spatula. These were then re-suspended in 260 mL of PAW-616P and PAW-821TP, respectively, filtered using an 0.2 μm Millipore filter (Nylon membrane, USA) prior to use.

Chemical analyses conducted on the samples included the measurement of pH, sulfide (Trüper and Schlegel, 1964), sulfate (ion chromatography with conductivity detector/anion column), ammonium, nitrite (ion chromatography with UV detector/anion column), and organic acids (ion chromatography with UV detector/organic acids column), as detailed else where (Park et al., 2011).

DNA Isolation

DNA was extracted from the PAS samples using a bead-beating procedure outlined by the manufacturer of the FastDNA® Spin Kit for Soil (MP Biomedicals). The extracted DNA was further purified by cesium chloride density gradient centrifugation. The concentration of DNA was quantified using the Qubit Fluorometer, and Quant-iT™ dsDNA HS Assay Kit (Invitrogen). A total of 20.5 and 25.8 μg of CsCl-purified DNAs were obtained from PAS-821TP and PAS-616P, respectively. The purified DNAs

were then used for pyrosequencing of 16S rRNA gene (16S) amplicons and for metagenome sequencing.

Pyrosequencing of 16S Amplicons

Amplification of 16S genes was with non-barcoded 16S primers 926Fw (AAACTYAAAKGAATTGRCGG) and 1392R (ACGGGCGGTGTGTRC) in the first PCR and with FLX titanium amplicon primers 454_RL_X and 454T_FwB in the second PCR. The latter primers have the sequences for 926Fw and 1392R as their 3′ ends. Primer 454T_RL_X has a 25 nucleotide A-adaptor (CCATCTCATCCCTGCGTGTCTCCGAC) and a 10 nucleotide multiplex identifier barcode sequence X. Primer 454T_FwB has a 25 nucleotide B-adaptor sequence (CTATGCGCCTTGCCAGCCCGCTCAG). The first PCR was run for 5 min at 95°C, followed by 25 cycles of 30 s at 95°C, 45 s at 55°C, and 90 s at 72°C and finally 10 min at 72°C. The PCR products were used as templates for a second PCR of 10 cycles under the same conditions. PCR products were checked on an agarose gel and purified with a QIAquick PCR Purification kit (Qiagen). The amounts of purified 16S amplicons were then normalized to 20 μl of 20 ng/μl and sent for pyrosequencing to the Genome Quebec and McGill University Innovation Centre (Montreal, QC). Pyrosequencing was performed in a Genome Sequencer FLX Instrument, using a GS FLX Titanium Series Kit XLR70 (Roche Diagnostics Corporation). The 16S sequence reads were analyzed with Phoenix 2 (Soh et al., 2013).

Metagenome Sequencing

Metagenome sequencing was performed with both 454- and Illumina-platforms at the Genome Quebec and McGill University Innovation Centre. For the 454 sequencing, single-end shotgun DNA libraries were prepared from 1.0 μg of purified DNA and sequenced with the 454 Life Sciences GS-FLX genome sequencer using titanium chemistry and standard library construction procedures (Roche Applied Science, Laval, Quebec, Canada).

For the Illumina sequencing, DNA libraries were prepared using the TruSeq DNA Sample Prep Kit v1 (Illumina, San Diego, CA) as per the manufacturer's instructions starting with 2 μg of purified DNA. The libraries were loaded onto the flow cell, one per lane using a cBot (Illumina). Sequencing to obtain paired end 150 bp reads was performed on a HiSeq 2000 instrument (Illumina) as per the manufacturer's instructions.

Metagenomic Analysis and Assembly

All 454 and Illumina metagenomic raw reads were subjected to quality control (QC) and an assembly process using the in-house developed software described by Saidi-Mehrabad et al. (2013) and Tan et al. (2013). Ribosomal RNA genes were identified with Meta-RNA (Huang et al., 2009) from 454 QC reads. All raw 454 and Illumina sequence data were submitted to the Short Read Archive under accession numbers SRX559897, SRX559898, SRX559901, and SRX559902.

Gene Abundance Analysis

Genes for large hydrogenase subunits (referred to as *hydL* for [FeFe]-hydrogenase and *hynL* for [NiFe] hydrogenase) and the house keeping gene *rpoB*, the gene for the RNA polymerase β subunit, were sought in the six-frame translated metagenomic contigs using hmmsearch with a cutoff *e*-value of e-5. The *hydL* (PF02906) and *hynL* (PF00374) hidden Markov models (HMMs) were downloaded from the Pfam database (Finn et al., 2014) and the *rpoB* (TIGR02013, TIGR03670) HMMs were downloaded from TIGRFAMs (Haft et al., 2013). In the aligned region, the translated amino-acid sequences and the corresponding nucleotide sequences were extracted and included in the further gene abundance and phylogenetic analyses.

The QC metagenomic reads were mapped against the *hydL*, *hynL,* and *rpoB* nucleotide gene sequences using bowtie2 (Langmead and Salzberg, 2012) and the mapping files were processed using Picard with "MarkDuplicates" options (http://broadinstitute.github.io/picard/) to remove PCR duplicates. The BEDTools (Quinlan and Hall, 2010) and in-house perl scripts were then used to calculate coverage information for the extracted genes. The coverages were subsequently normalized to the length of the respective genes to make sure that longer genes did not have higher coverage values just because of their length. The length-normalized coverages were then further normalized to the total number of *rpoB* genes in the metagenomic samples, yielding an approximation of the number of genes per genome for each extracted hydrogenase genes.

Phylogenetic Analysis

Phylogenetic trees were constructed for *hynL* genes obtained from the PAS-616P and PAS-821TP metagenomes with a multi-step approach using reference alignments and trees in order to minimize errors and biases introduced by the fragmentary and non-overlapping nature of the metagenomic sequences (Brazelton et al., 2012). The reference multiple sequence alignment was constructed from multiple sequence alignments generated in other work (Vignais et al., 2001) by using mafft version 7.245 with "–merge" option (Katoh and Standley, 2013). Unaligned metagenomic fragments were added to the reference alignment profile using mafft with "–add" option in order to avoid alteration of the relative positions of residues in the reference alignment. Next, a bootstrapped maximum-likelihood tree was constructed from the reference-only alignment using the "-f a" algorithm in RAxML version 8.2.3 (Stamatakis, 2014). The reference-only tree with the highest-likelihood was used as a constraint tree ("-r" flag in RAxML) for 100 inferences from the full alignment (including metagenomic fragments) by RAxML, and bootstrap support values were drawn on the highest-likelihood tree (Brazelton et al., 2012).

RESULTS

Chemical Characteristics of Pipe Samples

The pipe associated water (PAW) of the 616P and 821TP cutouts had a neutral pH and a sulfate concentration of 0.01 mM (Table S1). No sulfide was observed in either water sample. Higher concentrations of sulfate were observed in suspended PAS samples PAS-616P (0.33 mM) and PAS-821TP (0.09 mM). Passage through the SBS injection point increased the sulfide concentration from 0 mM in PAS-616P to 90 mM in PAS-821TP and the ferrous iron concentration from 1.0 mM

in PAS-616P to 1080 mM in PAS-821TP (Table S1). Acetate (0.85 mM), butyrate (0.20 mM), and propionate (0.17 mM) were detected in PAW-616P, but not in PAS-616P. Acetate (0.80 mM) and propionate (0.10 mM) were also detected in PAS-821TP, but not in PAW-821TP (Table S1). These data indicate that the chemical compositions of PAS-616P and PAS-821TP were different (reflecting SBS injection) and that these differed in turn from those of the corresponding planktonic samples.

Community Compositions from 16S Amplicon Sequencing

The 454 pyrosequencing platform produced 20,451 good 16S reads for the two PAS samples (**Table 1**). These were grouped into 141 operational taxonomic units (OTUs) at a sequence dissimilarity cut-off of 5%. The average Good's coverage of 99.7% suggested that the majority of the phylotypes present in the samples had been identified. A total of 76 taxa was found in the two samples. The microbial community diversity of PAS-616P was slightly lower than that of PAS-821TP, as reflected by the estimated OTUs (Chao) and Shannon index (**Table 1**). PAS-616P had 75% *Archaea* and 20% *Deltaproteobacteria*, whereas PAS-821TP had 50% *Archaea* and 46% *Deltaproteobacteria* (**Table 1**). The increase in *Deltaproteobacteria*, ferrous iron, and sulfide from PAS-616P to PAS-821TP, likely resulted from SBS injection.

Methanogenic archaea can contribute to MIC in anoxic pipeline systems together with SRB and acetogens. Methanogenic orders in the two PAS samples included the hydrogenotrophic *Methanobacteriales* and *Methanomicrobiales*, as well as the *Methanosarcinales*. This included the *Methanobacteriaceae* (68.5 and 48.2%), *Methanocalculus* (0.05 and 0.01%), and the methylotrophic genus *Methanolobus* (6.4 and 1.6%) in PAS-616P to PAS-821TP, respectively (Table S2).

Five *Deltaproteobacterial* orders, *Desulfovibrionales*, *Desulfobacterales*, *Desulfuromonadales*, *Myxococcales,* and *Syntrophobacterales*, were detected in the 16S amplicons (**Figure 1A**). Of these the first two represent potential SRB. *Desulfomicrobium* was the dominant genus within the *Desulfovibrionales* with proportions of 14.1 and 28.9% in PAS-616P and PAS-821TP amplicon libraries, respectively (Table S2). *Desulfomicrobium* species use hydrogen or simple organic compounds, including formate, ethanol, lactate, pyruvate, malate, or fumarate, as electron donors for sulfate respiration. However, in the absence of sulfate they grow fermentatively on some of these same organic substrates (Rabus et al., 2015). Within the *Desulfobacterales*, *Desulfocapsa* was the dominant taxon with proportions of 5.3 and 16.3% in PAS-616P and PAS-821TP, respectively (Table S2). A unique feature of this genus is its ability to grow by disproportionation of thiosulfate, sulfite, or sulfur (in the presence of ferric iron) in the absence of molecular hydrogen. In the presence of molecular hydrogen, reduction of sulfur and of sulfur oxyanions dominates over disproportionation (Finster, 2008). *Desulfuromonadales* of the genus *Desulfuromonas* were detected at 0.14 and 0.01% in the PAS-616P and PAS-821TP amplicons, respectively (Table S1).

Hence a survey of the 16S amplicons of the CsCl-purified DNAs confirmed earlier findings (Park et al., 2011), which indicated a decrease in methanogenic taxa and an increase in sulfate- and sulfite-reducing and sulfite-disproportionating taxa when passing from upstream to downstream of the SBS injection site.

Community Compositions from Metagenomic 16S Genes

Metagenome sequencing of the PAS samples was performed using both 454 and Illumina platforms. Because of the limitation of fragment sizes in Illumina sequencing, only the 454 16S rRNA gene sequences were used for the analysis of taxonomic profiles of the two metagenomes. A total of 270,379 and 337,937 reads with mean lengths of 659 and 654 bp were obtained for the PAS-616P and PAS-821TP metagenomes, respectively (**Table 2**). Among these 470 and 703 reads were identified as 16S fragments in the PAS-616P and PAS-821TP metagenomes (**Table 2**). This corresponds to one 16S read per 575 and 480 total reads, respectively. Taxonomic assignments of these 16S reads revealed that *Deltaproteobacteria* (58.3%) and *Archaea* (27.9%) dominated in the PAS-616P metagenome (**Table 2**). A larger fraction of *Deltaproteobacteria* (76.1%) and smaller fraction of *Archaea* (3.4%) was found in the PAS-821TP metagenome (**Table 2**). Hence these changed similarly as observed for the 16S amplicon libraries (**Table 2**).

Methanobacteriales (24.3%) and *Methanosarcinales* (3.6%) were the dominant methanogenic orders in the PAS-616P metagenome, while only *Methanobacteriales* (3.4%) were identified in the PAS-821TP metagenome (**Figure 1**). These included the *Methanobacteriaceae* and the genus *Methanolobus* (Table S2). The SRB were represented by the orders *Desulfovibrionales* (40.2%), *Desulfobacterales* (17.4%), and *Desulfuromonadales* (0.2%) in the PAS-616P metagenome, whereas only *Desulfovibrionales* (50.4%) and *Desulfobacterales* (23.8%) were detected in the PAS-821TP metagenome (**Figure 1B**). *Desulfomicrobium* and *Desulfocapsa* were the dominant genera in the orders *Desulfovibrionales* and *Desulfobacterales* in these two metagenomes (Table S2).

The frequency of 16S genes representing *Epsilonproteobacteria* increased from the PAS-616P to the PAS-821TP metagenome from 0.64 to 5.6% (**Table 2**). This increase was also seen in the 16S amplicon libraries (**Table 2**: from 0.02 to 0.19%). The smaller values of these fractions reflect the fact that the primers used did not optimally amplify this class (An et al., 2013). The *Epsilonproteobacteria* consisted mainly of the genera *Sulfuricurvum* (0.43%) and *Arcobacter* (0.21%) in the PAS-616P metagenome and of *Sulfuricurvum* (4.4%) and *Sulfurovum* (0.85%) in the PAS-821TP metagenome (Table S2). *Sulfuricurvum* and *Sulfurovum* are known to oxidize sulfide to sulfate (Kodama and Watanabe, 2004). This group of mainly chemolithotrophic bacteria may also reduce sulfur (S^0) to sulfide using H_2 as the electron donor (Gevertz et al., 2000).

Abundance of Hydrogenase Genes in PAS Metagenomes

Determining the prevalence of the *hynL* and *hydL* genes for the large subunits of [NiFe]- and [FeFe]-hydrogenase indicates the genetic potential of microbial communities for consuming or

TABLE 1 | Summary of 16S amplicon sequence data and derived diversity parameters for CsCl-purified DNAs from PAS samples also used for metagenomic analysis.

Sample name	Number of QC reads[a]	Archaea (%)	Delta[b] (%)	Observed OTUs (5% cutoff)	Estimated OTUs (Chao)[c]	Shannon's H index[c]	Good's coverage (%)	Number of taxa
PAS-616P	10,772	75	20	99	128	1.28	99.7	59
PAS-821TP	9679	50	46	96	155	1.44	99.6	53
Combined	20,451	63	32	141	ND	ND	99.7	76

[a] *The accession numbers for the PAS-616P and PAS-821TP reads are SRX1427962 and SRX1430039, respectively.*
[b] *Delta is Deltaproteobacteria.*
[c] *Diversity indices were calculated using a normalized number of 9679 reads.*

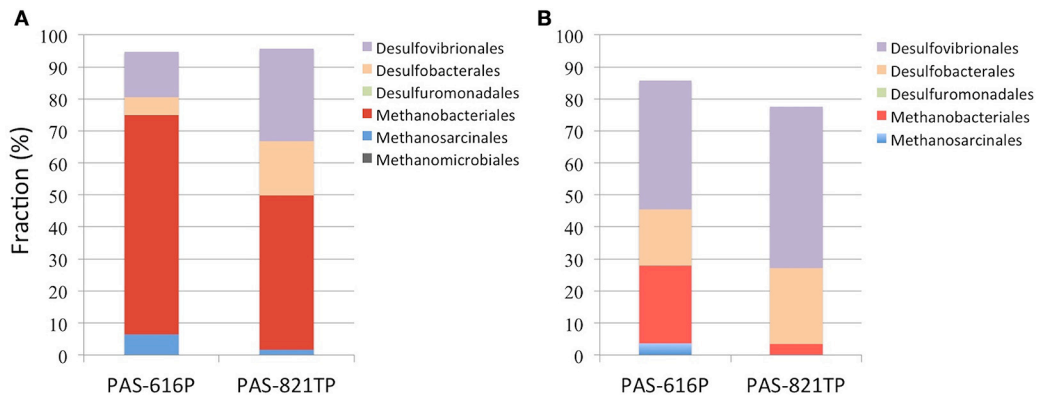

FIGURE 1 | Order-level composition of *Archaea* and *Deltaproteobacteria* derived from (A) 16S amplicons and (B) 16S sequences of 454 metagenomes. The deltaproteobacterial orders *Myxococcales* and *Syntrophobacterales* are not obvious due to their low abundance in both libraries. The increased fractions of archaeal orders in **(A)** are likely caused by PCR bias.

producing H_2. In order to assess the effect of SBS treatment in the brackish water-transporting pipeline system on this potential, we performed hmmsearches for potential homologs of the *hynL* and *hydL* genes against contigs of the merged 454 and Illumina assemblies of the PAS-616P and PAS-821TP metagenomes. The aligned region of assembled contigs was extracted and the unassembled metagenomic reads that passed the QC were then mapped. To get the abundance profile of either hydrogenase gene, the total length of the mapped reads was calculated and normalized by the length of the respective genes. For comparative purposes, the length-normalized coverage was further normalized by dividing by the total number of *rpoB* genes (43,059 in the PAS-616P and 35,808 in the PAS-821TP metagenomes), yielding the fraction of genes per genome (abundance) for each of the extracted hydrogenase gene types (**Table 3**). The hydrogenase gene sequences, obtained from hmmsearches, were taxonomically identified as the best hit in blastp searches of the NCBI database. The phylogenetic association of these genes is also given as relative abundance (**Table 3**: %).

The PAS-616P and PAS-821TP metagenomes for samples taken upstream and downstream of the SBS injection point in the pipeline system, respectively, had a 10-fold higher abundance of *hynL* genes for [NiFe]-hydrogenase (**Table 3**: 0.505 and 0.532) than of *hydL* genes for [FeFe]-hydrogenase (0.044 and 0.057). The abundance of both types of hydrogenase genes was similar in the PAS-616P and PAS-821TP metagenomes (**Table 3**). The phylogenetic association of *hydL* genes was similar with most (**Table 3**: 64 and 51%) belonging to the *Firmicutes*, consistent with a function in H_2 production in both metagenomes. In contrast, archaeal *hynL* genes decreased from 22.0 to 7.3%, whereas deltaproteobacterial *hynL* genes

TABLE 2 | Summary of 454 metagenomic data obtained for CsCl purified DNAs from the PAS samples.

Parameter	Metagenome		16S amplicon	
	PAS-616P	PAS-821TP	PAS-616P	PAS-821TP
Reads	270,379	337,937	10,772	9679
16S rRNA (>180 bp)	470	703	10,772	9679
Archaea	27.87	3.41	74.99	49.86
Bacteria	72.13	93.88	25.01	50.14
Actinobacteria	0.85	0	0.31	0.05
Bacteroidetes	0.85	1.00	0.21	0.37
Chloroflexi	5.32	3.56	1.61	1.44
Firmicutes	1.06	1.56	0.21	0.07
Deltaproteobacteria	58.30	76.10	19.84	45.90
Epsilonproteobacteria	0.64	5.55	0.02	0.19
Spirochaetes	0.21	0.28	0.07	0.27

Fractions (%) of taxa at the kingdom, phylum or class level from metagenomic 16S reads are compared with those obtained from 16S amplicon sequencing.

TABLE 3 | Taxonomic origin and abundance of *hydL* genes for [FeFe]-hydrogenase and of *hynL* genes for [NiFe]-hydrogenase in the PAS metagenomes.

Gene and taxon	PAS-616P	(%)	PAS-821TP	(%)
hydL for [FeFe]-hydrogenase	0.044	100.00	0.057	100.00
Bacteroidetes	0.003	7.49	0.011	19.10
Deltaproteobacteria	0.006	13.04	0.006	10.68
Firmicutes	0.028	64.35	0.029	50.88
Spirochaetes	0.002	3.92	0.004	6.31
hynL for [NiFe]-hydrogenase	0.505	100.00	0.532	100.00
Actinobacteria	0.008	1.68	0.002	0.34
Archaea	0.111	21.98	0.039	7.38
Bacteroidetes	0.008	1.52	0.010	1.87
Betaproteobacteria	0.008	1.68	0.001	0.28
Chloroflexi	0.014	2.72	0.007	1.36
Deltaproteobacteria	0.279	55.19	0.321	60.45
Epsilonproteobacteria	0.000	0.06	0.041	7.63
Firmicutes	0.001	0.24	0.003	0.57
Gammaproteobacteria	0.013	2.51	0.011	2.16

The numbers are fractions relative to those for rpoB[1]. The fractions (%) of the total of these are also given.

[1]A total of 43,059 and 35,808 rpoB gene fragments were detected in the merged 454 and Illumina assemblies.

increased from 55.2 to 60.4% in transitioning from the PAS-626P to the PAS-821TP metagenome (**Table 3**). These changes were similar to those observed for metagenome-derived 16S (**Table 2**). Bisulfite injection caused a large increase in *hynL* genes affiliated with *Epsilonproteobacteria* from 0.06 to 7.63% (**Table 3**), matching the increase in metagenome-derived 16S (from 0.64 to 5.6%). This indicates this class to have a strong potential for H_2 oxidation.

Phylogeny and Functional Diversity of *hynL* Genes

hynL genes for [NiFe]-hydrogenase detected in the PAS metagenome assemblies were compared with nearest homolog sequences from the literature in phylogenetic trees, in which genes with high similarity were collapsed into the same clade. Four groups of *hynL* genes, representing [NiFe]-hydrogenases with distinct physiological roles (Vignais et al., 2001), were all observed in the PAS-616P and PAS-821TP trees (**Figures 2**, **3**). Their phylogenetic distribution among *Deltaproteobacteria* (mostly SRB), *Archaea* (mostly methanogens), and *Epsilonproteobacteria* (mostly sulfur-metabolizing bacteria; SMB) is indicated in Table S3.

Group_1 includes the H_2-oxidizing membrane-bound [NiFe] hydrogenases, most of which leave protons on the external side of the membrane while transporting electrons to an internally located *b*-type cytochrome. Group_1 [NiFe] hydrogenases were represented in the PAS-616PL tree in clades 1-1 to 1-20 (**Figure 2**). Nine of these harbored multiple contigs (indicated in brackets) with clades 1-12_Desulfocapsa_(13), 1-15_Desulfomicrobium_(19), and

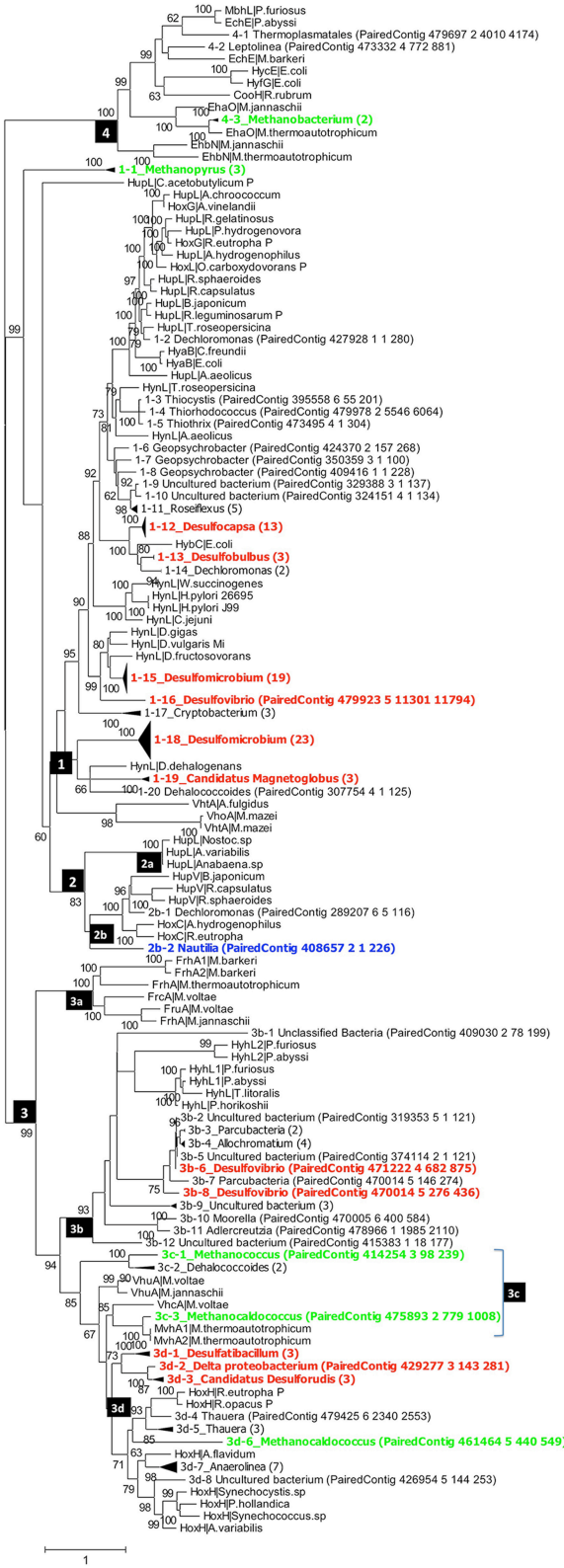

FIGURE 2 | Phylogenetic analysis of putative *hynL* genes for the large subunit of [NiFe]-hydrogenase detected in the PAS-616P metagenome. Contigs with high similarity and the same taxonomic origin were collapsed into,

(Continued)

FIGURE 2 | Continued
the same clade and the number of contigs in each clade is indicated in brackets. Clades for putative methanogens, SRB, and chemolithoautotrophic SMB are highlighted in green, red, and blue. Maximum-likelihood bootstrap support values of more than 60 are shown. The numbers in the black boxes are different types of [NiFe] hydrogenases, identified by Vignais et al. (2001) as 1, 2 (2a, 2b), 3 (3a, 3b, 3c, 3d), and 4.

1-18_Desulfomicrobium_(23) being most heavily populated. The abundance (the number of sequences divided by 43,059 observed *rpoB* sequences) of Group_1 clades was 0.365 with *Deltaproteobacteria hynL* genes (0.283) outnumbering those for *Archaea* (0.062). No Group_1 *hynL* genes for *Epsilonproteobacteria* were observed (Table S3). Group_1 *hynL* genes were represented in the PAS-821TP tree in clades 1-1 to 1-18 of which eight harbored multiple contigs (**Figure 3**), including 1-6_Desulfocapsa_(8), 1-7_Desulfobulbus_(2), 1-11_Desulfomicrobium_(18), and 1-17_Desulfomicrobium_(16) for the *Deltaproteobacteria*, 1-14_Methanopyrus_(3) for the *Archaea* and 1-8_Sulfuricurvum_(14) for the *Epsilonproteobacteria*. Relative to the PAS-616P metagenome, the PAS-821TP metagenome had a decreased abundance of *hynL* genes from *Archaea* (Table S3: 0.018) and an increased abundance of *hynL* genes from *Delta-* and *Epsilonproteobacteria* (Table S3: 0.363 and 0.034). The latter included *Sulfurovum*, in addition to the more dominant *Sulfuricurvum*. Filamentous *Gammaproteobacteria*, potentially oxidizing sulfide or sulfur (Chernousova et al., 2009; van der Meer et al., 2010), were observed in both metagenomes, i.e., 1-2_Thiothrix and 1-3_Thiothrix in the PAS-821TP metagenome. Hence, the continuous injection of bisulfite decreased the presence of archaeal *hynL* genes, while increasing *hynL* genes for sulfur-metabolizing *Epsilonproteobacteria*, which may use Group_1 [NiFe]-hydrogenases to oxidize H_2 for reduction of sulfur (Campbell et al., 2006; Peters et al., 2015).

Group_2a including cyanobacterial [NiFe]-hydrogenases (Peters et al., 2015) was not significantly present. Group_2b includes regulatory [NiFe] hydrogenases, which can induce expression of *hyn* genes, when H_2 is present. These were represented in a single clade 2b-1_Sulfuricurvum_(3) in the PAS-821TP metagenome, suggesting that expression of Group_1 *hyn* genes in *Epsilonproteobacteria* is regulated. The abundance of regulatory Group_2b *hynL* genes was lower than of Group_1 *hynL* genes (Table S3: 0.006 and 0.036, respectively).

Group_3a [NiFe]-hydrogenases are cytoplasmic enzymes involved in reduction of coenzyme F420 in methanogenic *Archaea*. This group was found in low abundance in the PAS-821TP metagenome (Table S3). Group_3b [NiFe]-hydrogenases had a diverse phylogenetic distribution, which included the SMB *Allochromatium* (both metagenomes) and the SRB *Desulfovibrio* (PAS-616P only). Group_3c includes methyl viologen-reducing [NiFe]-hydrogenases (MvhAgD), which function in the reduction of heterodisulfide with H_2 in methanogens (Peters et al., 2015). This group included *hynL* genes from *Methanocaldococcus* (**Figures 2**, **3**) and was more abundant in the PAS-616P than in the PAS-821TP

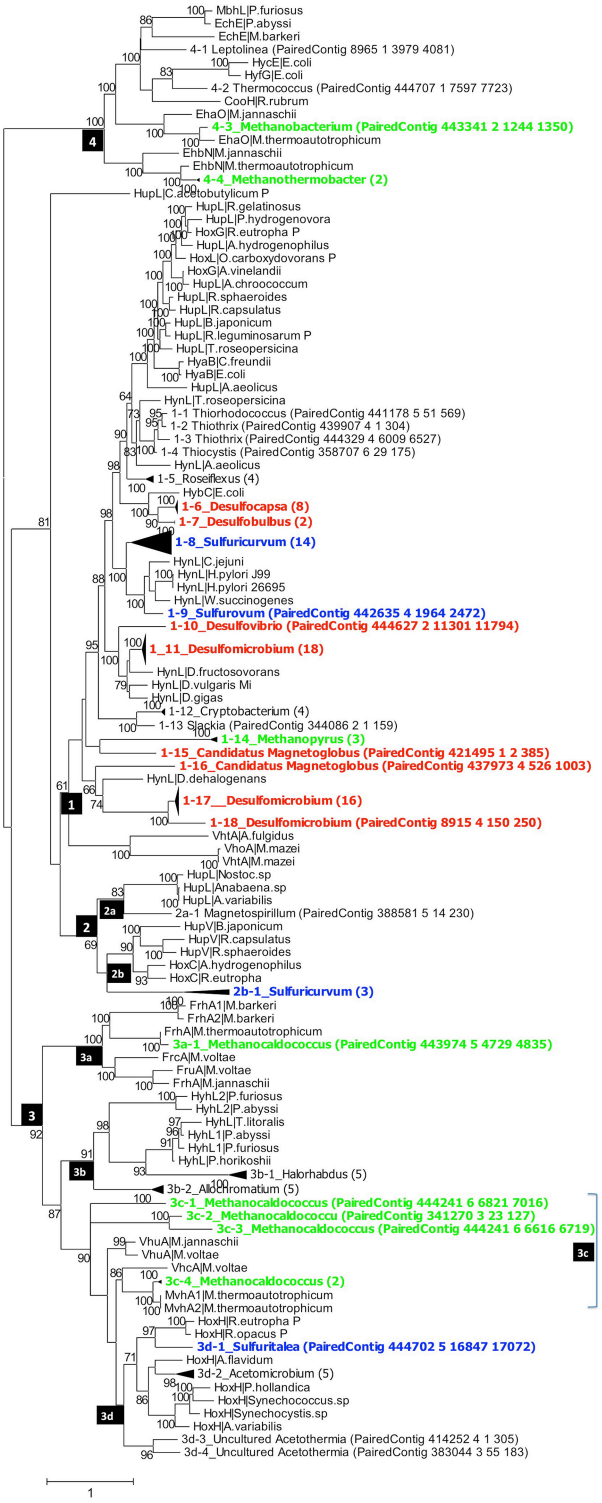

FIGURE 3 | Phylogenetic analysis of putative *hynL* genes for the large subunit of [NiFe]-hydrogenase detected in the PAS-821TP metagenome. Contigs with high similarity and same taxonomic origin were collapsed into the same clade and the number of contigs in each clade is indicated in brackets. Clades for putative methanogens, SRB, and *(Continued)*

FIGURE 3 | Continued
chemolithoautotrophic SMB are highlighted in green, red, and blue. Maximum-likelihood bootstrap support values of more than 60 are shown. The numbers in the black boxes are different types of [NiFe] hydrogenases, identified by Vignais et al. (2001) as 1, 2 (2a, 2b), 3 (3a, 3b, 3c, 3d), and 4.

metagenome (Table S3: 0.026 and 0.012, respectively). Group_3d includes bidirectional heteromultimeric [NiFe]-hydrogenases (HoxHY), which associate with NADH dehydrogenase for energy generation in aerobic bacteria (Peters et al., 2015). The *hydL* genes from the *Betaproteobacteria Sulfuritalea* in the PAS-821TP metagenome also belonged to this group (**Figure 3**: 3d-1_Sulfuritalea). In the PAS-616P metagenome Group_3d included *Firmicutes,* such as *Candidatus Desulforudis*.

The [NiFe] hydrogenases included in group 4 are typically responsible for cytoplasmic, flavodoxin-dependent H_2 production in *Bacteria,* and methanogenic *Archaea*. The latter were represented through clade 4-3_Methanobacterium in both metagenomes. The abundance of Group_4 *hynL* genes of the genus *Methanobacterium* decreased in transitioning from the PAS-616P to the PAS-821TP metagenome (Table S3). This genus includes hydrogenotrophic methanogens with potential for iron corrosion (Dinh et al., 2004).

DISCUSSION

The continuous injection of bisulfite into a pipeline system transporting brackish water with little sulfate (**Table 1**), but high concentrations of bicarbonate (0.5–1.5 g/L; Park et al., 2011) had a significant effect on microbial community composition of the pipe wall. Analysis of metagenomic 16S reads, obtained by 454 sequencing, indicated that SBS decreased the fraction of methanogenic *Archaea* (*Methanobacteriaceae* and *Methanolobus*), but increased the fraction of *Deltaproteobacteria* (*Desulfomicrobium* and *Desulfocapsa*) and *Epsilonproteobacteria* (*Sulfuricurvum* and *Sulfurovum*), as indicated in **Table 2** and Table S2.

The more extensive dataset on the distribution of *hydL* and *hynL* genes in metagenomic reads, obtained by 454 and Illumina sequencing, confirmed the shift in taxonomic distribution seen in 454 16S reads while allowing more detailed assigment of taxa involved in H_2 metabolism. First of all *hydL* genes for [FeFe]-hydrogenase were approximately ten-fold less frequent than *hynL* genes for [NiFe] hydrogenases. Most *hydL* genes were affiliated with *Firmicutes* (**Table 3**), which were only a minor fraction (**Table 2**: 1.1–1.6%) in both metagenomes. We focused, therefore, on analysis of the phylogenetic distribution of *hynL* genes. This includes *Archaea*, which have *hynL* but do not have *hydL* genes, but may exclude *Firmicutes*, i.e., only 0.24–0.57% of all *hynL* genes was affiliated with this phylum.

Injection of bisulfite decreased the abundance of *hynL* genes from methanogenic *Archaea* of Group_1 (0.062–0.018, *Methanopyrus* in both metagenomes), Group_3c (0.026–0.012) and Group_4 (0.023–0.006), while increasing the abundance of *hynL* genes from *Deltaproteobacteria* of Group_1 (0.283–0.363), but not of Group_3b (0.006–0) and Group_3d (0.020–0). Group_1 *hynL* genes were mostly affiliated with the genera *Desulfomicrobium* and *Desulfocapsa* in both metagenomes. Other Group_1 *hynL* genes found in both metagenomes included those from *Candidatus Magnetoglobus*, which are affiliated with the *Deltaproteobacteria* class and may also be SRB (Abreu et al., 2007). Group_1 *hynL* genes from *Dechloromonas* and *Dehalococcoides*, which use H_2 for inorganic or organic dechlorination, disappeared upon bisulfite injection (**Figures 2**, **3**).

Injection of bisulfite strongly increased the abundance of Group_1 *hynL* genes from the *Epsilonproteobacteria Sulfuricurvum* and *Sulfurovum* (from 0 to 0.034) and of Group_2b *hynL* genes from *Sulfuricurvum.* This class also includes the phylogenetically related genera *Sulfurimonas*, *Sulfurospirillum*, *Campylobacter,* and *Arcobacter*, as well as the more distantly related *Nautilia* (Han et al., 2012). Most *Epsilonproteobacteria* are chemolithoautotrophs, which oxidize reduced sulfur species (sulfide, sulfur, and thiosulfate) with O_2 (under microaerophilic conditions) and nitrate (Kodama and Watanabe, 2004; Campbell et al., 2006). Many also reduce sulfur with H_2 (Campbell et al., 2006), a reaction first documented for *Arcobacter* strain FWKO_B (Gevertz et al., 2000). This is in contrast to *Betaproteobacteria* of the genus *Sulfuritalea* (**Figure 3**: 3d-1), which oxidize both H_2 and reduced sulfur species with O_2 and/or nitrate, but do not reduce sulfur with H_2 (Kojima and Fukui, 2010). Because O_2 is absent following SBS injection, Group_1 [NiFe]-hydrogenase of the *Epsilonproteobacteria* likely functioned in reduction of sulfur with H_2 with the Group_2b regulatory proteins ensuring that the enzyme is expressed under these conditions. The Group_2b HoxBC [NiFe] hydrogenase sensor was first discovered in *Alcaligenes eutrophus* (now *Ralstonia eutrophus*), where HoxBC functions in H_2 recognition and transmits the signal to HoxJ, which modulates response regulator HoxA to activate transcription of Group_1 *hyn* genes (Lenz and Friedrich, 1998).

As indicated previously (Park et al., 2011), injection of bisulfite removes O_2 by chemical reaction:

$$HSO_3^- + ½O_2 \rightarrow H^+ + SO_4^{2-} \quad (1)$$

Both bisulfite (when dosed in excess) and sulfate can be reduced by SRB of the genus *Desulfomicrobium*, using cathodic H_2 (or low potential electrons) from steel (Fe^0) as the electron donor:

$$Fe^0 + 2H^+ \rightarrow Fe^{2+} + H_2 \quad (2)$$

$$HSO_3^- + 3H_2 \rightarrow HS^- + 3H_2O \quad (3)$$

$$SO_4^{2-} + H^+ + 4H_2 \rightarrow HS^- + 4H_2O \quad (4)$$

Alternatively, HSO_3^- is disproportionated into sulfide or sulfur and sulfate by *Deltaproteobacteria* of the genus *Desulfocapsa*:

$$4HSO_3^- \rightarrow HS^- + 3SO_4^{2-} + 3H^+ \quad (5)$$

$$3HSO_3^- \rightarrow S^0 + 2SO_4^{2-} + H_2O + H^+ \quad (6)$$

Assuming little H_2 production by fermentation of organics, the balance between reduction (Reactions 2 and 3) and disproportionation (Reactions 5 and 6) will depend on the availability of cathodic hydrogen (or of low potential electrons in an EMIC scenario). The generated sulfur (S^0) is then used by *Epsilonproteobacteria*, which were clearly stimulated by injection of bisulfite and which may also use cathodic H_2:

$$S^0 + H_2 \rightarrow HS^- + H^+ \quad (7)$$

Reactions (2) and (7) can be combined with formation of FeS from Fe^{2+} and HS^- to:

$$Fe^0 + S^0 \rightarrow FeS \quad (8)$$

which indicates that *Epsilonproteobacteria* may gain energy for growth by accelerating the reaction between metallic iron and elemental sulfur, which would take place more slowly in their absence. Both FeS and S^0 were significant components of the pipeline scale downstream from the SBS injection point (Park et al., 2011). The transported brackish water had little organic carbon (Table S1; Park et al., 2011), which could have served as an alternative electron donor for sulfur reduction. Indeed, *Deltaproteobacteria* of the genus *Desulfuromonas*, which specialize in this activity, were found in low fractions (Table S2: up to 0.14%) and decreased upon SBS injection.

Hence, the metagenomic studies presented here have uncovered a potential role for *Epsilonproteobacteria* in MIC in pipelines subjected to injection of bisulfite for the scavenging of oxygen, which is commonly used. Clearly injection of excess bisulfite should be avoided and the emergence of *Epsilonproteobacteria*, which were nearly absent upstream from the SBS injection point, may serve as an indicator of increased MIC threat.

AUTHOR CONTRIBUTIONS

DA: Contributed to design of the study and isolation and purification of DNA for 16S amplicon and metagenomic analysis, performed data analysis of 16S amplicons and metagenomes, interpretation of data, and drafting the paper. XD: Conducted metagenomic data analysis which included processing of QC and assembly of reads, generating gene abundance, and phylogeny trees. AA: Participated in data analysis of 16S amplicons and drafting the paper. HP: Contributed to sample collections. MS: Contributed to metagenomic data analysis. GV: Contributed to design of the study, interpretation of data, drafting the paper, final approval of the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00028

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Use of Homogeneously-Sized Carbon Steel Ball Bearings to Study Microbially-Influenced Corrosion in Oil Field Samples

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Microbially-influenced corrosion (MIC) contributes to the general corrosion rate (CR), which is typically measured with carbon steel coupons. Here we explore the use of carbon steel ball bearings, referred to as beads (55.0 ± 0.3 mg; Ø = 0.238 cm), for determining CRs. CRs for samples from an oil field in Oceania incubated with beads were determined by the weight loss method, using acid treatment to remove corrosion products. The release of ferrous and ferric iron was also measured and CRs based on weight loss and iron determination were in good agreement. Average CRs were 0.022 mm/yr for eight produced waters with high numbers (10^5/ml) of acid-producing bacteria (APB), but no sulfate-reducing bacteria (SRB). Average CRs were 0.009 mm/yr for five central processing facility (CPF) waters, which had no APB or SRB due to weekly biocide treatment and 0.036 mm/yr for 2 CPF tank bottom sludges, which had high numbers of APB (10^6/ml) and SRB (10^8/ml). Hence, corrosion monitoring with carbon steel beads indicated that biocide treatment of CPF waters decreased the CR, except where biocide did not penetrate. The CR for incubations with 20 ml of a produced water decreased from 0.061 to 0.007 mm/yr when increasing the number of beads from 1 to 40. CRs determined with beads were higher than those with coupons, possibly also due to a higher weight of iron per unit volume used in incubations with coupons. Use of 1 ml syringe columns, containing carbon steel beads, and injected with 10 ml/day of SRB-containing medium for 256 days gave a CR of 0.11 mm/yr under flow conditions. The standard deviation of the distribution of residual bead weights, a measure for the unevenness of the corrosion, increased with increasing CR. The most heavily corroded beads showed significant pitting. Hence the use of uniformly sized carbon steel beads offers new opportunities for screening and monitoring of corrosion including determination of the distribution of corrosion rates, which allows estimation of the probability of high rate events that may lead to failure.

Keywords: microbially influenced corrosion, weight loss, carbon steel, coupons, beads, sulfate-reducing bacteria, methanogens, acetogens

INTRODUCTION

Corrosion of carbon steel is caused by physical, chemical, and microbiological factors (Beech and Sunner, 2004; Gieg et al., 2011; Enning and Garrelfs, 2014). It is a serious and expensive problem, which can lead for instance to failure of pipelines. When corrosion is uneven localized pits can form. These can be narrow and deep or shallow and wide (https://www.nace.org/Pitting-Corrosion/) and may eventually span the entire pipeline wall causing a failure. Because pitting corrosion is unpredictable pipelines must be regularly inspected to identify sections of decreased wall thickness, which may have experienced pitting corrosion. The surrounding areas may then be subjected to excavation to replace the corroded sections and prevent a future leak event. In the laboratory and in the field flat metal coupons are typically used to evaluate general and pitting corrosion by metal weight loss and by surface examination of the coupons, respectively. This allows definition of the pitting factor (P) as the ratio of the corrosion rate of the deepest pit (mm/yr) divided by the general corrosion rate (mm/yr).

The contribution of microbes to corrosion is often referred to as microbially-influenced corrosion (MIC). Although a wide variety of microbes may contribute the sulfate-reducing bacteria (SRB) are considered the main culprits. SRB can contribute to chemical MIC (CMIC) by reducing sulfate to sulfide using organic electron donors (organic acids, alcohols, or oil components), as shown for lactate in Equation 1. The produced sulfide can then react with carbon steel to form hydrogen (Equation 2), which is then also used by SRB as electron donor for sulfate reduction (Equation 3):

$$2CH_3CHOHCOO^- + SO_4^{2-} + H^+ \rightarrow 2CH_3COO^- + 2CO_2 + HS^- + 2H_2O \quad (1)$$

$$H^+ + HS^- + Fe^0 \rightarrow FeS + H_2 \quad (2)$$

$$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O \quad (3)$$

Some SRB may be capable of directly using electrons derived from Fe^0 (Equation 4) or using these through a H_2 intermediate (Equations 3, 5):

$$4Fe^0 + SO_4^{2-} + 9H^+ \rightarrow HS^- + 4Fe^{2+} + 4H_2O \quad (4)$$

$$4Fe^0 + 8H^+ \rightarrow 4Fe^{2+} + 4H_2 \quad (5)$$

The latter is the classical mechanism through which SRB were thought to act (Von Wolzogen Kühr and Van der Vlugt, 1934). However, recent evidence has been presented that some SRB may be able to catalyze direct electron transfer from Fe^0, as per Equation 4 (Dinh et al., 2004; Enning et al., 2012). This mechanism has been referred to as electrical MIC (EMIC). EMIC electron transport from Fe^0 to cells may include electron-conductive corrosion products, e.g., FeS (Enning et al., 2012). Other hydrogenotrophic microbes, such as methanogens or acetogens may also contribute to MIC (Daniels et al., 1987; Dinh et al., 2004; Mand et al., 2014) through direct electron transfor or hydrogen intermediate mechanisms.

In studying the mechanism of corrosion or the corrosivity of field samples we have found that the use of carbon steel ball bearings (carbon steel beads) offers advantages for measuring general corrosion rates, because of their small size and therefore small weight variation. These beads can be used during incubations in serum bottles with or without shaking or can be packed in tubing or small columns for study of corrosion under flow conditions. Methods and results for these applications are presented here.

MATERIALS AND METHODS

Carbon Steel Beads and Coupons

Beads of a36 carbon steel (Thomson Precision Ball, Rockwell hardness C60 to C67, Grade 200, $\emptyset = 3/32 = 0.0938$ in; tolerance $\pm$ 0.001 in) were purchased from Motion Canada in Calgary, Alberta, Canada. Because of their use as ball bearings these are very similarly sized (55.0 $\pm$ 0.3 mg, $\emptyset = 0.238 \pm 0.001$ cm, A = 0.178 cm^2). Coupons of ASTM a366 carbon steel (2.0 $\times$ 1.0 $\times$ 0.1 cm) were cut at the Engineering Workshop of the University of Calgary. Beads and coupons were pretreated according to National Association of Corrosion Engineers (NACE) protocol RP0775-2005. This involved briefly sanding the coupons with grit size 400 sand paper. Beads were rolled between two sheets of grit size 400 sand paper for 1–2 min. Coupons or beads were then treated for 2 min with inhibited HCl and then for 2 min with 1.2 M $NaHCO_3$. The beads and coupons were washed with deionized water (dH_2O), then with acetone and allowed to dry. Inhibited HCl was made by dissolving 10.6 g/L of dibutylthiourea in 37% (w/w) HCl and diluting with an equal volume of dH_2O. The weight of the pretreated beads or coupons was then determined thrice and the average value was used as the starting weight. The combined weight of the pretreated beads was determined prior to incubation. Individual bead weights prior to incubation were assumed to be same as the average.

Following the use of beads or coupons in a corrosion experiment these were again treated with NACE protocol RP0775-2005 to remove adhering corrosion products. Following treatment with inhibited HCl and with $NaHCO_3$ and washing with dH_2O and with acetone, the dried beads were rolled between two Kimwipes (Kimtech Science Model S-8115) for 1 min to remove remaining loosely-associated corrosion product. The combined weight of coupons or beads was then determined thrice and the average used to calculate the weight loss (ΔW in g). The corrosion rate CR (mm/yr) was then calculated as:

$$CR = 87,600 \times \Delta W/(DxAxT) \quad (6)$$

Where D is the density of carbon steel (7.85 g/cm^3), A is the surface area of the coupon or of the beads and T is the incubation time in h. The factor 87,600 converts the measured CR from cm/h to mm/yr. Weights of individual beads were also determined to characterize the change in weight distribution following corrosion.

Physical, Chemical, and Microbial Characterization of Field Samples

Samples were obtained from an oil field in Oceania in December 2013 and January 2014 (2013/2014; **Table 1**) and in December 2014/January 2015 (2014/2015; **Table 2**). A schematic of the field indicating three distinct groups of samples is shown in **Figure 1**. The field has an *in situ* temperature of 70°C and produces light oil with an American Petroleum Institute (API) gravity of 40°. Samples of liquids were collected in 1 L glass or polyethylene containers, filled to the brim to exclude air as much as possible, whereas samples of solids were collected in Ziploc bags. Samples were stored and shipped at ambient temperature. Average transport time from the date of collection to arrival at the University of Calgary (UofC) was 2 weeks. Samples were stored in a Coy anaerobic hood in an atmosphere of 90% (v/v) N_2 and 10% (v/v) CO_2 (N_2-CO_2) immediately following arrival. This included samples of produced water (PW, which contained some produced oil), samples of central processing facility (CPF) water and of injection water (CPW and IW, which were oil free) and samples of solids and sludges (SS), retrieved from tank bottoms or from pipelines as pigging solids. Extracts from SS samples were made by contacting 15 g of SS with 15 ml of dH_2O in the anaerobic hood, vortexing for 5 min and allowing the solids to settle by gravity. The supernatant was used as the SS extract.

Twenty one water samples (1 L) were also obtained from two fields in NE British Columbia and NW Alberta in which coiled tubing (CT) was used for removal of fracturing plugs during completion of horizontal wells for shale gas production operations.

Samples were subjected to general physical and chemical characterization. This included determination of the pH and of the salinity in molar equivalent of NaCl (Meq) with an Orion pH meter (model 370) and using the same meter with an Orion conductivity cell (model 013005MD), respectively. The concentration of dissolved sulfide was measured with the diamine method (Trüper and Schlegel, 1964). The concentrations of sulfate and acetate were determined by high performance liquid chromatography (HPLC). Sulfate was analyzed by ion chromatography using a conductivity detector (Waters 423) and IC-PAK anion column with borate/gluconate buffer at a flowrate of 2 ml/min (4 × 150 mm, Waters). Acetate was determined using an HPLC equipped with a UV detector (Waters 2487 Detector) and an organic acids column (Alltech, 250 × 4.6 mm) eluted with 25 mM KH_2PO_4 buffer at pH 2.5.

Most probable numbers (MPNs) of lactate-utilizing SRB and of glucose-fermenting acid-producing bacteria (APB) were determined from triplicate dilution series using 48-well microtiter plates (Shen and Voordouw, 2015). For MPNs of SRB, 0.1 ml of sample was inoculated into 0.9 ml of Postgate B medium, and then serially diluted 10-fold to 10^{-8} in the same medium in triplicate wells. The plate was immediately covered with a Titer-Tops membrane and incubated at 32°C inside the anaerobic hood. Wells were scored as positive when a black FeS precipitate was evident. For MPNs of APB, the sample was serially diluted in Phenol Red Dextrose medium (ZPRA-5, DALYNN Biologicals, Calgary, Alberta, Canada) using the same procedure as described for SRB. Growth of APB results in a color change of the medium from red to orange/yellow. MPN values were calculated by comparing the positive pattern to a probability table for triplicate MPN tests.

Comparison of Corrosion Rates by Weight Loss and Iron Dissolution

In addition to measuring corrosion rate by weight loss, it was also measured as the release of ferrous iron measured spectrophotometrically with the ferrozine assay. Acid (1 N HCl) was used to dissolve ferrous iron from corrosion products, typically FeS and $FeCO_3$, through a series of timed assays. Samples (20 ml) were placed in 50 ml serum bottles together with 20 acid pre-treated iron beads (1102 mg, 3.56 cm^2). For samples OC11_SS and OC16_PS 20 ml of mixed solids, oil and water were used. Acid pretreatment was as per NACE protocol RP0775-2005. Sodium sulfide (20 µl of 1 M Na_2S) was added and the serum bottles were then closed with butyl rubber stoppers. This procedure was done in a Forma anaerobic hood with an atmosphere of 85% N_2, 10% CO_2, and 5% H_2. The samples were then incubated for 15–18 days at room temperature while lying flat on the platform of an orbital shaker, shaking at 150 rpm. At the end of the incubation each serum bottle was treated as follows. At $t = 0$ the stopper was removed, a stirring bar was added and a volume of 1.78 ml of 12 N HCl was injected, giving a final concentration of 1 N. At $t = 1, 2 \ldots 10$ min 50 µl of sample was removed and added to 450 µl of a solution of 0.7 M hydroxylamine in 1 N HCl and left to react for 15 min at room temperature. Following that 100 µl of this solution was added to 900 µl of ferrozine-HEPES solution at $t = 16$, 17, …..25 min incubated for a further 15 min after which the absorbance values were measured at 562 nm (A_{562}) in 1 min intervals. The concentrations of dissolved iron were measured from a standard line for samples of ferric chloride in 1 N HCl, which were treated identically. The data (dissolved iron concentration vs. time, 1–10 min) were used to extrapolate the dissolved iron concentration at $t = 0$ and this value was used to calculate the weight of iron remaining at time zero (W_0) and the general corrosion rate. The period from 10 to 15 min was used to remove the beads from the solution and washing these briefly with water, 1 M $NaHCO_3$ (2 min) and then again briefly with water. They were then immersed in acetone (1 min) and placed on Kimwipes to dry. They were rolled briefly between Kimwipes to remove any remaining loose corrosion product. The beads were then weighed to determine weight loss. The determined weight loss for beads incubated in acid for 10 min (ΔW_{10}) was corrected for weight loss during the 10 min acid treatment, by using the formula: $\Delta W_0 = \Delta W_{10} \times C_{Fe,0}/C_{Fe,10}$. The corrosion rate (mm/yr) was then calculated as CR = ΔW_0/ATD, where A was the surface area of the beads (3.55 cm^2), T was the incubation time and D was the density of iron (7.85 g/cm^3).

Corrosion Rates of Carbon Steel Beads or Coupons Inoculated in the Field

Eight 120 ml serum bottles were sent to the field with either five carbon steel beads or two carbon steel coupons (2.0 × 1.0 × 0.1 cm) each. The beads and coupons were pre-treated as per NACE protocol RP0775-2005 and weighed. The bottles had an N_2-CO_2

TABLE 1 | Water chemistry, microbial numbers and corrosion rates of samples of produced water (PW), central processing facility water (CPW), injection water (IW), and solids and sludges (SS) obtained from an oil field in Oceania in 2013/2014.

Sample Name	Sample appearance	pH	NaCl (Meq)	Sulfate (mM)	Acetate (mM)	APB/ml[a]	SRB/ml[a]	CR (mm/yr) wt loss	CR (mm/yr) Fe diss
OC1_PW	Clear water with oil	7.45	0.25	0.93	4.23	1.5×10^3	<30	ND[b]	0.024
OC2_PW	Same as OC1	7.67	0.23	0.55	7.97	2.4×10^5	<30	ND[b]	0.031
OC3_PW	Brown water with oil	5.81	0.49	1.77	4.44	4.3×10^3	<30	0.032	0.027
OC4_PW	Same as OC1	6.51	0.25	0.58	13.19	4.3×10^6	<30	0.024	0.029
OC5_PW	Same as OC1	7.11	0.3	0.87	6.82	2.4×10^7	<30	0.014	0.024
OC6_PW	Same as OC1	7.4	0.17	0.74	5.68	<30	<30	0.022	0.024
OC7_PW	Same as OC1	7.75	0.1	1.01	2.11	4.3×10^5	<30	0.026	0.015
OC12_PW	Same as OC1	7.47	0.18	0.82	5.22	4.3×10^5	<30	0.017	0.02
OC13_PW	Same as OC1	7.46	0.21	0.68	4.51	2.4×10^6	<30	0.025	0.024
OC14_PW	Same as OC1	7.35	0.2	0	4.34	4.3×10^3	2.4×10^5	0.017	0.014
	Average	7.2	0.24	0.8	5.85			0.022	0.023
	Standard deviation	0.6	0.1	0.44	3.03			0.006	0.006
OC8_CPW	Yellow water; no oil	6.54	0.18	6.29	67.67	<30	<30	0.003	0.005
OC9_IW	Clear water; no oil	6.92	0.15	2.45	33.98	<30	<30	0.005	0.008
OC10_IW	Same as OC8	6.92	0.2	2.72	52.15	<30	<30	0.003	0.01
OC18_CPW	Same as OC9	7.29	0.16	1.89	7.15	<30	<30	ND[b]	ND[b]
OC15_IW	Same as OC8	7.01	0.2	0.84	6.37	<30	<30	0.023	0.021
OC17_IW	Same as OC8	7.33	0.21	0.91	5.09	<30	<30	0.013	0.019
	Average	7	0.18	2.52	28.7			0.009	0.013
	Standard deviation	0.29	0.02	2	26.9			0.009	0.007
OC11_CPSS	Black oily solids	7.08	0	0	0.65	9.3×10^4	2.4×10^8	0.038	ND[b]
OC16_CPSS	Black oily solids	6.8	0	0	6.09	2.4×10^6	4.3×10^7	0.033	ND[b]
	Average	6.94	0	0	3.37			0.036	
	Standard deviation	0.2	0	0	3.85			0.004	

CRs were determined with 20 carbon steel beads in a volume of 20 ml.
[a]MPNs of APB and SRB; lack of positive wells was scored as <30/ml because of the 0.1 ml volume used for 10-fold dilutions.,
[b]ND is not determined.

TABLE 2 | Corrosion rates of carbon steel coupons and beads in serum bottles filled with samples of produced water (PW) or central processing facility water (CPW) at an Oceania oil field in 2014/2015[a].

Sample	Carbon steel	Weight (g)	Back pressure (ml)	Corrosion rate (mm/yr)
OC19_CPW	Coupons	2.654	24	0.018
OC20_CPW	Coupons	–	ND[b]	ND[b]
OC21_PW	Coupons	2.654	33	0.011
OC22_CPW	Coupons	2.654	51	0.014
OC19_CPW	Beads	0.276	37	0.122
OC20_CPW	Beads	0.276	48	0.089
OC21_PW	Beads	0.276	32	0.108
OC22_CPW	Beads	0.276	42	0.155

CRs were determined for coupons or beads in a volume of 50 ml.
[a]Samples were shipped to the UofC and incubation was continued with shaking for a total of 45 days.
[b]Not determined as serum bottle broke during transport.

headspace and contained 10 mg of sodium bisulfite as oxygen scavenger. Sterile plastic syringes (60 ml), needles, gloves and sampling instructions were sent together with the serum bottles to assure aseptic sampling. Field personnel added 50 ml of sample to each of the 120 ml serum bottles and these were sent to the UofC. Once received the samples were placed on a shaker at 30°C and incubated for a total of 45 days, including shipping time. At the end of the incubation, coupons and beads were treated with NACE protocol RP0775-2005.

Microscopic Examination

Following treatment with NACE protocol RP0775-2005, the carbon steel beads were viewed under a dissecting microscope. LED lights were used to illuminate the beads in order to increase contrast and improve detail. An Olympus SZ61 microscope fitted with an Infinity 2 Camera was used to take digital pictures of the carbon steel beads in various positions.

RESULTS

Characteristics and Corrosion Rates of Oceania Oil Field Samples Collected in 2013/2014

Samples were obtained from an oil field in Oceania from which oil was produced by water injection (**Figure 1**). Samples included produced waters, waters from the CPF, and tank bottom sludges (**Figure 1**, **Table 1**). Water tanks of the CPF were

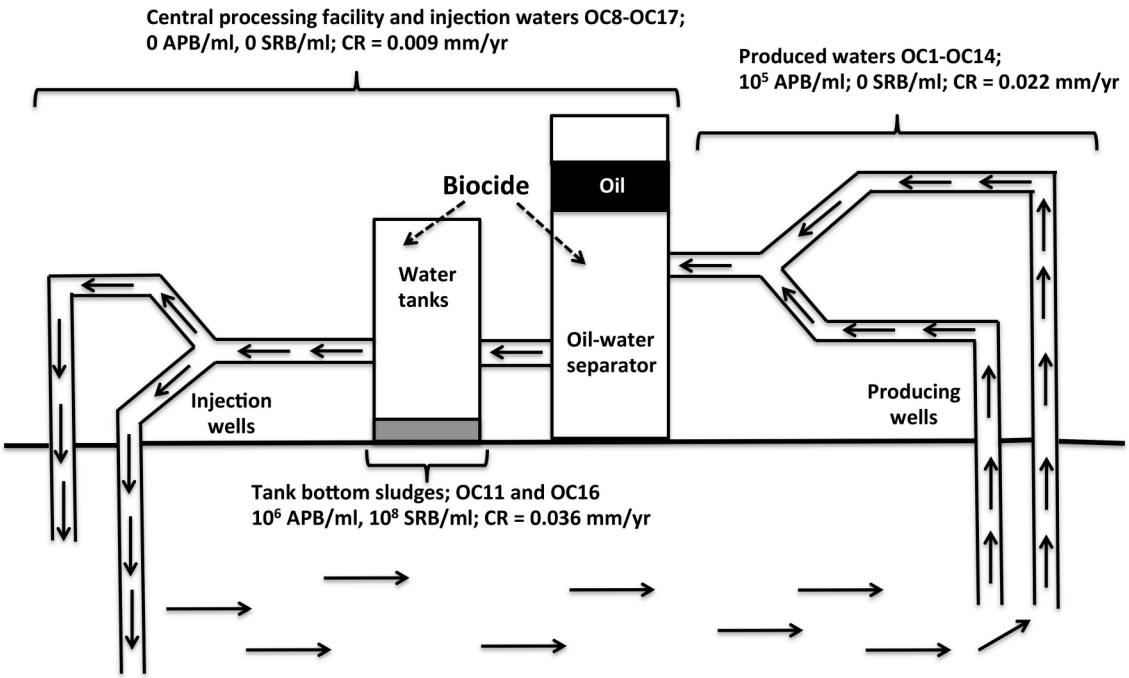

FIGURE 1 | Schematic of Oceania oil field from which samples OC1 to OC18 were obtained (Table 1). Samples of produced waters contained some oil, as these were collected upstream from the oil-water separator. Weekly biocide addition to water tanks of the central processing facility eliminated planktonic APB, but tank bottom sludges retained high numbers of APB and SRB and had the highest general CR.

subjected to weekly biocide treatment to prevent MIC. Produced water samples collected in 2013/2014 (**Table 1**: OC1_PW to OC14_PW) had a near neutral pH (7.2), a low ionic strength (0.24 Meq of NaCl), a low sulfate (0.80 ± 0.44 mM), and a much higher acetate concentration (5.8 ± 3.0 mM). Water samples from the CPF and injection water samples (**Table 1**: OC8_CPW to OC17_IW) had a similar pH and ionic strength. However, these had higher concentrations of sulfate (2.5 ± 2.0 mM) and of acetate (28.7 ± 26.9 mM). Aqueous extracts of solids and sludges from tank bottoms of the CPF (**Figure 1**, **Table 1**: OC11_CPSS and OC16_CPSS) had a neutral pH, no salt, 0 mM sulfate, and 3.4 ± 3.8 mM acetate. All produced water samples, except OC6_PW, had a significant MPN of up to 2.4×10^7 APB/ml. These samples had no significant MPNs of SRB with the exception of sample OC14_PW, which had 2.4×10^5 SRB/ml. Water samples from the CPF and injection waters had no significant MPNs of APB or SRB. The elimination of planktonic APB from CPF and injection waters was likely the result of biocide addition to the CPF. However, this did not appear to affect the MPNs of extracts from solids and sludge samples OC11_CPSS and OC16_CPSS, which had 10^5–10^6 APB/ml and 10^7–10^8 SRB/ml (**Table 1**). This suggested that biocide did not effectively penetrate in tank bottom solids.

Corrosion rates (CRs) were measured by incubating 20 ml of sample with 20 pre-treated carbon steel beads (55.0 mg each) in 50 ml serum bottles under anaerobic conditions, while shaking at room temperature. The incubations were terminated by addition of 12 N HCl to a final concentration of 1 N. CRs were determined both from the measured concentration of dissolved and dissolving iron and from the measured weight loss, extrapolated to $t = 0$ (the time when acid was added). A typical spreadsheet for collection and calculation of corrosion data for sample OC6 is shown in Table S1. The dissolved iron concentration prior to acid treatment was measured to be 0.19 mM. Treatment with 1 N HCl gave an increase in dissolved iron with time as indicated in Table S1, giving an intercept of 3.03 mM. We interpret the intercept as due to the near instantaneous dissolution of corrosion products and the slope as due to the slower dissolution of metallic iron. Hence the actual concentration of Fe in corrosion product was 3.03–0.19 = 2.84 mM, corresponding to 3.25 mg in 20 ml and a CR of 0.024 mm/yr. Following incubation of iron beads in 1 HCl for 10 min, the beads were neutralized, washed, and dried. A weight loss of $\Delta W_{10} = 5.50$ mg was measured at 10 min (Table S1). This was corrected for dissolution of metallic iron by noting that the concentrations of dissolved iron were 3.03 and 5.60 mM at 0 and 10 min, respectively. Hence, the corrected weight loss at 0 min was $\Delta W_0 = 5.50 \times 3.03/5.60 = 2.98$ mg, which corresponded to a weight loss CR of 0.022 mm/yr. Hence CRs measured by iron dissolution and weight loss were in good agreement, as found for most other samples (**Table 1**). Poor agreement between the two methods to determine CR was observed when the concentration of dissolved iron in the sample could not be accurately determined, as for the two multi-phase sludges where large variations in dissolved iron were seen depending on whether aqueous or oily sub-samples were taken. We will, therefore, only refer to weight loss CRs in the results presented below.

The average weight loss CR for 8 produced waters was 0.022 ± 0.006 mm/yr, whereas that for 5 CPF and injection waters was lower at 0.009 ± 0.009 mm/yr (**Table 1**). The highest average CR was observed for the two solids samples at 0.036 ± 0.004 mm/yr. Hence, bacterial numbers and average CRs appeared to be related with highest CRs seen in the two solids and sludge samples which had high MPNs of both APB and SRB (**Figure 1**; **Table 1**).

Effect of the Number of Carbon Steel Beads Per Unit Volume On the Measured Corrosion Rate

Serum bottles containing 20 ml of sample OC2_PW and either 1, 2, 5, 10, 20, 30, or 40 carbon steel beads were incubated and corrosion rates were determined by weight loss. The corrected weight loss CRs obtained following treatment with 1 N HCl for 10 min are indicated in **Figure 2**. The results indicated that the measured CR decreased from 0.061 to 0.007 mm/yr, when the number of beads used in the analysis increased from 1 (55 mg of Fe) to 40 (2200 mg of Fe). Interestingly, the weight of carbon steel per unit volume is not routinely stated in corrosion studies. Having a larger weight of carbon steel in a small volume may stimulate the back reaction of the anodic dissolution of iron, decreasing the observed weight loss:

$$Fe^{2+} + 2e \rightarrow Fe^{0} \quad (7)$$

In view of the strong dependence of number of beads per unit volume on CR we have typically used only 5 beads per 20–50 ml. We also note that the decrease of CR with increased numbers of beads per unit volume (**Figure 2**) indicates that erosion corrosion from beads hitting each other during incubation with shaking did not contribute to the observed CR.

Comparison of Corrosion Rates Obtained with Carbon Steel Beads and Coupons

Capped and crimped serum bottles (120 ml) with either 2 coupons or 5 beads, 10 mg of sodium bisulfite and a headspace of N_2-CO_2 were shipped to the field, where they were filled with 50 ml sample. When the serum bottles were received at the UofC a backpressure of 32–51 ml was measured (**Table 2**), indicating that the crimped rubber stoppers provided a good seal. At the end of the 45-day incubation period the samples were pre-treated as per NACE protocol (RP0775-2005) and weighed to measure corrosion rates.

The average CR of six carbon steel coupons in three samples was 0.0142 mm/yr, whereas the average CR for 20 carbon steel beads (five in each of four samples) was 0.1185 mm/yr (**Table 2**). Hence the CRs measured with beads were on average eight-fold higher than those measured with coupons. Beads and coupons had a similar surface area of 3.24 and 2.80 cm^2/g, respectively. This small difference was corrected for in the calculation of CR by Equation (6). A higher weight of carbon steel per unit volume for the incubation with coupons, as compared to beads (**Table 2**: 2.654 vs. 0.276 g) may have contributed to the decreased CR with coupons. Increased CRs with beads could be due to the fact that spherical beads make contact with the surrounding medium on all sides, whereas coupons (when not mounted in suspended coupon holders) tend to have a more exposed side (contacting the solution) and a less-exposed side (contacting the glass bottom of the serum bottles). Beads and coupons were made from a36 and a366 mild low carbon steel with carbon contents of 0.26 and 0.08% (w/w), respectively. These materials are similar, but not identical.

The CRs measured with beads in 2014/2015 samples (**Table 2**) were 5.4-fold higher than those measured in 2013/2014 samples (**Table 1**). This increase was partly caused by using a lower number of beads per unit volume (5/50 ml) in 2014/2015, than in 2013/2015 (20/20 ml). This increased the CR by an estimated 3.6-fold (**Figure 2**). Some of the difference may also have been caused by initiating corrosion experiments on site and by the longer incubation time of 45 days for the 2014/2015 samples. Interestingly we found that the standard deviation (SD in mg) of the average residual bead weight for the five beads present in incubations of 2014/2015 samples increased with decreasing average bead weight (**Figure 3**). This indicated the weight loss to become increasingly uneven as corrosion progressed.

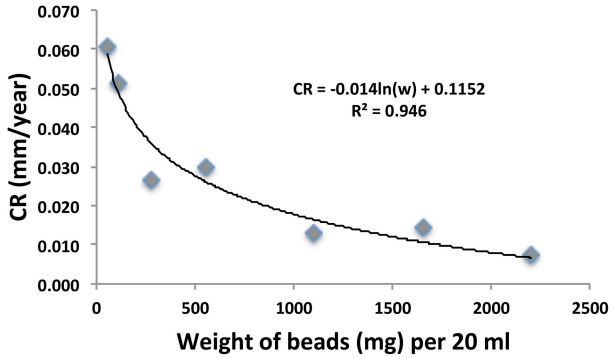

FIGURE 2 | Relation of corrosion rate (CR in mm/yr) and the weight of 55 mg carbon steel beads used in anaerobic incubations of sample OC2_PW in serum bottles, as described in the text. The numbers of beads were from left to right 1, 2, 5, 10, 20, 30, and 40 in a constant volume of 20 ml.

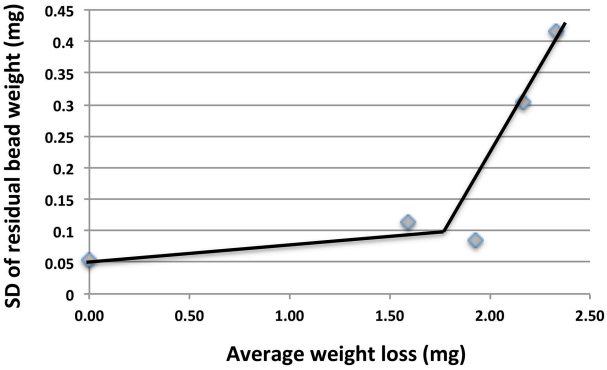

FIGURE 3 | Plot of standard deviation (SD) of residual bead weights vs. average weight loss for incubations in Table 2. The increase in SD with increased weight loss indicates unevenness of the corrosion.

Higher corrosion rates for beads as opposed to coupons were also observed with 21 samples obtained from coiled tubing (CT) operations in shale gas fields in NW British Columbia and NE Alberta. These samples had MPNs of APB, which varied from zero to 9.3×10^6/ml, but no SRB, i.e., similar to the MPNs for most samples in **Table 1**. Samples (50 ml/120 ml serum bottle with an N_2-CO_2 head space) were incubated with either five beads for 39 days or with three coupons for 32 days. Coupons ($1.3 \times 0.8 \times 0.4$ cm) were cut from CT carbon steel. The average weight loss corrosion rate for all 21 samples was 0.054 ± 0.049 mm/yr (range 0.008–0.185 mm/yr) for coupons and 0.214 ± 0.261 mm/yr (range 0.09–1.31 mm/yr) for beads. Images of highly and moderately corroded beads are shown in **Figure 4**. Hence, the CRs measured with beads exceeded those measured with coupons by an average of 4.0-fold. These differences may be caused by systematic factors as indicated already. The CT samples also contained variable amounts of sand (0.4–8 g/L), used during fracturing, which may have contributed to erosion corrosion during incubation with shaking.

Corrosion of Carbon Steel Beads with Acids

Incubation of 50 pre-treated carbon steel beads in five 20 ml 1.5×15 cm Hungate tubes (10 beads/tube) each containing 15 ml of 1 N anoxic HCl and an N_2-CO_2 headspace for 51 h gave an average residual bead weight of 41.4 ± 8.9 mg (average $\pm$ SD for 50 beads), indicating highly uneven corrosion with an average CR of 16.7 ± 3.1 mm/yr. Indeed, extensive pitting was evident on the surface of highly corroded beads (**Figure 5**).

Corrosion Under Flow Conditions

Carbon steel beads (60, or 30 with 30 similarly sized glass beads) were packed in 1 ml syringe columns, which were subjected to injection of 10 ml/day of the effluent of a chemostat, containing actively growing SRB in defined medium with 10 mM lactate and 10 mM sulfate. The chemostat effluent had 10 mM acetate, 5 mM of residual sulfate, and 5 mM sulfide, indicating effective oxidation of lactate to acetate and CO_2 and reduction of sulfate to sulfide as per Equation (1). The medium had excess sulfate allowing in principle reduction of sulfate with Fe^0 as the electron donor in the syringe columns as per Equation (4). Up-flow injection of chemostat effluent into a carbon steel- and glass-bead containing column for 256 days gave an overall general corrosion rate of 0.11 mm/yr. The residual weight of the beads ranged from 36.5 to 51.4 mg, with an average of 44.0 ± 3.3 mg. The average weight of beads prior to corrosion was 55.0 ± 0.3 mg. The significant 11-fold increase in SD from 0.3 to 3.3 mg indicated uneven (pitting) corrosion. The most heavily corroded beads had deep pits (not shown). Short-term injection (46–60 days) of the same chemostat effluent into columns containing only carbon steel beads gave general corrosion rates of 0.016–0.030 mm/yr. We are currently trying to find conditions giving increased corrosion to allow rapid determination of the effect of treatment with biocides or corrosion inhibitors on corrosion under flow conditions.

DISCUSSION

The study of MIC involves determining (i) the contribution of microorganisms to general and pitting corrosion rates in the laboratory and in the field (ii) the structure and function of multiple species biofilms in catalyzing corrosion, and (iii) the mechanisms through which pure cultures or consortia of microorganisms are able to use metallic iron (Fe^0) as electron donor for their metabolism.

Determination of CRs is typically done with coupons by electrochemical methods (Dexter et al., 1991) or by determining weight loss. The latter method is popular in MIC studies, where long incubation times are often required. As an example, Ilhan-Sungur et al. (2007) used galvanized carbon steel coupons ($2 \times 2 \times 0.05$ cm), covered with a small layer of zinc with the edges treated with epoxy primer. Twenty six coupons were exposed to a 1 L culture of the SRB *Desulfovibrio* sp. and the CR for eight sets of triplicate coupons was found to decrease from 0.035 to 0.001 mm/yr over 31 days. Rajala et al. (2015) incubated single carbon steel coupons ($0.5 \times 8 \times 0.1$ cm) with 250 ml samples of anoxic ground water from a radioactive waste storage site for 3–8 months and found CRs of 10^{-3}–10^{-4} mm/yr. In contrast, when coupons were exposed to actual or simulated marine conditions, which included high sulfate concentrations and periodic exposure to air, much higher CRs of up to 1 mm/yr were observed (Enning et al., 2012; Marty et al., 2014). Marty et al. (2014) protected the coupon edges from corrosion by treatment with acrylic polyurethane to prevent edge effects. In our laboratory general weight loss CRs of coupons have been determined for field samples (as in this study), enrichments or cultures of pure strains (Park et al., 2011; Mand et al., 2014; Okoro et al., 2014).

General CRs have been classified by Al-Shamari et al. (2013) as low (<0.0254 mm/yr), moderate (0.0254–0.1245 mm/yr), high (0.127–0.254 m/yr), and severe (>0.254 mm/yr). Our results indicate that general CRs can be determined accurately with carbon steel beads over a wide range from 0.001 to 10 mm/yr. The absence of edge effects and the fact that beads have no preferred side of contact with glass during incubation with shaking are advantages over the use of coupons. Determination of precise general CRs allows assessment whether biocide addition decreases general CRs (**Figure 1**, **Table 1**). Because higher CRs are measured when less carbon steel is added to a corrosion assay (**Figure 2**), it is important that the weight of carbon steel added per unit volume is standardized. It is of interest in this regard that high weight loss CRs (0.70 mm/yr) catalyzed by marine EMIC-catalyzing SRB were achieved by incubating a $10 \times 10 \times 1$ mm coupon (~785 mg of iron) in 1.4 L of medium (Enning et al., 2012). At 11 mg/20 ml this is 1/5 of the lowest value in **Figure 2** (1 bead of 55 mg/20 ml). Using the fitted curve in **Figure 2** we estimate that CRs in **Table 1** increase by about five-fold at such a low weight per unit volume with the highest value observed (**Table 1**: 0.038 mm/yr) increasing to 0.190 mm/yr. Hence, standardization of weight per unit volume is important to allow other factors, which may cause differences in CR, such as surface to volume ratio and type of carbon steel to be assessed.

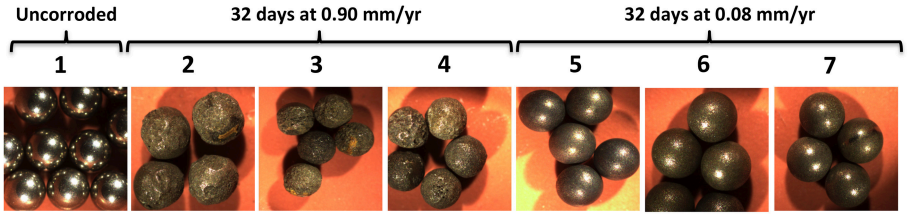

FIGURE 4 | Images of carbon steel beads (Ø= 0.238 cm). Photographs were taken of (1) pretreated beads prior to incubation and following exposure to (2–4) highly and (5–7) moderately corrosive conditions, as indicated.

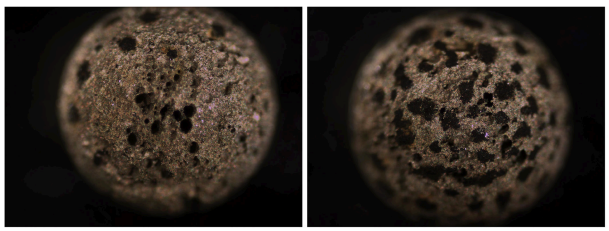

FIGURE 5 | Images of heavily corroded carbon steel beads subjected to 1 N of HCl for 51 h under anoxic conditions with shaking giving an average CR = 16.7 ± 3.0 mm/yr. Extensive pitting is evident. Images of pretreated beads not subjected to incubation are shown in **Figure 4**.

The flat surfaces of coupons can be examined for pitting corrosion by optical microscopy to find the deepest pit (Johnston and Voordouw, 2012), by scanning electron microscopy (SEM) of fixed, dehydrated coupons (Nemati et al., 2001; Hubert et al., 2005; Enning et al., 2012), or by SEM- or optical microscopy-mediated profilometry (Liang et al., 2014). SEM-associated methodologies allow analysis of corrosion product composition. The biofilm formed on coupons has been analyzed by sequencing of PCR-amplified 16S rRNA genes, fluorescence *in situ* hybridization (FISH) or other methods (Zhang et al., 2003; Zhu et al., 2003; Rajala et al., 2015). Profilometry of spherical beads is clearly difficult, as this requires rotation to be able to scan the entire surface. However, limited sections of the surface can be seen (**Figure 5**) and analyzed, as has been done for corroded ball bearings (Squires and Radcliffe, 1983). A unique feature of the homogeneous size of beads is that it allows measurement of the corrosion of multiple simultaneously incubated specimens. Increasing unevenness of the corrosion can then be evaluated as the increase in SD of the weight distribution of multiple beads (**Figure 3**). The most heavily corroded beads in this distribution tend to have significant pits or other surface features (**Figures 4**, **5**). The microbial community composition of adherent biofilms can be evaluated by transferring beads into microfuge tubes and proceeding directly with DNA isolation.

The study of the effective use of metallic iron (Fe^0) as electron donor for microbial metabolism is facilitated by using forms of iron with a large surface-to-volume ratio and a reactive surface. Early studies on the co-metabolism of Fe^0 by SRB growing on lactate and sulfate used steel wool as the substrate with a surface to volume ratio of 100 cm^3/g (Cord-Ruwisch and Widdel, 1986). Likewise an early demonstration of Fe^0 corrosion by methanogens used iron powder of which the properties were not stated (Daniels et al., 1987). More recent studies on the isolation and characterization of SRB, methanogens and acetogens, which use Fe^0 effectively as electron donor for the reduction of sulfate to sulfide, of CO_2 to methane or of CO_2 to acetate, have made use of Fe^0 granules with a diameter of 0.1–0.2 cm (Dinh et al., 2004; Mori et al., 2010; Uchiyama et al., 2010; Kato et al., 2014). The rate of anodic dissolution of ferrous iron was typically reported in these experiments, but the CR was not. The use of homogeneously sized carbon steel beads in such studies would allow calculations of precise CRs and an initial evaluation of the potential for pitting corrosion from evaluation of the increase in SD of the weight distribution. Using beads in culturing may allow the identification of surface activity, which cannot be evaluated with less defined powders or granules. Highly pitted carbon steel beads, which may serve as an even better substrate for microbial growth, can be easily generated by prolonged acid treatment (**Figure 5**).

In conclusion, the use of carbon steel beads offers additional opportunities for screening and monitoring of corrosion, including MIC, in oil field samples. This includes determination of the distribution of corrosion rates, characterized by a mean and an SD allowing estimation of the probability of occurrence of rates high enough to lead to failure.

AUTHOR CONTRIBUTIONS

GV—planned experiments, monitored progress and suggested revisions to protocols, drafted the final manuscript. PM—water chemistry analysis of oilfield samples from Oceania, conducted corrosion experiments with these samples. TP—conducted the carbon bead experiments in upflow bioreactors and associated water analysis and corrosion rate measurements. MS—did experiments with coiled tubing samples including water analysis, corrosion experiments, and data interpretation. YS—conducted all MPN assays for all samples in the study. AV—did experiments on corrosion of carbon steel beads in varied concentrations of acids. JV—assisted with upflow bioreactor carbon bead experiments and acid experiments with carbon steel beads. AS—coordinated collection and shipment of Oceania field samples to the lab. Provided facility operational details to assist with data interpretation. Contributed to manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00351

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Microbial Methane Production Associated with Carbon Steel Corrosion in a Nigerian Oil Field

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Microbially influenced corrosion (MIC) in oil field pipeline systems can be attributed to many different types of hydrogenotrophic microorganisms including sulfate reducers, methanogens and acetogens. Samples from a low temperature oil reservoir in Nigeria were analyzed using DNA pyrotag sequencing. The microbial community compositions of these samples revealed an abundance of anaerobic methanogenic archaea. Activity of methanogens was demonstrated by incubating samples anaerobically in a basal salts medium, in the presence of carbon steel and carbon dioxide. Methane formation was measured in all enrichments and correlated with metal weight loss. Methanogens were prominently represented in pipeline solids samples, scraped from the inside of a pipeline, comprising over 85% of all pyrosequencing reads. Methane production was only witnessed when carbon steel beads were added to these pipeline solids samples, indicating that no methane was formed as a result of degradation of the oil organics present in these samples. These results were compared to those obtained for samples taken from a low temperature oil field in Canada, which had been incubated with oil, either in the presence or in the absence of carbon steel. Again, methanogens present in these samples catalyzed methane production only when carbon steel was present. Moreover, acetate production was also found in these enrichments only in the presence of carbon steel. From these studies it appears that carbon steel, not oil organics, was the predominant electron donor for acetate production and methane formation in these low temperature oil fields, indicating that the methanogens and acetogens found may contribute significantly to MIC.

Keywords: microbially influenced corrosion, microbial community, pyrosequencing, methanogen, acetogen, biofilms

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INTRODUCTION

Oil production is one of the most important factors contributing to the economic growth of Nigeria (Ogwumike and Ogunleye, 2008). According to the Organization of the Petroleum Exporting Countries (OPEC), the oil and gas sector accounts for close to 35% of the gross domestic product of Nigeria (OPEC Annual Statistical Bulletin, 2015[1]). The majority of the development of this resource occurs in the Niger delta area in southwestern Nigeria.

[1]Organization of Petroleun Exporting Countries (OPEC). Annual Statistical Bulletin (2015). Available at: www.opec.org.

Microbial activity in oil reservoirs is common and can impact oil and gas production. In fact, some problems associated with hydrocarbon recovery can be attributed to microbial activity (Voordouw, 2011). In particular, microorganisms may be responsible for different mechanisms that lead to pipeline corrosion failures. Pipeline corrosion can have serious economical implications including production shutdown and the need for infrastructure replacement (Jones, 1996). Corrosion linked to the activity of microorganisms is termed microbially influenced corrosion (MIC) and is often caused by hydrogenotrophic microorganisms. These include the sulfate-reducing bacteria (SRB), which are able to reduce sulfate to sulfide. It has long been proposed that these bacteria use the hydrogen formed on the surface of pipeline metal to reduce sulfate (Von Wolzogen Kühr and Van der Vlugt, 1934). More recently, SRB that are able to directly use iron as electron donor for sulfate reduction have been discovered (Enning et al., 2012). Furthermore, SRB cause increased sulfide levels in petroleum product, known as souring (Gieg et al., 2011). The microbially produced sulfide can precipitate with iron as iron sulfides; semiconductive minerals that play a role in steel corrosion (Enning et al., 2012). In addition to SRB, methanogenic archaea and acetogenic bacteria have also been reported to be capable of steel corrosion. Hydrogenotrophic methanogens and acetogens are able to use hydrogen on the surface of steel pipelines to reduce bicarbonate to either methane or acetate, respectively (Daniels et al., 1987; Mori et al., 2010; Mand et al., 2014). Recently, strains capable of using the steel directly as an electron donor have also been isolated (Dinh et al., 2004; Uchiyama et al., 2010; Kato et al., 2014; Siegert et al., 2015). Consortia involving these microorganisms have been implicated in MIC in numerous environments (Zhang et al., 2003; Davidova et al., 2012; Usher et al., 2014, 2015).

The production facility we studied was the Obigbo oil field, a site recently used for MIC studies (Okoro et al., 2014). The Obigbo reservoirs are injected with low-sulfate groundwater for pressure support to stimulate oil production. The production fluids from this site are then transported to the Bonny Oil and Gas Terminal (BOGT) for export. The Obigbo reservoir temperature is close to 40°C and concentrations of sulfate in formation water have historically been low (0–7 ppm) (Okoro et al., 2014). As a consequence of the low availability of sulfate from both the injection as well as the formation water, neither souring, nor MIC caused by SRB is considered a severe threat. Hence this field does not employ nitrate injection as a means to control SRB activity. Instead, previous research has demonstrated that methanogenic archaea are not only present, but active within the Obigbo reservoir and production facilities (Okoro et al., 2014). The role these microorganisms are playing remained unspecified, since in addition to the growth using carbon steel, these methanogens may also be able to produce methane through the syntrophic breakdown of oil organics within the field (Widdel and Rabus, 2001; Gieg et al., 2014). Syntrophy involves the breakdown of hydrocarbons by fermentative microorganisms to form compounds such as formate, acetate or hydrogen (Sieber et al., 2012). Subsequently, these compounds are used by methanogenic archaea to produce methane (Sieber et al., 2012; Gieg et al., 2014). The interactions between different groups of microorganisms ensures that the concentration of small intermediary compounds remains low, and therefore syntrophy is a mutually beneficial manner of growth (Morris et al., 2013).

As part of regular maintenance at this field, pipelines are mechanically cleaned using brushes and pigs to rid the carbon steel surfaces of sludge, scale and deposits that may have formed (Videla, 2002). These scrapings, or pipeline pigging samples, consist of formation sands, organic hydrocarbons, inorganic minerals, corrosion products such as iron sulfides and also microbial biofilms. This resulting scale/wax/debris can therefore be a valuable sample; often overlooked in favor of easy to obtain water samples, to determine corrosion products, corrosion risks and corrosion mechanisms by microorganisms (Wrangham and Summer, 2013). A previous microbial study of the Obigbo field indicated that biological activity exists in such deposits and it is important to understand how these solids scraped from pipeline walls, may be enhancing MIC (Okoro et al., 2014).

In our study, we obtained both water samples and pipeline pigging solids samples from the Obigbo field. The objective was to determine the potential for biocorrosion in these samples and further understand how hydrogenotrophic acetogenic and methanogenic microorganisms may be playing a role in MIC.

MATERIALS AND METHODS

Sampling Procedure

The production site that we have studied, the Obigbo oil field, is located onshore in the Niger delta region, near the city of Port Harcourt (Onwusiri et al., 2003). Sampling from the Obigbo field was done in 2013 and samples were shipped at ambient temperature to the University of Calgary, Calgary, AB, Canada for chemical and molecular analyses. Water samples were collected in 1 L bottles (Nalgene, Rochester, NY), filled to the brim and tightly capped to exclude oxygen ingress. In total, five water samples and two solids samples were collected. Sampling points are indicated in **Figure 1**. Of these samples, two were produced waters (PW) (1_PW, 2_PW), sampled from the surface facilities. A third sample was injection water (IW), the ground water sample injected into the reservoir for pressure maintenance (3_IW). A water sample associated with pigging operations, after the pig run, (4_PigWater) was also sampled, along with a sample of the water that is exported to the BOGT, 5_Crude. The pipeline solids (PS) samples were collected in plastic bags and were labeled 6_PS and 7_PS. Both of these were the solids resulting from a pigging operation and were in pipelines transporting product to the BOGT. Upon arrival at the laboratory, samples were stored in an anaerobic hood (Coy Laboratory Products, Grass Lake, MI) containing an atmosphere of 90% (vol/vol) N_2, 10% (vol/vol) CO_2 (N_2-CO_2), (Praxair, Calgary, AB) at room temperature.

Chemical Analyses

Chemical components in water, solids and oil samples may serve as indicators of corrosion or as a gauge for potential corrosion risk. Upon arrival at the University of Calgary, subsamples were taken and stored at −20°C, and were used for chemical analyses. Water samples were analyzed as sampled. Solids samples (5 g)

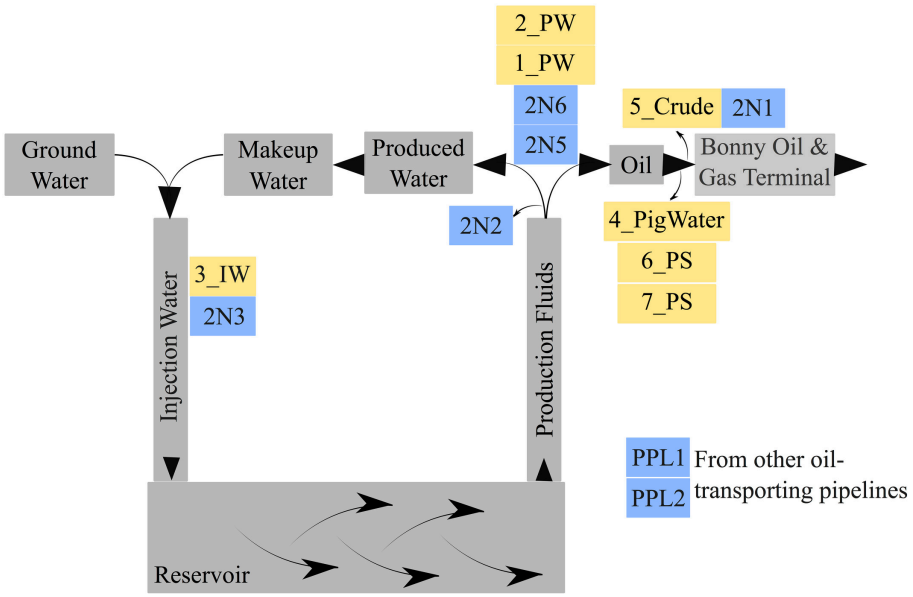

FIGURE 1 | Schematic diagram of the Obigbo oil field in Nigeria. Samples received in 2013 are shown in yellow. 1_PW and 2_PW are produced waters, 3_IW is an injection water, 4_PigWater is water associated with a pigging operation, and 5_Crude is an oil and water mixture that is exported to an oil and gas terminal. 6_PS and 7_PS are pipeline pigging solids. Samples received from sampling in 2011 are indicated in blue. 2N2, 2N5, and 2N6 are production waters, 2N3 is an injection water and 2N1 is an oil and water mixture exported to an oil and gas terminal. PPL1 and PPL2 are pipeline pigging solids.

were mixed with 10 mL MilliQ (Millipore, Etobicoke, ON) water, vortexed at high speed for 10 min and allowed to settle. The resulting supernatant was used for colorimetric assays; 1.5 mL of the supernatant was centrifuged at 13,300 rpm for 5 min; the supernatant was filtered through a 0.45 μm nylon filter (Millipore, Etobicoke, ON) and the filtrate was used for all liquid chromatography measurements.

Sulfide and ferrous iron concentrations were assessed using colorimetric assays (Trüper and Schlegel, 1964; Park et al., 2011). Ammonium concentrations were assayed using the indophenol method (American Public Health Association, 1992). The pH was measured using a pH meter (Orion VERSA STAR, Thermo Scientific, Beverly, MA). Conductivity was measured with a commercial probe and converted into molar equivalents of NaCl (Orion Conductivity Probe, Thermo Scientific, Beverly, MA).

Other chemical analyses were performed as previously described (Mand et al., 2014). To measure acetate concentrations, a Waters 515 model (Milford, MA) high-performance liquid chromatograph (HPLC) equipped with a Waters 2487 model UV detector set at 220 nm and an organic acid column (250 × 4.6 mm, Alltech Prevail) eluted with 25 mM KH_2PO_4 (pH of 2.5) were used. Samples (1 mL of each water sample, 1 mL of each filtrate for pigging solids samples) were centrifuged at 13,300 rpm for 5 min and 300 μL of the supernatant was acidified using 20 μL of 1 M H_3PO_4. 50 μL of this solution was injected and eluted at a flow rate of 1 mL/min. Sulfate concentrations were monitored using a Waters 600 model HPLC equipped with a Waters 432 conductivity detector and an IC-PAK anion column (150 × 4.6 mm, Waters) eluted with 24% (v/v) acetonitrile, 2% butanol and 2% borate-gluconate concentrate. Following centrifugation 100 μL of the sample supernatant was added to 400 μL of the acetonitrile solution; 50 μL was injected into the HPLC and eluted at 2 mL/min.

The presence of oil organics in pipeline solids samples was estimated by extraction with dichloromethane (DCM) and analysis using a gas chromatograph equipped with mass spectrometry (GC-MS) (7890A GC system, 5975C detector, Agilent, Santa Clara, CA) (Gieg et al., 2008; Agrawal et al., 2012). 10 mL of DCM was added to 1 g of pipeline solids sample 6_PS. This mixture was thoroughly mixed by vortexing at high speed to ensure that oil organics would become dissolved into the organic phase. Subsequently, the organic phase was dried over sodium sulfate and was concentrated to 1 mL under a headspace of N_2. 1 μL of this was injected as previously described (Agrawal et al., 2012).

Methanogenic Incubations in Synthetic Oil Field Brine

Microbial methanogenesis was monitored in sterile 120 mL serum bottles. 50 mL of Coleville synthetic brine K (CSBK) medium (Callbeck et al., 2013), was dispensed into each bottle under a headspace of N_2-CO_2. CSBK medium contains (in g/L): NaCl (1.5), KH_2PO_4 (0.05), NH_4Cl (0.32), $CaCl_2{\cdot}2H_2O$ (0.21), ($MgCl_2{\cdot}5H2O$ (0.54) and KCl (0.1). After autoclaving under N_2-CO_2, a 30 mL solution of 1 M $NaHCO_3$ was added, along with 1 mL of trace elements (Widdel and Bak, 1992), and 1 mL of a 1 M solution of Na_2S. The pH was adjusted to be between 7.2 and 7.4. 2.5 mL of each Obigbo field water sample was added, amounting to a 5% inoculum. Two grams of each solids sample (as sampled) was added for the pigging sample incubations. Each

incubation was done with and without carbon steel coupons (5 × 0.5 × 0.1 cm, American Society for Testing and Materials, ASTM, a366 containing 0.015% carbon) present, at 30°C with agitation at 100 rpm. Coupons were cleaned according to a National Association of Corrosion Engineers (NACE) standard protocol, and weighed thrice before incubation (NACE, 2013). Methane was measured as previously described, where 0.2 mL of the headspace was sampled periodically using a syringe that had been pre-flushed with N_2-CO_2 and injected into a gas chromatograph (Hewlett-Packard Model 5890) at 150°C, equipped with a flame-ionizing detector set to 200°C and a stainless steel column (0.049 × 5.49 cm, Porapak) (Mand et al., 2014). Following 8 weeks of incubation, coupons were again cleaned according to protocol, weighed thrice and the calculated weight loss was used to determine general corrosion rates (Park et al., 2011; NACE, 2013).

Pipeline Pigging Solids Incubation Experiments

Pipeline solids samples were incubated anaerobically both in the presence and absence of carbon steel beads. The field sample was a powdery solid, with no aqueous phase when received. It was mixed with equal volumes of anaerobic, sterile MilliQ water before incubation. Close to 1 g of sample was mixed with 2.04 g of steel beads (Carbon steel precision ball, diameter 3/32 ± 0.001 inches, weight 0.055 g, Grainger, Richmond Hill, ON). These beads were sanded with 400 grit sandpaper and cleaned according to standard protocol and weighed thrice before incubation (NACE, 2013). Samples were incubated in 25 mL Hungate tubes, under an atmosphere of N_2-CO_2, at 30°C with agitation at 100 rpm. Methane was measured periodically by sampling 0.2 mL of the headspace using a syringe pre-flushed with anaerobic gas (N_2-CO_2) and injection into a GC-FID, as described above. Following incubation, the beads were cleaned according to standard protocol and corrosion rates were determined from metal weight loss. One replicate, representing each condition was used for DNA extraction and subsequent pyrotag sequencing, as described below.

Microbial Community Analysis by Pyrosequencing

Immediately upon arrival in the laboratory, 300 mL of each liquid field sample was filtered through a 0.2 μm membrane filter to collect biomass. These filters were frozen at −80°C for use in DNA extraction. At the same time, 1 g of each solids sample was also frozen at −80°C. Genomic DNA was isolated using a bead-beating procedure, as outlined by manufacturer instructions (FastDNA Spin Kit for Soil, MP Biomedicals, Santa Ana, CA). DNA was eluted in 75 μL of 10 mM Tris-Cl pH 8.5 buffer and quantified with the Invitogen Qubit fluorometer, using the Quant-iT dsDNA HS Assay Kit (Invitrogen, Burlington, ON). The V6-V8 regions of the 16S rRNA genes were amplified using a two-step PCR amplification using the TopTaq PCR Kit (Qiagen, Toronto, ON), as described earlier (Park et al., 2011). The first step (30 cycles) was done using 16S rRNA primers 926F (AAACTYAAAKGAATTGRCGG) and 1392R (ACGGGCGG TGTGTRC). These primers are suitable for the amplification of both bacterial and archaeal DNA. The second amplification step (10 cycles) used the first PCR product as a template and was done using FLX titanium amplicon primers 454_RA_X (primer 926F with a 25-nucleotide A-adaptor sequence: CGTATCGCCTCCCTCGCGCCATCAG and a 10 nucleotide multiplex identifier barcode sequence) and 454T_FwB (primer 1392R with a 25-nucleotide B-adaptor sequence: CTATGCGCCTT GCCAGCCCGCTCAG). The final 16S rRNA PCR amplicons were confirmed using agarose gel electrophoresis and subsequently purified using the QIAquick PCR Purification Kit (Qiagen, Toronto, ON). After measuring final concentrations of each PCR product using the Invitogen Qubit fluorometer again, amplicons were sent to Genome Quebec and McGill University Innovation Centre (Montreal, QC) for 16S rRNA pyrosequencing.

Analyses of data were done with the Phoenix2 software package, where data were subjected to stringent quality control (QC) checks (Soh et al., 2013). This included matching primer sequences, matching adaptor sequences, and removing chimeric sequences. Phoenix2 used a cutoff quality score for each sequence of 27 and a minimum length of each sequence of 200 base pairs (Kunin et al., 2010; Schloss et al., 2011). The remaining QC sequences were clustered into operational taxonomic units (OTUs) using average neighbor clustering at a distance of 5%. OTUs that contained less than 0.01% of total QC reads were filtered out. Each remaining OTU was subsequently assigned to a taxon by comparison with the non-redundant 16S rRNA small subunit SILVA database, release 108, using RDP classifier (SSU ref NR 108; http://www.arb-silva.de/no_cache/download/archive/release_108/Exports). A tree dendrogram was formed using the unweighted pair group method algorithm (UPGMA), where distances between communities was calculated using the Bray-Curtis coefficient in the Mothur software package (Schloss et al., 2009). The tree dendrogram was visualized using the MEGA4.2.2 program (Tamura et al., 2011).

Culture-Based Microbial Enumeration

The number of lactate-utilizing SRB and glucose-fermenting acid-producing bacteria (APB) present in all samples were assayed by inoculating commercial medium for SRB and APB growth (DALYNN, Calgary, AB) in a single inoculation series. One mL of each field water sample, or 1 mL of each pipeline solids sample mixture (0.5 mL sterile anaerobic MilliQ water and 0.5 g solids sample) was added to 9 mL of medium and mixed well. 1 mL from this tube was added to another tube containing 9 mL of medium until a dilution series up to 10^{-10} for each sample was achieved. The culturable population was estimated from the highest dilution showing growth after 30 days of incubation at 30°C. Growth for SRB was estimated from black precipitate formation in vials, due to iron sulfide formation. Growth of APB was estimated from a color change caused by the phenol red indicator in the medium, due to a change in pH caused by glucose fermentation to acidic products.

Comparison with a Sample from a Canadian Oil Field

Corrosion testing was also done using a produced water sample from a low-temperature (30°C) Canadian oil field. 120 mL serum bottles containing 50 mL of CSBK medium, as described above, and a headspace (70 mL) of N_2-CO_2 were used. These were inoculated with a produced water sample and an oil sample from the Medicine Hat Glauconitic C (MHGC) field, in Medicine Hat, AB, Canada (Voordouw et al., 2009). Serum bottles were amended and inoculated with: 1 mL oil only, 1 mL oil and 2.5 mL produced water (M_PW) or 2.5 mL M_PW only. Bottles containing the combination of oil and M_PW and bottles containing M_PW only were also incubated in the presence of carbon steel coupons (5 × 1 × 0.1 cm, ASTM a366 containing 0.015% carbon), prepared as described above (NACE, 2013). The headspace was sampled periodically to measure methane. Samples of the aqueous phase were also taken over time to measure acetate concentrations. Following the 6 week incubation at 30°C with agitation at 100 rpm, coupons were removed and cleaned and corrosion rates were determined using the resulting metal weight loss, as described above.

Accession Numbers

All samples were assigned a sample code for pyrotag sequencing, found in Tables S1, S2. The sequences from the raw reads are available from the Sequence Read Archive from the National Center for Biotechnological Information (NCBI), (under the following accession numbers): V30_1349 (SRR1508449), V30_1350 (SRR1508450), V30_1351 (SRR1508451), V30_1352 (SRR1508452), V30_1353 (SRR1508453), V30_1354 (SRR1508454), V39_1847 (SRR2189969), and V39_1850 (SRR2189970).

RESULTS

Sample Characteristics

All chemical analyses of the field samples are summarized in **Table 1**. The pH of all Obigbo samples was between 6.85 and 8.32. The molar equivalents of NaCl in these samples ranged from 1 to 220 mM. Sulfate levels were low in all water samples, but there was 8.9 and 11.6 mM found in the 6_PS and 7_PS pigging solids extracts, respectively. It is possible that this sulfate may be resulting from sulfide oxidation. A similar trend was seen for sulfide concentrations and ferrous iron concentrations. Almost no sulfide was present in the water samples, but 2.7–3.1 mM was found in the pigging solids extracts, as aqueous dissolved sulfide. The lack of sulfide in water samples may be due to oxidation during sampling or transport. Ferrous iron was not detected in water samples, but 160–690 μM was found in the pigging solids extracts. All samples contained very small amounts of ammonium (0–80 μM), acetate (0–1650 μM) and propionate (0–200 μM).

Microbial Community Composition

A comprehensive microbial community composition was obtained using DNA sequencing technology. DNA was not successfully isolated from the 3_IW sample. This may have been caused by the low concentration of biomass in this water, as DNA isolation was also unsuccessful from this sample during 2011 sampling (Okoro et al., 2014). For the remaining field samples, a total of 6941 quality reads were obtained following 454-pyrotaq sequencing (Table S1). From this, it was seen that *Archaea* dominated the samples and the majority of these were methanogens (**Figure 2**). The pipeline pigging solids samples in particular harbored many methanogens. For example, the genus *Methanobacterium* formed between 33.0 and 33.3% of the total reads obtained for the two solids samples. *Methanobacterium* was also found in the pigging water sample, 4_PigWater, but in a smaller fraction (3.6%). Additionally, the genus *Methanosaeta* was abundant in the pigging solids samples, comprising 24.9–30.8% of all reads for these two samples. Other groups of methanogens such as the genus *Methanolobus* formed small portions of the population throughout all samples (4.6–18.7%). Noticeably, the genus *Methanocalculus* was more abundant in some water samples (4_PigWater: 9.0%) than in the pipeline solids samples (6_PS: 0.63%; 7_PS: 0.83%).

The produced waters from this field had other genera as the main community components (**Figure 2**). In the 1_PW sample, the genus *Azovibrio* formed 27.4% of the reads. The 2_PW sample was instead dominated by the genera *Marinobacter* (39.6%) and *Petrobacter* (36.3%), while *Thauera* were predominant in the 5_Crude sample (11.6%). All four of these genera are potential nitrate-reducing bacteria and are capable of hydrocarbon degradation. Small fractions of the field sample populations may belong to hydrocarbon degrading SRB as well. For instance, the genus *Desulfotigum* was found in 0.16–0.76% of the reads from the produced water and pigging solids samples. The genus *Acetobacterium* was also found in a small

TABLE 1 | Chemical characteristics of the low temperature (40°C) Obigbo oil field water and pigging solids samples.

Sample	pH	NaCl (mM)	Sulfate (mM)	Sulfide (mM)	Ferrous Iron (μM)	Acetate (μM)	Propionate (μM)
1_PW	8.13	174	0.09	0.02	0	0	0
2_PW	8.01	157	0	0.03	0	450	0
3_IW	7.66	1	0.036	0.02	0	0	0
4_PigWater	7.78	220	0	0.01	0	1650	0
5_Crude	8.32	199	0.005	0.02	0	0	0
6_PS	6.93	28	8.87	3.14	160	30	120
7_PS	6.85	32	11.57	2.71	690	40	200

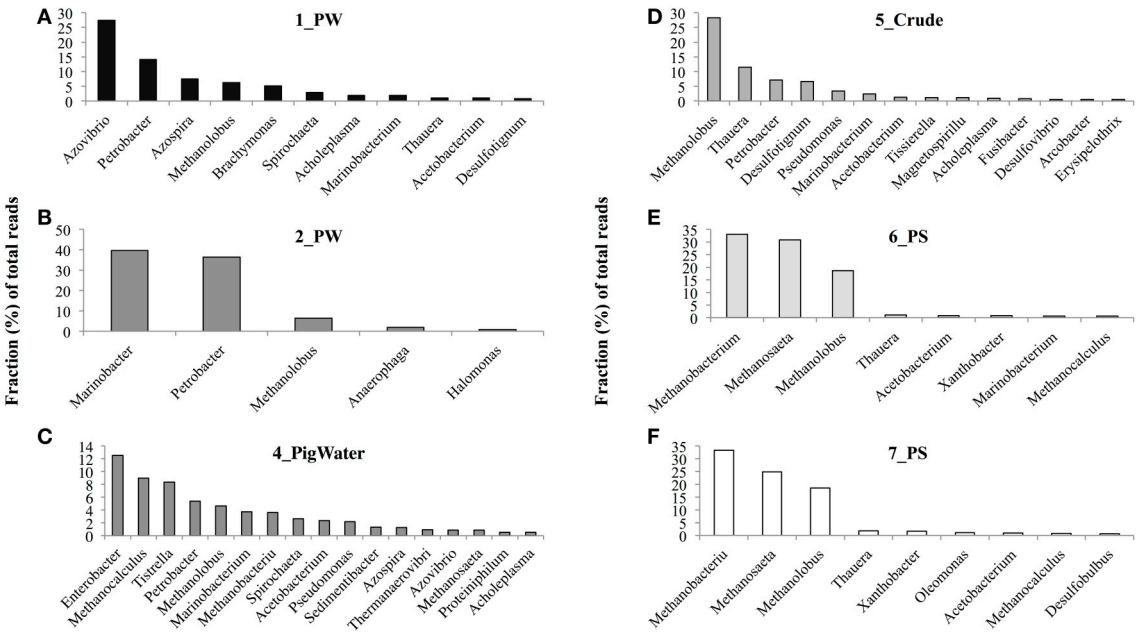

FIGURE 2 | Predominant genera identified in the Obigbo field samples, as determined by 454 pyrotag sequencing. Only those with a representation of 0.5% or higher in produced water 1_PW **(A)**, produced water 2_PW **(B)**, pigging sample water 4_PigWater **(C)**, crude oil sample 5_Crude **(D)**, pipeline pigging sample 6_PS **(E)**, and pipeline pigging sample 7_PS **(F)** are shown.

fraction (0.79–2.4%) of the reads for these samples. Cultured relatives of this organism are capable of metabolizing a variety of substrates and interestingly can convert CO_2 and H_2 into acetate using the acetyl-coenzyme A pathway (Schiel-Bengelsdorf and Dürre, 2012).

As mentioned, the Obigbo field has been previously sampled for microbial activity testing and microbial community composition analysis (Okoro et al., 2014). Sample sites and sample descriptions from this 2011 sampling trip can be found in **Figure 1** and Table S3. The microbial communities present in the current field samples were compared with the previous study (**Figure 3**). The produced water samples from this study did not cluster with similar samples from the previous study, which is an indication that the microbial communities have changed over time at this site. Furthermore, the produced water samples (1_PW and 2_PW), which were taken from different parts of the production system, did not cluster together, possibly indicating that fluid processing during production leads to shifts in microbial populations. Also, the 4 solids samples, although taken from a different location each year, did not cluster together, demonstrating a change in the microbial community composition of pipeline biofilm associated microorganisms.

Culture-based incubations that target only certain culturable microorganisms are often correlated with corrosion in oil field systems. The numbers of lactate-oxidizing SRB and glucose-fermenting APB in each sample were estimated using a culturing assay, which is often used by pipeline operators in oil fields to assess microbial growth. APB were found in all Obigbo samples and ranged from 10^5 to 10^7 cells/mL (or g) of sample. SRB in the pipeline pigging solids samples (10^8 cells/g) were higher than in the water samples (10^1–10^5 cells/mL). The low counts of APB (10^5) and SRB (10^1) in the 3_IW sample indicate low biomass concentrations, as was the case during previous studies (Okoro et al., 2014).

Obigbo Field Sample Incubations under Methanogenic Conditions

To test whether the different groups of hydrocarbon degrading organisms present in the field samples (**Figure 2**) were active and potentially involved in MIC, samples were incubated in a basal salts medium under an atmosphere of N_2 and CO_2, where in addition to the CO_2 in the atmosphere, the hydrocarbons associated with the field samples served as carbon and energy sources. Incubation without the addition of carbon steel coupons yielded very low methane production (**Figure 4A**). Less than 12 μM methane was produced in each water sample enrichment. A maximum 126 ± 4.8 μM and 293 ± 183 μM methane was produced for pipeline solids samples 6_PS and 7_PS, respectively, but these values are very low compared to parallel incubations where steel coupons were present.

When the field samples were incubated with steel coupons, methane production could be correlated with corrosion rates (**Figure 4B**). A Pearson product-moment correlation coefficient was calculated to assess the relationship between methane production and corrosion rates. There was a positive correlation between the two variables ($r = 0.95$, $n = 8$, $p = 0.71$). The lowest methane concentration produced was in the incubation with the injection water sample (3_IW: 326 ± 55 μM)

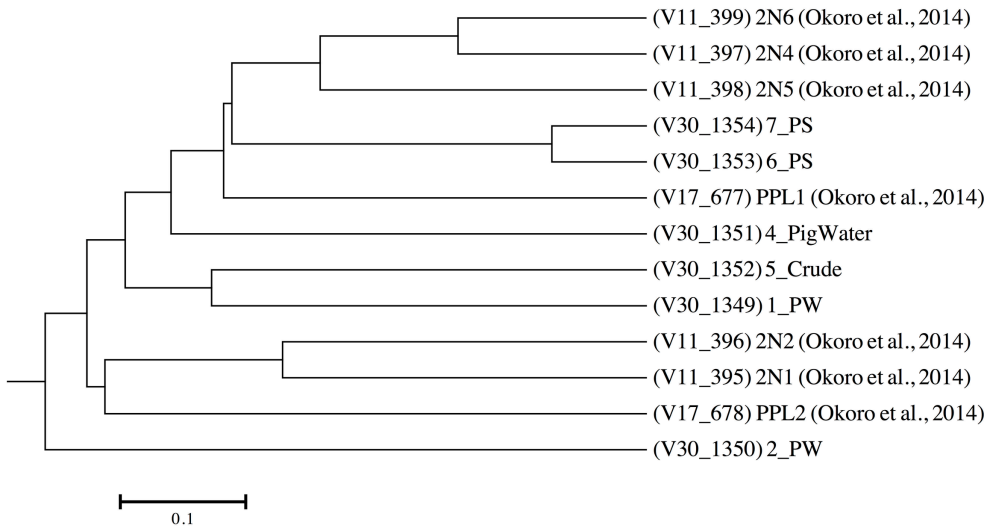

FIGURE 3 | Tree dendrogram showing the relationships between Obigbo field samples used in the present study (1_PW, 2_PW, 4_PigWater, 5_Crude, 6_PS, and 7_PS) and those used in a previous study (2N1, 2N2, 2N4, 2N5, 2N6, PPL1, and PPL2) (Okoro et al., 2014).

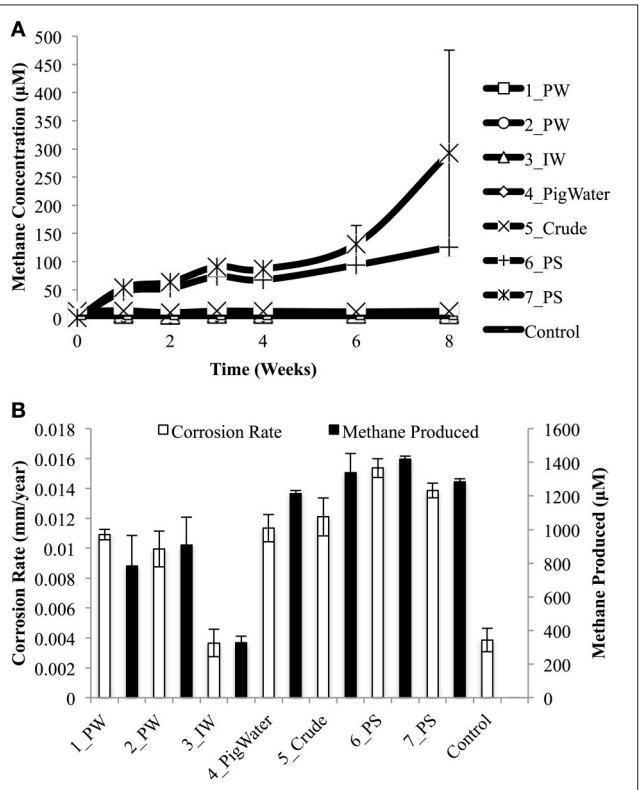

FIGURE 4 | Methane production over an 8 week incubation of Obigbo field samples in a synthetic oil field brine (CSBK) medium in the absence of carbon steel coupons (A). Maximum methane production and associated metal weight loss corrosion rates for the incubation of field samples in CSBK medium in the presence of carbon steel coupons **(B)**. Data represent results from two separate incubations containing two coupons each.

and this also showed the lowest corrosion rate (0.0037 ± 0.0013 mm/year). Conversely, the highest methane production was seen for incubations involving the pipeline pigging samples (6_PS: 1419 ± 26 µM and 7_PS: 1284 ± 24 µM). These incubations also showed the highest corrosion rates (6_PS: 0.0154 ± 0.0009 mm/yr and 7_PS: 0.0139 ± 0.0007 mm/yr). The control incubations containing medium only, with coupons showed no methane production and a very low corrosion rate (0.0039 ± 0.0011 mm/yr), similar to the injection water sample.

Further incubations were done using a pipeline pigging sample under methanogenic conditions. When the pigging solids sample, 6_PS, was incubated with carbon steel beads, up to 438 ± 18 µM methane was produced after 95 days of incubation (**Figure 5A**). In contrast, when the sample was incubated without any beads present, methane production was negligible. The corrosion rate obtained following this incubation was only 0.0021 ± 0.0001 mm/year, which is similar to the abiotic control (0.0039 ± 0.0010 mm/year) (**Figure 5B**). Acetate was not measured during these incubations, as very little aqueous phase was available for sampling.

Following the incubation period, 454-pyrotag sequencing was used to assess the community that emerged during this enrichment (Table S2). Of the reads obtained for the 6_PS incubation containing carbon steel beads, 55.98% were assigned to the order *Clostridiales,* which only formed 1.97% of the original 6_PS sample. This includes the genus *Acetobacterium*, which is known for the ability to make acetate from bicarbonate and steel (Kato et al., 2014; Mand et al., 2014). The fraction of the order *Methanosarcinales,* which contains the acetotrophic genus *Methanosaeta,* remained relatively high (19.75%) after incubation with steel, compared to the original field sample (50.75%).

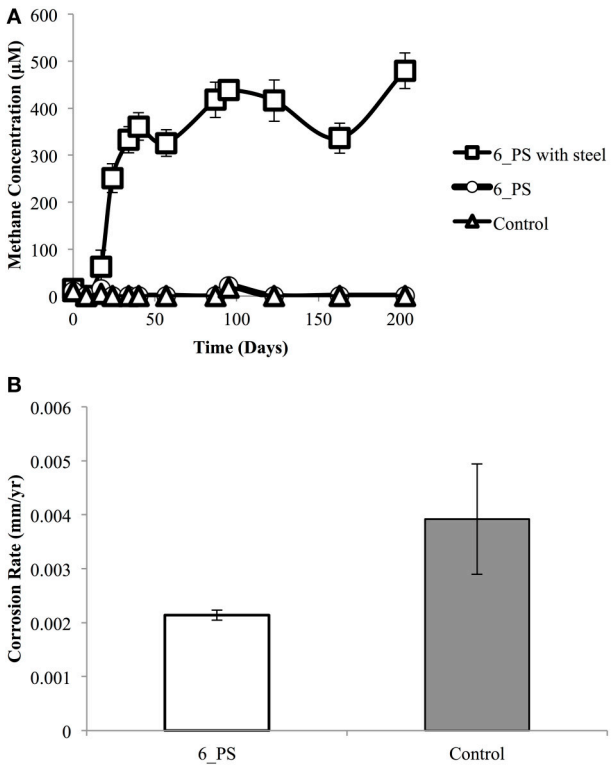

FIGURE 5 | Incubation of pigging solids sample 6_PS in the presence and absence of carbon steel beads. Methane production was monitored over time **(A)**. Corrosion rates were determined following incubation **(B)**. Data are averages of three different incubations for each condition.

MHGC Field Sample Incubations under Methanogenic Conditions

To further understand the potential MIC scenarios seen with Obigbo incubations, samples from a different field were also used. A produced water sample from the MHGC field (M_PW) containing a methanogenic population (*Methanoculleus,* 70.2%) was used. This sample also contained the acetotrophic methanogen (*Methanosaeta,* 2.6%) and acetogenic bacteria (*Acetobacterium,* 0.13%). M_PW was incubated with and without oil, in the presence and in the absence of carbon steel coupons, using CSBK medium. When the M_PW sample (inoculum) was incubated by itself in medium, neither acetate nor methane was produced. These negative results were mirrored in the incubation of MHGC oil by itself in CSBK medium, without M_PW. Furthermore, when the M_PW and the MHGC oil were incubated together in CSBK medium, no microbial activity was witnessed. Acetate formation was only seen when a carbon steel coupon was added to the M_PW incubation (maximum production of 309 ± 5 μM) and when a carbon steel coupon was added to the produced water/oil incubation (maximum production of 192 ± 30 μM) (**Figure 6A**). Acetate production appeared to start earlier than methane production. Methane production was also only seen in incubations containing carbon steel. Methane production appeared to reach a plateau after 3 weeks of incubation, where a maximum of 1250 ± 141 μM was seen in the incubation of M_PW, oil and steel and a maximum of 1588 ± 427 μM was seen in the incubation of M_PW and steel (**Figure 6B**). Based on the concentrations of produced methane and acetate (**Figures 6A,B**) and assuming that iron was the reductant to make these from CO_2, we calculate a theoretical corrosion rate of 0.028 mm/year in the absence of oil which is in good agreement with the experimental value (0.0196 ± 0.0016 mm/year) (**Figure 6C**). In the presence of oil we calculate a theoretical corrosion rate of 0.021 mm/year, which is higher than the measured 0.0088 ± 0.0005 mm/year (**Figure 6C**).

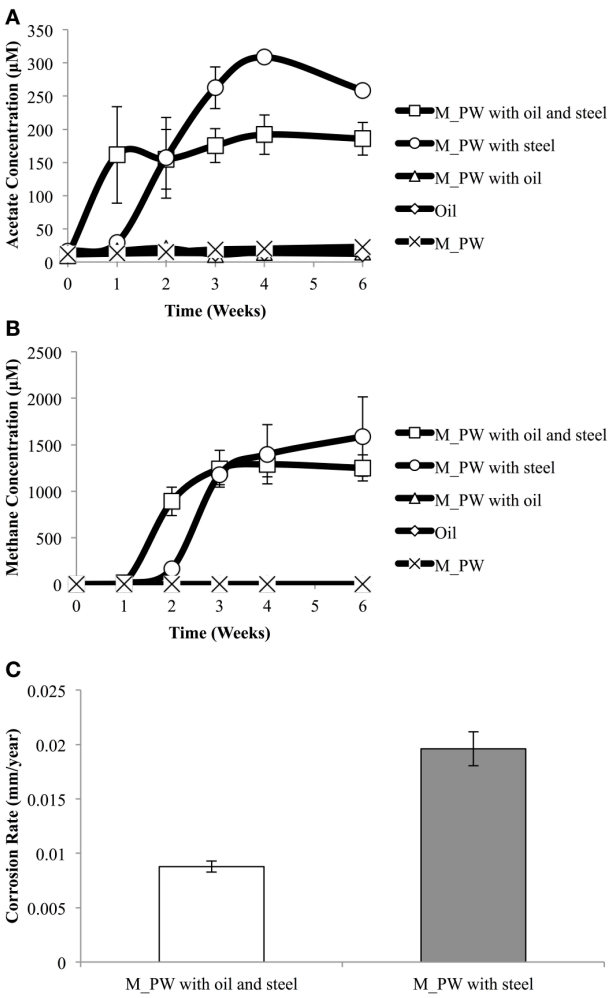

FIGURE 6 | A produced water sample from the MHGC field was incubated in a synthetic oil field brine (CSBK) medium with oil, with a metal coupon, with oil and a coupon, or by itself. Acetate **(A)** and methane **(B)** production were measured over time. Corrosion rates **(C)**, as indicated were measured following incubation. Data represent the values from two different incubations for each condition.

DISCUSSION

Microbial Communities and Potential for Biocorrosion

A previous study showed that the Obigbo oil field was dominated by methanogenic archaea, however it remained unclear how

these may be thriving (Okoro et al., 2014). Our results confirm the presence of methanogens, specifically the genus *Methanobacterium*, an organism catalyzing methanogenesis using either hydrogen and bicarbonate or formate ($4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$ or $4HCO_2^- + H_2O + H^+ \rightarrow CH_4 + 3HCO_3^-$) and also the genus *Methanosaeta*, which uses acetate to form methane ($CH_3COO^- + H^+ \rightarrow CO_2 + CH_4$). There are several roles these microorganisms may play in MIC. Electrons from steel (iron) and protons from water form hydrogen on steel surfaces and the hydrogenotrophic methanogens use this hydrogen for their metabolism and thereby accelerate steel corrosion (Daniels et al., 1987). Other methanogens play a role in corrosion because of their ability to use electrons from iron directly to reduce bicarbonate (Dinh et al., 2004). Lastly, methanogens are able to use metabolic products produced by other, potentially corrosive organisms, such as the SRB (Zhang et al., 2003) or the acetogenic bacteria (Mand et al., 2014) seen in previous studies and thereby may contribute indirectly to MIC.

In incubations containing carbon steel coupons, methane production could be correlated to metal weight loss corrosion rates. There was a positive correlation between the maximum concentration of methane produced and corrosion rates measured for the 7 field samples and one abiotic control. Therefore, methane production was probably a result of using the iron, either through hydrogen production on the surface of the steel or as a direct electron donor to reduce bicarbonate and form methane.

Another potentially corrosive taxon in the microbial community of the Obigbo samples included the genus *Desulfotigum,* found in 0.16–0.76% of the population of the produced water and pigging solids samples. This toluene-degrading SRB was found in highest proportion in the sample associated with crude oil (5_Crude), where toluene may be abundant (Ommedal and Torsvik, 2007). In addition to producing sulfide, it can contribute to corrosion by degrading oil organics into potentially corrosive organic acids, similar to another report of SRB producing potentially corrosive organic acids (Lyles et al., 2014). There were many other bacterial genera capable of hydrocarbon degradation present in the samples as well. These include *Thauera* and *Petrobacter,* capable of producing organic acids as metabolic intermediates (Biegert et al., 1996; Salinas et al., 2004). Even modest concentrations of some organic acids, such as acetic acid, may be corrosive toward carbon steel (Liu et al., 2008), and in this way, these organisms may contribute to metal weight loss and therefore MIC at the Obigbo site. Furthermore, simple intermediates formed by these microorganisms could serve as metabolites for methanogenesis, in a process known as syntrophy (Gieg et al., 2014).

We were able to show that despite the presence of microorganisms known to catalyze syntrophic hydrocarbon degradation, the presence of methanogenic archaea and the availability of easy to degrade oil components in field samples, insignificant methane production was seen. This shows that methane production resulting from the syntrophic biodegradation of hydrocarbons was not occurring.

Pipeline Pigging Solids Samples

The two pipeline pigging solids samples (6_PS and 7_PS) were quite similar to one another. This was first noted in the sample chemistry. The solids samples contained the highest levels of sulfate potentially due to sulfide oxidation, which could lead to SRB growth, the highest concentrations of sulfide, a potential indicator of SRB activity and the highest concentrations of ferrous iron, a potential indicator of steel corrosion (**Table 1**).

The microbial communities of the two pipeline solids samples also harbored many of the same microorganisms (**Figures 2E,F**, **3**). These were, in turn, quite different from the remaining water samples, including the pigging run water sample, 4_PigWater (**Figure 2C**). This may be an indication that the abundance and types of microorganisms found in the pipeline solids and potentially involved in corrosion are different from that in the planktonic population found in the pipeline fluid water samples.

When the community data were further examined, it was interesting to witness that between 70.8 and 93.5% of the microbial community of the water samples was classified as *Bacteria*, while the pigging samples were dominated by *Archaea* (79.5–85.6%). Therefore, the biofilm populations associated with pipeline surfaces, contained the highest proportion of methanogenic *Archaea* (**Figure 2**).

Microorganisms may predominantly live in biofilms on pipeline surfaces, instead of as planktonic free-flowing cells for a variety of reasons. A biofilm is a multifaceted structure, formed of different microbial cells embedded in a complex extracellular polymeric substance (EPS), and may be the preferred way for microbial growth in some natural environments (Costerton et al., 1995; Hall-Stoodley et al., 2004; Harrison et al., 2005). The cells within a biofilm are less vulnerable to eradication by chemical means or biopredation than free-swimming cells because they are protected by the EPS matrix and corrosion product matrix (Beech and Sunner, 2004). For example, when culture-based microbial enumerations were done, the highest counts of SRB and APB were found in the pigging solids samples, possibly because these cells were protected from chemical (e.g., biociding) and mechanical (e.g., pigging) processes. Living in this matrix is also beneficial for microbial cells because it serves to trap nutrients that may otherwise be lost in a fast-flowing pipeline. Evidence for this is suggested in the chemical data, which shows a higher concentration of ions, potential microbial nutrients, in the solids samples than the water samples (**Table 1**). In conclusion, it appears that pigging solids samples were more indicative than water samples for biocorrosion potential. In addition to taking aqueous samples when testing for microbes in the oil and gas industry, solids should also be sampled to obtain comprehensive microbial community data (Cote et al., 2014).

Comparison with a Canadian Low-Temperature Oil Field

The MHGC field is a low temperature oil reservoir located near Medicine Hat, AB, Canada (Voordouw et al., 2009; Agrawal et al., 2012). The reservoir temperature is only 30°C, somewhat lower than the Obigbo field (40°C), but both are conducive to microbial growth. The MHGC field uses water flooding to produce oil, and the injection water contains

a small amount (average 0.8 mM) of sulfate. Contrary to the Obigbo field, this field is flooded with nitrate to curb negative SRB activity (Voordouw et al., 2009; Agrawal et al., 2012). 454 pyrotag sequencing has shown that the majority of the microbial populations found in produced waters are methanogenic *Archaea* (Callbeck et al., 2013), similar to the Obigbo field.

Incubations of Obigbo pipeline pigging solids showed methane production in the presence of steel and a community composition enriched in hydrogenotrophic methanogens, acetogenic bacteria (*Acetobacterium)* and acetotrophic methanogens (*Methanosaeta*). These incubations were unable to uncover the role of acetate-producing organisms in MIC. When a produced water sample from the MHGC field was used to repeat the experiment, we were able to show methane and acetate production due to the presence of carbon steel coupons. Pyrotag sequencing of these samples revealed the same, potentially corrosive acetogenic bacteria (*Acetobacterium*) and acetotrophic methanogens (*Methanosaeta*). Since these same organisms were also found in the Obigbo pigging solids samples, these results suggested a role for these microorganisms in MIC at the Obigbo site.

While we have previously shown in pure culture studies that the acetate requirement of corrosive SRB may be fulfilled by corrosive acetogens (Mand et al., 2014), similar syntrophic roles may also be played in methanogenic consortia. In the present study it appears that in addition to corrosive hydrogenotrophic methanogens, corrosive acetogens may also produce acetate, which can then lead to acetotrophic methane production. The acetate may also serve as a carbon source for hydrogenotrophic methanogenesis.

CONCLUSIONS

While many studies into biocorrosion continue to focus on microorganisms traditionally associated with MIC such as the SRB, we have shown here that oil field samples may contain an abundance of methanogenic archaea and hydrogenotrophic acetogens, which may also be active in steel corrosion. We were able to show that these groups were most active when incubated in the presence of carbon steel, indicating that the steel was preferred as the electron donor over oil organics by the methanogenic and acetogenic consortia present in these samples. Furthermore, we have also shown here, through genomic studies, that the microbial populations of pipeline solids samples may not be the same as the microbial population of liquid samples. In this study, it appears that the microbial communities most closely associated with pipeline surface biofilms were potentially more active in MIC than planktonic microorganisms associated with the water samples. This work emphasizes the need to obtain representative field samples in order to obtain accurate and useful MIC data.

AUTHOR CONTRIBUTIONS

JM and HP designed and carried out the study, collected data, performed the analysis and wrote the manuscript. CO, BL, SS, LC, and GV contributed to data interpretation and preparation of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01538

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Metabolic Capability of a Predominant *Halanaerobium* sp. in Hydraulically Fractured Gas Wells and Its Implication in Pipeline Corrosion

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Microbial activity associated with produced water from hydraulic fracturing operations can lead to gas souring and corrosion of carbon-steel equipment. We examined the microbial ecology of produced water and the prospective role of the prevalent microorganisms in corrosion in a gas production field in the Barnett Shale. The microbial community was mainly composed of halophilic, sulfidogenic bacteria within the order *Halanaerobiales*, which reflected the geochemical conditions of highly saline water containing sulfur species ($S_2O_3^{2-}$, SO_4^{2-}, and HS^-). A predominant, halophilic bacterium (strain DL-01) was subsequently isolated and identified as belonging to the genus *Halanaerobium*. The isolate could degrade guar gum, a polysaccharide polymer used in fracture fluids, to produce acetate and sulfide in a 10% NaCl medium at 37°C when thiosulfate was available. To mitigate potential deleterious effects of sulfide and acetate, a quaternary ammonium compound was found to be an efficient biocide in inhibiting the growth and metabolic activity of strain DL-01 relative to glutaraldehyde and tetrakis (hydroxymethyl) phosphonium sulfate. Collectively, our findings suggest that predominant halophiles associated with unconventional shale gas extraction could proliferate and produce sulfide and acetate from the metabolism of polysaccharides used in hydraulic fracturing fluids. These metabolic products might be returned to the surface and transported in pipelines to cause pitting corrosion in downstream infrastructure.

Keywords: halophilic, *Halanaerobium*, thiosulfate reducing bacteria, guar gum, biocorrosion, hydraulic fracturing

INTRODUCTION

It is estimated that the percentage of total natural gas supplies from geological shale formations will increase from approximately 20% (2010) to 50% by 2025 in the United States (Davis et al., 2012). Such remarkable growth of shale gas production is largely attributed to the advancement of hydraulic fracturing technologies (Vengosh et al., 2014). Hydraulic fracturing involves the process of injecting large volumes of water, proppant and a myriad of chemical additives into the deep subsurface with high pressure to liberate natural gas from shale formations (Gregory et al., 2011).

Microbial activity (e.g., biogenic sulfide production) associated with produced water can lead to gas souring and corrosion of production facilities (Davis et al., 2012). Since biocorrosion can lead to disruptions of gas pipelines and storage tanks, considerable efforts have been made by the oil and gas industry to monitor and control deleterious microbial activities during hydraulic fracturing operations (Struchtemeyer et al., 2012; Gaspar et al., 2014; Kahrilas et al., 2014).

Despite treatments of the fracturing fluid with biocides and the extreme physicochemical conditions (e.g., high salinity, Supplementary Table S1) in the fractured deep subsurface, a stream of recent studies (see Supplementary Table S1) has revealed that similar dominant microorganisms were associated with produced water from various hydraulically fractured shale formations in the United States (Davis et al., 2012; Struchtemeyer and Elshahed, 2012; Murali Mohan et al., 2013a; Strong et al., 2013; Wuchter et al., 2013; Cluff et al., 2014). Typically, the microbial community was mostly composed of halophiles including acid-producing bacteria and multiple lineages of sulfidogenic microorganisms (Davis et al., 2012; Struchtemeyer and Elshahed, 2012; Murali Mohan et al., 2013a,b; Cluff et al., 2014). Most of the dominant halophilic species were affiliated with the genus *Halanaerobium*, which can sometimes account for over 99% of the microflora in produced water (Murali Mohan et al., 2013a; Cluff et al., 2014). Members of the genus *Halanaerobium* are well known for fermentation of carbohydrates and sulfide production through thiosulfate reduction (Ravot et al., 1997, 2005). Therefore, *Halanaerobium* is clearly an important genus that might play vital roles in the biodegradation of organic matter and production of sulfide in fractured shale formations (Akob et al., 2015).

Numerous organic chemical additives are typically found in hydraulic fracturing fluids (Stringfellow et al., 2014; Kekacs et al., 2015) that could serve as electron donors to support microbial growth and sulfidogenic processes (Youssef et al., 2009; Strong et al., 2013). Among them, guar gum is a principal component of fracturing fluids and is commonly used as a thickening agent in the oil and gas industry (Lester et al., 2013; Stringfellow et al., 2014). Recently, activated sludge and microbial mats were proposed as efficient biological treatments to aerobically degrade such high-molecular weight polysaccharides in flowback wastewater from hydraulic fracturing (Lester et al., 2013; Akyon et al., 2015). Additionally, other studies have indicated that concentrations of dissolved organic carbon in produced water decreased rapidly via abiotic or biotic processes in the subsurface (Strong et al., 2013; Cluff et al., 2014). However, the biodegradation of guar gum by the dominant anaerobic microorganisms in the fractured shale formation remains unknown. In this regard, we hypothesized that the dominant *Halanaerobium* species in the produced water can contribute to the decomposition of guar gum and the production of acetate and sulfide in the fractured deep subsurface.

Better understanding of the *in situ* metabolic activity of the prevalent *Halanaerobium* in complex subterranean environments may help in the control of these acid-producing and sulfidogenic organisms and further mitigate biocorrosion problems. However, culture-independent molecular approaches only provide phylogenetic information and genomic potential of the associated microbial community (Mohan et al., 2014). These molecular techniques such as high-throughput sequencing typically cannot distinguish between DNA from live organisms and dead cells (Cluff et al., 2014), potentially leading to biased conclusions on the *in situ* metabolic activity of indigenous microorganisms. In contrast, cultivation-based approaches can provide complementary insight on the physiology and possible interactions among the live, cultivable microbes. The efficacy of biocides against the indigenous microorganisms can also be critically evaluated against isolates or enrichment cultures to provide guidance on field treatment (Struchtemeyer et al., 2012; Gaspar et al., 2014; Santillan et al., 2015). Therefore, the integration of both culture-dependent and -independent techniques will help to gain a better understanding of the fate of organic matter in the fractured deep subsurface and the potential role of associated metabolites such as organic acids and sulfide in the corrosion of carbon-steel pipelines.

The objective of this study was to examine the microbial ecology of produced water associated with hydraulic fracturing and the potential roles of the dominant microorganisms in corrosion of gas pipelines. We characterized the geochemistry and microbial assemblages in produced water from a hydraulically fractured site in the Barnett Shale (Texas City, Texas, USA). One of the predominant organisms (*Halanaerobium* sp. strain DL-01) was subsequently isolated and its metabolic capability in the biodegradation of guar gum was further examined under both fermentative and thiosulfate-reducing conditions. Moreover, the efficacy of biocides against strain DL-01 was critically evaluated to potentially decrease the prevalence of *Halanaerobium* and thereby mitigate deleterious biocorrosion processes in shale gas production facilities.

MATERIALS AND METHODS

Site Description and Samples Collection

Samples of produced water were collected from a shale gas production field in the Barnett Shale near Arlington, Texas, USA in July and September 2012, respectively. Multiple wells (A1–A6) were hydraulically fractured in the sampling site and the schematic is represented in **Figure 1**. The upstream comingled produced water (UCPW) near the gas-water separator (**Figure 1**) was collected in a 1L polypropylene bottle closed without a headspace (Nalgene, Rochester, NY, USA). The temperature of the upstream sample was ∼37°C. Downstream water from the receiver (DRW) along the gas pipeline (**Figure 1**) was obtained in the similar manner. The samples for geochemical analyses and cultivation were stored at 4°C before use. For microbial community characterization, 250–300 mL samples were filtered in the field using Nalgene analytical filter funnels containing nitrocellulose filters (sterile, 0.45 μm, Nalgene Fisher Scientific, Pittsburgh, PA, USA). The filters were placed into sterile 50 mL centrifuge tubes and preserved with 1 mL of DNAzol (DNAzol DN127, Molecular Research Center, Inc., Cincinnati, OH, USA). The tubes containing the filters were placed on ice until returned to the laboratory (within

5 h) and then transferred to a freezer (−85°C) until DNA extraction.

Microbial Enumeration

A most probable number (MPN) procedure was used to enumerate sulfate-reducing (SRB), acid-producing (APB), and thiosulfate-reducing bacteria (TRB). For SRB and APB, commercially produced media (C&S Labs, Tulsa, OK, USA) were directly compared with media prepared in the lab upon return of samples. All MPN media formulations were adjusted to pH 7.0 and salinities of 5% and 10% (w/v) NaCl, based on available commercial medium salinities routinely employed at this site. The enumeration of SRB was conducted using RST-API medium (Tanner, 1989). The media for APB and TRB enumeration were prepared according to NACE (National Association of Corrosion Engineers) standard TM0194 and others (TM0194, 2004; Tanner et al., 2007). The original aqueous sample (1 mL) was inoculated into 9 mL of medium in Balch tubes with a sterile syringe flushed with N_2. Serial dilutions (10-fold) were made by transferring 1 mL of each dilution to separate Balch tubes containing 9 mL of fresh medium. MPN tubes were prepared in triplicate and incubated at 37°C for 4 weeks. Each group of bacteria was enumerated with the statistical table described previously (Banwart, 1981).

Microbial Community Characterization

Filters preserved in the field were thawed and DNA was extracted using the Promega Maxwell® 16 Tissue LEV Total RNA purification kit as described previously (Oldham et al., 2012). The pooled 16S rRNA gene library was prepared with primers S-D-Arch-0519-a-S-15 and S-D-Bact-0785-b-A-18 (Klindworth et al., 2012) modified to include a 16 bp M13 sequence to allow for the addition of a 12 bp barcode in a two-step PCR unique to each library (Hamady et al., 2008; Wawrik et al., 2012). The libraries were sequenced on the Illumina MiSeq platform using V2 PE250 chemistry. Paired reads were joined and then demultiplexed in QIIME (Quantitative Insights Into Microbial Ecology) software package (Caporaso et al., 2010). Chimeras were removed and Operational Taxonomic Units (OTUs) were assigned at 97% similarity using USEARCH 6.1.544 (Edgar, 2010). Taxonomy was assigned using the RDP (Ribosomal Database Project) naive Bayesian classifier (Wang et al., 2007) against the SILVA (r SSU, small subunit) database release 111 (Yilmaz et al., 2013). Raw sequences were submitted to the NCBI sequence read archive (SRA) database (Accession number: SRX1046644).

Enrichment and Isolation

Samples (10 mL) of upstream, comingled water (UCPW) and downstream water from the receiver (DRW) were inoculated into 160 mL serum bottles containing 50 mL of reduced marine mineral medium (Widdel and Bak, 1992) adjusted to 4 and 10% salinity. In order to test potential relevant electron donors in the fractured subterranean systems, the incubations received the following compounds as sources of energy and carbon: (1) gas condensate (1 μL); (2) 20 psi gas mixture of methane, ethane, butane, and propane (1:1:1:1); (3) 20 psi H_2:CO_2 (80:20); (4) a mixture of pyruvate (10 mM) and lactate (10 mM); (5) elemental iron (as granules). Sterile and substrate-free controls were provided. All enrichments were incubated at 31°C and monitored for growth by following sulfate consumption and microscopic cell counts. Incubations showing active growth were transferred, and enrichments obtained in 10% NaCl medium were used for isolations.

Initial isolations were performed using serial 10-fold dilutions in the same basal medium, (Widdel and Bak, 1992) containing 10% NaCl and glucose (20 mM) as a growth substrate at 31°C. After several subsequent dilutions, pre-purified cultures were used as inoculum for anaerobic culturing bottles containing the same glucose-based medium solidified with 2% agar. After 7 days of incubation, isolated colonies were picked from the culture bottles inside an anaerobic glove chamber. An isolate obtained in this manner and designated strain DL-1 was maintained in sulfate-free, reduced marine mineral medium containing 10% NaCl, glucose (20 mM) and yeast extract (0.001%). The purity of the isolate was checked by microscopy. Growth was tested with the following electron donors: galactose (20 mM), mannose (20 mM), guar gum (0.5% w/v), and cellulose (0. 2% w/v cut filter paper). Thiosulfate (10 mM) and sulfate (20 mM) were tested as electron acceptors. Growth was monitored by optical density measured at 600 nm (where possible), protein production and by sulfide production (see Analytic techniques).

Sequencing and Phylogenetic Analysis

Cells in stationary phase cells were collected by centrifugation at 8000 × *g* for 15 min at room temperature. Cell pellets were further treated with 20 μg/mL proteinase K (Promega) for 15 min at room temperature. Genomic DNA was extracted with the Maxwell 16 Tissue LEV total RNA purification kit as previously described (Oldham et al., 2012). The 16S rRNA gene of strain DL-01 was amplified using PCR Supermix (Invitrogen, Carlsbad, CA, USA) with universal primers fD1 and rP2 described previously (Weisburg et al., 1991). The sequence was determined by Sanger sequencing on ABI 3730 (Applied Biosystems, Foster City, CA, USA). The quality of the obtained sequence was verified and assembled with the program suite Sequencher version 5.1 (Gene Codes Corp., Ann Arbor, MI, USA). The assembled sequence (length = 1362 bp) was aligned with other closely related type strain sequences retrieved from the NCBI Genbank. The phylogenetic analysis was performed using MEGA 6.0 using the neighbor-joining method (Tamura et al., 2013) and bootstrap analysis with 1000 replicates. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DL-01 is KR612329.

Analytical Techniques

The anions sulfate and chloride were analyzed by ion chromatography as described previously (Lyles et al., 2013). Sulfate was determined in the samples pretreated using Dionex OnGuardII Ag/Na cartridges (Thermo Fisher Scientific, Sunnyvale, CA, USA) to remove halides as described by the manufacturer. The pH was measured in the field with pH strips (color pHast indicator strips pH 5–10; EM Science, Gibbstown, NJ, made in Germany). The concentration of thiosulfate

TABLE 1 | Geochemical characteristics of produced water from the upstream (UCPW) and downstream (DRW) of a shale gas production facility in Barnett Shale formations (Figure 1).

Samples	Sampling time	pH	Sulfate (mM)	Fe^{2+} (mM)	Sulfide (mM)	Thiosulfate (mM)	Salinity (Cl^- g/L)	Acetate (mM)
Upstream(UPCW)	July 2012	7.0	0.74	1.73	0.04	BDL[a]	117	NR[b]
	September2012	6.8	1.84	0.73	BDL	BDL	46.8	0.5
Downstream(DRW)	July 2012	6.5	0.79	NR	0.25	BDL	19.7	NR
	September2012	7.0	1.93	11.76	BDL	0.17	16.9	170

[a]*BDL, below detection level (5 ppm);* [b]*NR, data not recorded.*

was quantified using an iodometric CHEMetrics thiosulfate titration kit (CHEMetrics, Inc., Calverton, VA, USA). Dissolved ferrous iron and sulfide were measured by the ferrozine assay (Stookey, 1970) and methylene blue method (Tanner, 1989) as previously described. Protein was determined using the Thermo Scientific™ Pierce™ BCA™ Protein Assay (Thermo Scientific, Pittsburgh, PA, USA) according to manufacturer's instructions. Fermentation products such as formate, acetate, pyruvate, and lactate were measured by high performance liquid chromatography (HPLC, Dionex model IC-3000, Sunnyvale, CA, USA) as previously described. (Lyles et al., 2014) The wavelength of the UV absorbance detector was set at 210 nm and the mobile phase was 60% (vol/vol) KH_2PO_4 (25 mM, pH 2.5) and 40% acetonitrile. The pump was operated at a flow rate of 1 mL/min. In addition, portions of the samples were diluted in 30 mM oxalic acid in order to measure ethanol by gas chromatography with flame ionization detection (GC-FID) under the operating conditions described in Davidova et al. (2012).

Efficacy of Biocides against Strain DL-01

The efficacy of common biocides used in the oil and gas industry was determined against *Halanaerobium* sp. strain DL-01 under fermentative and thiosulfate-reducing conditions. The biocides tested included glutaraldehyde, tetrakis (hydroxymethyl) phosphonium sulfate (THPS) and benzyldodecyldimethylammonium chloride (a representative quaternary ammonium compound, abbreviated as QAC). Glucose (20 mM) was used as a substrate and strain DL-01 was grown in 10% NaCl marine mineral medium (Widdel and Bak, 1992) at 37°C. The assay was performed in Balch tubes containing 9 mL medium and 1 mL cells of strain DL-01. The inoculated media were exposed to different dosages of biocides (final concentrations varying from 0 mg/L to 500 mg/L depending on the minimum inhibitory concentration of each biocide) under fermentative conditions (no electron acceptor) and thiosulfate-reducing conditions (10 mM thiosulfate). The corresponding sterile (inoculated with heat-killed cells of strain DL-01) and inhibitor-free controls served as the basis for determining the efficacy of biocides. Optical density (600 nm wavelength) was measured over time to monitor the microbial growth. Additionally, acetate and sulfide were measured at the conclusion of the experiment as further evidence of microbial activity.

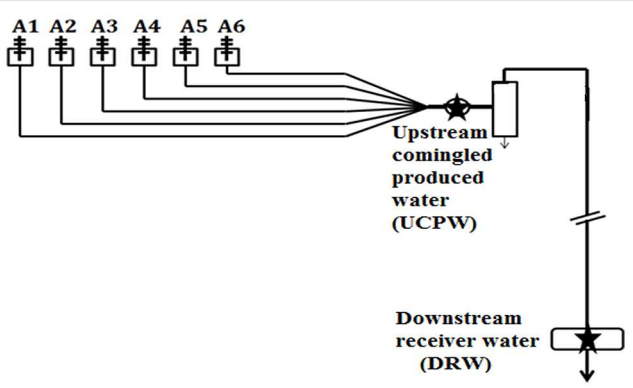

FIGURE 1 | Schematic diagram of the shale gas production facility in Barnett Shale. Six wells (A1–A6) were drilled in this site and the stars indicated the locations where upstream (UCPW) and downstream (DRW) samples were collected. The distance between the two locations is around 1 km. Severe corrosion was detected in the downstream pipeline where replacement was mandated for maintenance.

RESULTS

Geochemistry and Microbial Enumerations

The geochemistry of the produced water in the upstream (UCPW) and downstream (DRW) samples is summarized in **Table 1**. The pH in all samples was between 6.5 and 7.0. The content of Cl^- in the UCPW (4.68–11.7%) was much higher than the DRW sample (1.69–1.97%). The low salinity in the downstream produced water might be attributed to the introduction of external water into the system. Notably, dissolved ferrous iron (Fe^{2+}) in DRW (11.76 mM) was much higher than UCPW (0.73 mM) probably due to the corrosion that occurred in the downstream pipeline (DRW) (**Figure 1**). In addition to sulfate and sulfide, a low level of thiosulfate was detected in the DRW (0.17 ± 0.01 mM). These data suggest that sulfate and thiosulfate could be potential electron acceptors for sulfide production. Interestingly, the concentration of acetate in the DRW (170 mM) was much higher than the UCPW sample (0.5 mM). The number of cultivable acid producing bacteria in UCPW was 5×10^3 cells/mL when grown in medium containing 10% NaCl (Supplementary Table S2), which was close to the salinity of the original sample (11.7% NaCl). However, only minimal growth of thiosulfate and sulfate-reducing bacteria was observed. None of the tubes in the DRW sample showed growth.

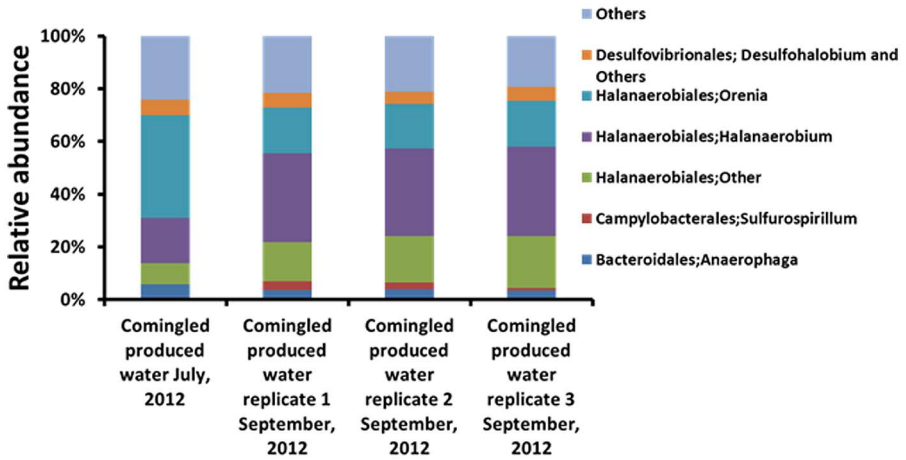

FIGURE 2 | Relative abundance of major taxa (Genus level classification) in upstream comingled produced water (UCPW). Three biological replicates (1, 2, and 3) were included in the later produced water collected in September, 2012.

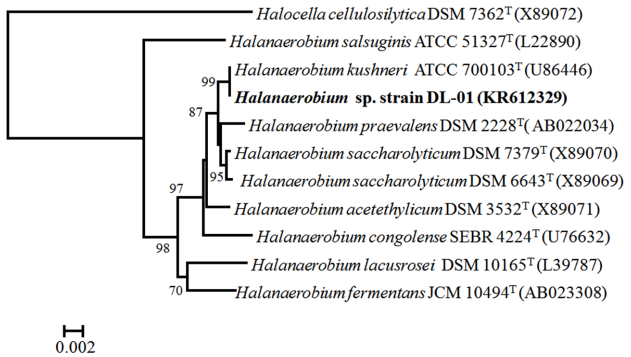

FIGURE 3 | Phylogenetic relationship of *Halanaerobium* sp. strain DL-01 (in bold) to other species within the genus *Halanaerobium*. The accession number of each strain is indicated in the parenthesis. The tree was constructed based on approximately 1362 bp 16S rRNA gene sequences. One thousand bootstrap replications were performed and only those greater than 700 are shown. Bar indicated 2 nucleotide substitutions per 1000 bp.

Microbial Community Characterization

No quantifiable or amplifiable DNA could be extracted from DRW samples. In contrast, an appreciable amount of DNA was obtained from UCPW sampled in both July and September, 2012. The microbial assemblages associated with the UCPW samples were characterized by high-throughput sequencing of PCR-amplified 16S rRNA gene libraries. Sequences affiliated with the order Halanaerobiales were numerically dominant (64.4–70.7%) in both UCPW samples (**Figure 2** and Supplementary Figure S1). Representative sequences from these OTUs were primarily affiliated with the genera *Orenia* and *Halanaerobium* (**Figure 2**). Interestingly, the relative abundance of OTUs most closely related to the genus *Halanaerobium* increased to become the most dominant taxon (from 17 to ∼33%) in the produced water collected in September, whereas the genus *Orenia* decreased from 39 to ∼17% (**Figure 2**). In addition, sequences affiliated with the order *Desulfovibrionales* only accounted for ∼5% of the total bacterial community (Supplementary Figure S1).

Isolation and Characterization

Microbial growth was initially observed with the UCPW inoculum when either pyruvate or lactate served as a substrate. Subsequent transfers revealed that the enrichment was also capable of growth with glucose as a carbon and energy source. Repeated transfer of the enrichment culture and eventual isolation of individual colonies on the same glucose-based medium solidified with 2% agar ultimately was used to obtain a pure culture. The colonies were round, smooth and opaque. Cells of strain DL-01 were short rods and usually appeared in pairs or have been assembled in string-like chains (Supplementary Figure S2). Isolate DL-01 could grow under a broad range of salinities ranging from 2 to 15% NaCl, and could ferment glucose, galactose, mannose and the polysaccharide, guar gum. Thiosulfate was found to be a suitable electron acceptor when strain DL-01 was grown with various carbohydrates including guar gum. Sulfate was not utilized as an electron acceptor when strain DL-01 was grown with guar gum. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain DL-01 was likely a member of the genus *Halanaerobium* and most closely related to the type strain of *Halanaerobium kushneri* ATCC 700103^T (**Figure 3**). Notably, the 16S rRNA gene sequence of strain DL-01 places it within OTU-3 (Supplementary Figure S3), suggesting that the dominant *Halanerobium* phylotype was isolated from UCPW.

Acetate and Sulfide Production by *Halanaerobium* sp. Strain DL-01

Strain DL-01 was capable of growth with guar gum as the sole carbon and energy source under both fermentative and

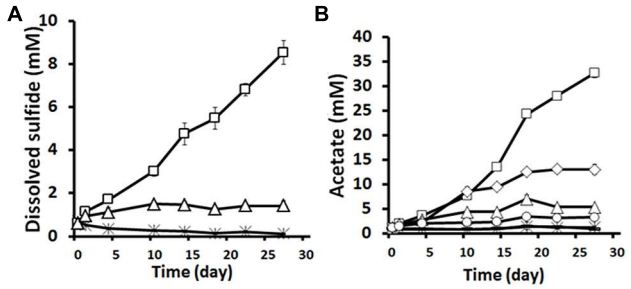

FIGURE 4 | Sulfide (A) and acetate (B) production by strain DL-01 when grown on 0.5% guar gum under fermentative and thiosulfate-reducing conditions. Legends: □, 0.5% guar gum+thiosulfate; Δ,no guar gum+thiosulfate; ⁎, sterile (0.5% guar gum+thiosulfate); ◇, 0.5% guar gum+no thiosulfate; ∘, no guar gum+no thiosulfate; −, (overlapping data with ⁎), sterile (0.5% guar gum+ no thiosulfate). Sterile indicates that cells of DL-01 were heat killed by autoclaving.

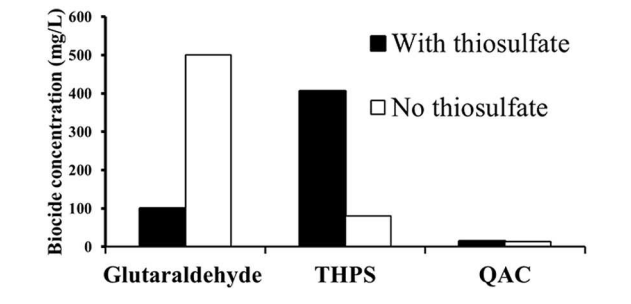

FIGURE 5 | Minimum inhibitory concentrations of biocides against *Halanaerobium* DL-01 based on microbial growth, sulfide, and acetate production relative to sterile controls, which the cells of DL-01 were heat-killed by autoclaving. Black and clear bars indicate the conditions with and without thiosulfate, respectively.

thiosulfate-reducing conditions. During the growth with guar gum and thiosulfate, 7.1 ± 0.3 mM dissolved sulfide (excluding background level) was accumulated in 27 days (**Figure 4A**). No significant sulfide production was detected in the sterile control and the control without guar gum. The growth with guar gum without an electron acceptor resulted in accumulation of acetate (11.8 ± 0.8 mM, **Figure 4B**). However, if thiosulfate was provided as an electron acceptor, twice the amount of acetate was produced during the same time period (**Figure 4B**). In addition, small amounts of formate (0.2–0.9 mM) and ethanol (1.1–3.4 mM) were also detected.

Efficacy of Biocides against Strain DL-01

The efficacy of biocides against strain DL-01was assessed on the basis of sulfide and acetate production (Supplementary Figures S4 and S5) and growth relative to sterile controls. A relatively low dosage of QAC (13.5 mg/L) was sufficient to completely inhibit acetate and sulfide production under both thiosulfate-reducing (Supplementary Figure S4B) and fermentative conditions (Supplementary Figure S4A). The efficacy of glutaraldehyde against strain DL-01 was much higher in the presence of thiosulfate (100 mg/L, Supplementary Figures S5A,B) than in its absence (500 mg/L, Supplementary Figure S4B). In contrast, THPS (81 mg/L) completely inhibited microbial growth and thus, acetate production when no thiosulfate was present (Supplementary Figure S5D). However, up to 406 mg/L THPS showed no inhibition on sulfide and acetate production when thiosulfate was present (Supplementary Figures S5C,D). Accordingly, the minimum inhibitory concentrations (the lowest dosage to completely inhibit microbial growth and activity) of the three biocides are summarized in **Figure 5**. The minimum inhibitory concentration of QAC (13.5 mg/L) was much lower than that of glutaraldehyde (500 mg/L in the absence of thiosulfate) and THPS (no inhibition up to 406 mg/L in the presence of thiosulfate).

DISCUSSION

Microbial activities associated with hydraulically fractured shale formations are of great concern to the oil and gas industry due to the potential for corrosion of pipelines, separators and storage tanks (Struchtemeyer and Elshahed, 2012). We characterized the geochemistry and microbial community of produced water from a shale gas production field in part of the Barnett Shale system. Typically, low levels of acetate (up to ∼0.9 mM) are detected in produced waters from hydraulically fractured sites (Orem et al., 2014). In contrast, we detected an extremely high concentration of acetate (170 mM) in DRW (**Figure 1**). While acetate can be introduced through hydraulic fracturing (Sydansk, 1988), microbial activity might play a substantial role in the production of acetate. Regardless of the sources of acetate, its accumulation in produced water can potentially exacerbate corrosion of carbon-steel equipment (Azambuja and Muller, 1994; Suflita et al., 2008). In addition, sulfidogenic activity might also contribute to the observed corrosion of carbon-steel in the DRW where sulfide (0.25 mM) and sulfate (0.74–1.93 mM) were detected. Although the rapid rate of microbial metabolism (Jørgensen, 1990) makes measurement of thiosulfate quite challenging, thiosulfate (0.17 ± 0.01 mM) was detected in the DRW produced water when measured in the field using a thiosulfate titration kit. Moreover, a recent study found that the major fraction of the total sulfur in produced water was present as non-sulfate compounds and thereby the importance of sulfidogenic potential of non-sulfate-reducing microorganisms was implied in the fractured subsurface (Murali Mohan et al., 2013a). In fact, fermentative, thiosulfate-reducing bacteria have been demonstrated to be important in catalyzing corrosion of carbon steel in oil production facilities (Magot et al., 1997; Liang et al., 2014).

Molecular characterization of the microbial assemblages in each sample revealed that the most abundant taxa in the upstream produced water were members of the order *Halanaerobiales*, which is consistent with previous studies on the microbial ecology of produced water from shale gas extraction (Davis et al., 2012; Struchtemeyer and Elshahed, 2012; Murali

Mohan et al., 2013a,b; Strong et al., 2013; Wuchter et al., 2013; Cluff et al., 2014). Such predominance of halophilic microorganisms suggested that the relative high salinity in the upstream produced water plays important roles in shaping the microbial community. Earlier studies have shown that members of the genus *Halanaerobium* increased dramatically over time in the produced water from the Marcellus and Barnett shale formations in the United States (Davis et al., 2012; Murali Mohan et al., 2013a; Cluff et al., 2014). Despite the limited sampling time points in this study (July and September, 2012), the relative abundance of *Halanaerobium* increased from 17 to ∼33% in the later produced water sample (**Figure 2**). Although sulfate-reducing bacteria within *Deltaproteobacteria* were not typically detected in previous studies (Murali Mohan et al., 2013a; Cluff et al., 2014), members of the order *Desulfovibrionales* were present in low relative abundance (∼5%; Supplementary Figure S1). The genera *Desulfohalobium* (Ollivier et al., 1991; Jakobsen et al., 2006) and *Desulfovermiculus* (Belyakova et al., 2006) were reported to be halophilic sulfate reducers, suggesting that sulfate reduction might also contribute to sulfidogenesis in subterranean systems with high salinity. Sequences affiliated with *Epsilonproteobacteria* (∼3%) were detected in the produced water sampled in September. The majority of the OTUs within the *Epsilonproteobacteria* were affiliated with the genus *Sulfurospirillum* (**Figure 2**), a group of metabolically versatile bacteria previously shown to reduce sulfur and thiosulfate (Kodama and Watanabe, 2007). The presence of multiple lineages of sulfidogenic organisms and sulfur species ($S_2O_3^{2-}$, SO_4^{2-}, and HS^-) suggested that active sulfur cycle involving thiosulfate might be occurring in the deep subterranean shale formations after hydraulic fracturing.

The ubiquity and abundance of members of the genus *Halanaerobium* found in high salinity production water suggests that they could play crucial roles in the cycling of carbon and sulfur in these environments (Murali Mohan et al., 2013b; Cluff et al., 2014). Cultivation of the abundant microbes in produced water is important to understand the physiology of these taxa and their potential role in corrosion. A numerically dominant organism was isolated from the produced water in this study and identified as *Halanaerobium* sp. DL-01. This isolate was highly similar to the most abundant OTU from the genus *Halanaerobium* within the microbial assemblage of upstream produced water (Supplementary Figure S3). Further phylogenetic analysis revealed that strain DL-01 was most closely related to *H. kushneri* isolated from high saline produced water from an oil reservoir in Central Oklahoma (Bhupathiraju et al., 1999). *H. congolense* (Ravot et al., 1997) and *H. salsugo* (Bhupathiraju et al., 1999) have also been isolated from oil fields, alluding to the ecological importance of the genus *Halanaerobium* in high saline produced water in oil and gas production facilities.

Many species of *Halanaerobium* are capable of fermenting carbohydrates to acetate and other organic acids (Ravot et al., 1997, 2005). We found that strain DL-01 could produce acetate through the fermentation of guar gum, the major gelling agent in the fracturing fluid to increase viscosity (Lester et al., 2013). Most importantly, strain DL-01 was able to generate sulfide from the reduction of thiosulfate, but it was unable to utilize sulfate. Thiosulfate and elemental sulfur may be used as electron acceptors by various *Halanaerobium* species but none of the validated described organisms are known to use sulfate in this manner (Oren, 2015). Of the numerous chemical additives introduced into the shale formation during hydraulic fracturing, the organic constituents in the fracturing fluid, such as hydrocarbon distillates and carbohydrate polymers (e.g., guar gum) (Cluff et al., 2014), could conceivably serve as electron donors to stimulate potentially deleterious microbial processes such as acetate and sulfide production. The fate of the organic matter in fracturing fluids injected into the deep subsurface is poorly understood, but a recent metagenomic study of produced water from shale gas extraction revealed a relatively high abundance of functional genes associated with metabolism of mono- and polysaccharides (Mohan et al., 2014). Furthermore, other studies have found that the concentration of dissolved organic matter in produced water decreased over time due to abiotic or biotic processes occurring in the deep subsurface (Cluff et al., 2014; Orem et al., 2014). Until the study described here, the numerically dominant *Halanaerobium* spp. in produced water had not been shown to biodegrade organic matter (e.g., guar gum) in the high saline water and contribute to the production of acetate and sulfide.

Since the corrosiveness of sulfide and acetate is well-known in the oil and gas industry (Liang et al., 2014), the collective findings led us to propose a possible corrosion scenario (Supplementary Figure S6) in the hydraulically fractured site in Barnett Shale. Fracturing fluids containing biodegradable polysaccharide polymers like guar gum (0.1–0.5%) get injected deep into shale formations (Lester et al., 2013). The abundant members of the genus *Halanaerobium* decomposed the guar gum, producing acetate, and sulfide (if thiosulfate is available). Eventually, the produced acetate and sulfide could be returned in the produced water and transported to downstream pipeline networks (Supplementary Figure S6). Acetate (170 mM) and sulfide (0.25 mM) detected in saline production waters could act synergistically (Singer et al., 2007) to corrode the downstream production facilities such as gathering pipelines and storage tanks.

To mitigate the potential corrosion caused by the abundant members of the genus *Halanaerobium*, the efficacy of three biocides was evaluated against *Halanaerobium* sp. DL-01. A relatively high dosage of glutaraldehyde (500 mg/L) was required to completely inhibit strain DL-01. This concentration was much higher than the minimum inhibitory concentrations for *Desulfovibrio alaskensis* strain G20 (12.5 mg/L) and a sulfate-reducing enrichment culture (100 mg/L) obtained from fracturing fluid (Struchtemeyer et al., 2012). Interestingly, exposure of *Pseudomonas fluorescens* to produced water can cause an increased resistance to glutaraldehyde (Vikram et al., 2014). It is unclear, however, whether the enhanced tolerance against glutaraldehyde in strain DL-01 was triggered by a similar mechanism. The presence of thiosulfate did increase the resistance of *Halanaerobium* DL-01 to THPS (no inhibition up to 406 mg/L). The ineffectiveness of THPS might be attributed to potential interaction between THPS and thiosulfate

(Williams and McGinley, 2010). Notably, QAC was found to be more efficient than glutaraldehyde and THPS (**Figure 5**) under both thiosulfate-reducing and fermentative growth conditions. Therefore, the preferential utilization of QAC might be considered to decrease the microbial activity of the dominant *Halanaerobium* in high salinity produced water. In addition, future work is needed to assess the synergistic effect of multiple biocides (Kahrilas et al., 2014) against strain DL-01 and the underlying mechanisms for the potential resistance to glutaraldehyde and THPS in high saline brines (Vikram et al., 2014).

It is generally believed that problematic microorganisms are directly associated with severe corroding sites when assessing risks of biocorrosion in the oil and gas industry. The research presented here implicated that the sulfidogenic microorganisms (e.g., *Halanaerobium* sp. DL-01) in the produced water after hydraulic fracturing could play an important role in the biodegradation of organic carbon such as guar gum to produce acetate and sulfide in the fractured deep shale formations. The produced acetate and sulfide could be transported to aboveground with the returning water (Supplementary Figure S6) and might greatly contribute to corrosion of carbon-steel gathering pipelines and other equipment in distal, downstream locations (Supplementary Figure S6). The findings on the efficacy of biocides against strain DL-01 should ultimately help select suitable biocides to decrease the prevalence of *Halanaerobium* spp. in the shale formations and thereby mitigate detrimental biocorriosion processes during hydraulic fracturing operations (Struchtemeyer et al., 2012).

AUTHOR CONTRIBUTIONS

RL spearheaded the work under the supervision of JS. RL also drafted the manuscript with JS responsible for overall interpretation, coordination and editing of the paper. ID contributed to the isolation of the culture. CM and BH helped on the sampling and geochemical analyses. BWS, BS, and KD performed and helped interpret the molecular data in the manuscript. All authors reviewed the manuscript and contributed to the final revision of the manuscript.

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Control of Microbial Sulfide Production with Biocides and Nitrate in Oil Reservoir Simulating Bioreactors

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Oil reservoir souring by the microbial reduction of sulfate to sulfide is unwanted, because it enhances corrosion of metal infrastructure used for oil production and processing. Reservoir souring can be prevented or remediated by the injection of nitrate or biocides, although injection of biocides into reservoirs is not commonly done. Whether combined application of these agents may give synergistic reservoir souring control is unknown. In order to address this we have used up-flow sand-packed bioreactors injected with 2 mM sulfate and volatile fatty acids (VFA, 3 mM each of acetate, propionate and butyrate) at a flow rate of 3 or 6 pore volumes (PV) per day. Pulsed injection of the biocides glutaraldehyde (Glut), benzalkonium chloride (BAC) and cocodiamine was used to control souring. Souring control was determined as the recovery time (RT) needed to re-establish an aqueous sulfide concentration of 0.8–1 mM (of the 1.7–2 mM before the pulse). Pulses were either for a long time (120 h) at low concentration (long-low) or for a short time (1 h) at high concentration (short-high). The short-high strategy gave better souring control with Glut, whereas the long-low strategy was better with cocodiamine. Continuous injection of 2 mM nitrate alone was not effective, because 3 mM VFA can fully reduce both 2 mM nitrate to nitrite and N_2 and, subsequently, 2 mM sulfate to sulfide. No synergy was observed for short-high pulsed biocides and continuously injected nitrate. However, use of continuous nitrate and long-low pulsed biocide gave synergistic souring control with BAC and Glut, as indicated by increased RTs in the presence, as compared to the absence of nitrate. Increased production of nitrite, which increases the effectiveness of souring control by biocides, is the most likely cause for this synergy.

Keywords: sulfate-reducing bacteria, nitrate, biocide, synergy, bioreactor

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INTRODUCTION

Souring of produced oil, water, and gas is caused by production of sulfide by sulfate-reducing bacteria (SRB). Sulfide is present as H_2S in all three phases and also as HS^- and as S^{2-} in the aqueous phase, depending on pH (Khatib and Salanitro, 1997; Gieg et al., 2011). Souring must be controlled due to the negative effects of hydrogen sulfide (H_2S) on oil and gas quality (Vance and Thrasher, 2005) and due to increased risks of sulfide on health and safety (Beauchamp et al., 1984) and on biocorrosion of carbon steel infrastructure (Enning and Garrelfs, 2014). Souring can be controlled by the application of biocides or of nitrate (Davidova et al., 2001; Bødtker et al., 2008) with biocides being used mostly for control in above-ground infrastructure (e.g., tanks and

pipelines) and nitrate being used mostly for control of souring in the reservoir. Other forms of control, like the use of bacteriophages to eliminate specific SRB, have also been advocated (Summer et al., 2011).

Biocides are organic chemicals designed to kill a broad spectrum of microorganisms. This broad spectrum activity, the possible persistence of biocides in the environment and the economics of biocide use all necessitate the choice of an optimal strategy that minimizes biocide use for a given application (Bradley et al., 2011; McGinley et al., 2011). The chemical structures and modes of action of some biocides, used in the oil industry and in this study, are indicated in **Table 1**. Glutaraldehyde (Glut) and tetrakishydroxymethyl phosphonium sulfate (THPS) are chemically-reactive biocides that kill microbes by irreversible chemical reactions, which inactivate the biocide. In contrast, benzalkonium chloride (BAC) and cocodiamine are physically-reactive biocides that kill bacteria by membrane disruption and cell lysis. These biocides remain active and toxic. THPS has no long alkyl chain R, like BAC and cocodiamine, so it is not also a physically-reactive biocide.

Souring control by nitrate injection has been extensively studied in the laboratory (Myhr et al., 2002; Hubert et al., 2003; Coombe et al., 2004; Grigoryan et al., 2008; Callbeck et al., 2011) and in the field (Jenneman et al., 1999; Sunde et al., 2004; Bødtker et al., 2008; Voordouw et al., 2009). Its mechanism involves the biocompetitive exclusion of SRB by heterotrophic nitrate-reducing bacteria (hNRB) (Davidova et al., 2001; Thorstenson et al., 2002; Hubert and Voordouw, 2007), the inhibition of SRB by nitrite (Reinsel et al., 1996; Sturman et al., 1999; Myhr et al., 2002; O'Reilly and Colleran, 2005) and the direct oxidation of sulfide with nitrate by sulfide-oxidizing NRB (soNRB) (Nemati et al., 2001a; Voordouw et al., 2002; Greene et al., 2003; Hubert et al., 2003). Compared with biocides, nitrate is cheaper, not broadly and persistently toxic, highly soluble in water and compatible with other chemicals. In spite of these advantages and its successful application in high temperature oil reservoirs, souring control in low-temperature oil reservoirs by continuous nitrate injection is more difficult (Voordouw et al., 2009). Following initial decreases, the concentration of produced sulfide recovered to pre-nitrate injection levels. This recovery was proposed to be due to the formation of zones of NRB in the near injection wellbore region (NIWR) and of SRB deeper in the reservoir (Voordouw et al., 2009; Callbeck et al., 2011). With excess electron donors, as expected in an oil field, and a favorable temperature in the deeper zone, SRB will continue to produce sulfide to cause souring with continuous supply of sulfate from injection water. Thus, souring control with nitrate can be transient in low-temperature reservoirs.

This paper addresses the question whether souring control by continuous injection of nitrate under low temperature conditions can be improved by combining this with pulsed injection of biocides. The use of biocides to prevent souring in reservoir-simulating bioreactors has not been extensively explored. We start this study, therefore, with a determination of the effectiveness of biocides in preventing souring in bioreactors in the absence of nitrate as a prerequisite for the evaluation whether continuous injection of nitrate and pulsed injection of biocides can be synergistic.

MATERIALS AND METHODS

Media and Enrichment Cultures

CSBA medium with volatile fatty acids (VFA, 3 mM each of acetate, propionate and butyrate) and either 2 mM sulfate (CSBA-S) or 2 mM sulfate and 2 mM nitrate (CSBA-SN) were used (Hubert et al., 2003; Callbeck et al., 2011). Sulfate and nitrate were added as the sodium salts. VFA are widely present in oil field produced waters and are easily oxidized by oil field hNRB and SRB. SRB in enrichments from the Medicine Hat Glauconitic C (MHGC) field (Voordouw et al., 2009) do not use acetate and incompletely oxidize propionate to acetate and CO_2 and butyrate to two acetate. Under these conditions 12 mM acetate can be formed from the incomplete oxidation of 3 mM VFA (Grigoryan et al., 2008). hNRB use all VFA components with similar kinetics to reduce nitrate to N_2 with nitrite as an intermediate (Grigoryan et al., 2008; Callbeck et al., 2011). The equations shown in **Table 2** indicate that 1.33 mM propionate and 2 mM butyrate can each reduce 1 mM sulfate (equations 1 and 2), whereas 0.42 mM acetate, 0.24 mM propionate, and 0.17 mM butyrate suffice to each reduce 0.67 mM of nitrate. Hence, 3 mM VFA has sufficient reducing power to completely reduce 2 mM nitrate and 2 mM sulfate, which would require 0.42 mM acetate, 1.57 mM propionate and 2.17 mM butyrate, respectively, when equations 1–5 apply. If hNRB also reduce nitrate initially through incomplete oxidation of propionate and butyrate, then 0.83 mM propionate and 1.25 mM butyrate would be used to reduce 1 mM nitrate each (equation 6 and 7, respectively), leaving 2.17 mM propionate and 1.75 mM butyrate for sulfate reduction, which would still be enough for the complete reduction of both nitrate and sulfate. Hence, 3 mM VFA represents an excess of electron donors in both CSBA-S and in CSBA-SN medium. The headspace of all media was gassed with 90% (vol/vol) of N_2 and 10% CO_2 (N_2-CO_2), as described by Callbeck et al. (2011). Initial SRB enrichments used 10 mL of MHGC produced water and 90 mL of modified CSBA-S medium, containing 10 mM sulfate and 8 mM VFA in a 150 mL serum bottle with an N_2-CO_2 headspace. The culture was inoculated into bioreactor columns after two transfers in modified CSBA-S medium (Callbeck et al., 2011).

Bioreactor Setup and Establishment of SRB Biofilms

Either plastic (60 mL, 12.2 × 2.7 cm) or glass (30 mL, 9.8 × 2 cm) syringes without piston were used as bioreactor columns. These were packed from the bottom to the top with a 1 mm layer of glass wool, a 3 mm polymeric mesh, sand (Sigma-Aldrich, 50–70 mesh particle size), a 3 mm polymeric mesh and a 1 mm layer of glass wool (Callbeck et al., 2011; Xue et al., 2015). The packed columns were closed with a rubber stopper perforated with a syringe needle, using zip ties on the outside. Three-way Luer-Lock valves were connected to the bottom inlet and the top syringe needle outlet to allow sampling of the influent and effluent streams. The packed and assembled dry columns were autoclaved. A peristaltic

TABLE 1 | Some biocides commonly used in the oil industry, which were used in this study.

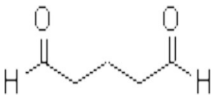
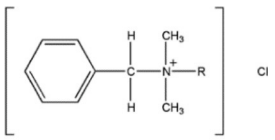
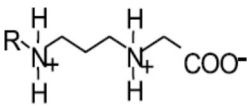
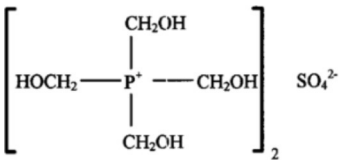
Biocide	Chemical structure	Mode of action	References
Glutaraldehyde (Glut)		Chemically-reactive: aldehyde groups cross-link amino-groups in proteins and nucleic acids	Gorman et al., 1980; Bartlett and Kramer, 2011; McGinley et al., 2011
Benzalkonium chloride (BAC)	 R= C_{12}~C_{16}	Physically-reactive: quaternary ammonium cationic surfactant, the long alkyl chain R solubilizes cytoplasmic membranes and causes cell lysis	Ferrer and Furlong, 2001; Ioannou et al., 2007; Ferreira et al., 2011; Oh et al., 2014
Cocodiamine	 R= C_{24}-C_{28}	Same as BAC	Greene et al., 2006
Tetrakis hydroxymethyl phosphonium sulfate (THPS)		Reacts to denature proteins; damages membranes, interrupting proton flux and the ADP-ATP energy cycle; inhibits sulfate reduction by SRB	Jones et al., 2010, 2012

TABLE 2 | Stoichiometries for microbially-mediated oxidation of VFA by sulfate or nitrate[a].

SO_4^{2-}	Propionate	$4C_3H_5O_2^- + 3SO_4^{2-} + 3H^+ \rightarrow 4C_2H_3O_2^- + 3HS^- + 4CO_2 + 4H_2O$	(1)
	Butyrate	$2C_4H_7O_2^- + SO_4^{2-} \rightarrow 4C_2H_3O_2^- + HS^- + H^+$	(2)
NO_3^-	Acetate	$5C_2H_3O_2^- + 8NO_3^- + 13H^+ \rightarrow 10CO_2 + 4N_2 + 15H_2O$	(3)
	Propionate	$5C_3H_5O_2^- + 14NO_3^- + 19H^+ \rightarrow 15CO_2 + 7N_2 + 22H_2O$	(4)
	Butyrate	$5C_4H_7O_2^- + 20NO_3^- + 25H^+ \rightarrow 20CO_2 + 10N_2 + 30H_2O$	(5)
	Propionate	$5C_3H_5O_2^- + 6NO_3^- + 6H^+ \rightarrow 5C_2H_3O_2^- + 3N_2 + 5CO_2 + 8H_2O$	(6)
	Butyrate	$5C_4H_7O_2^- + 4NO_3^- \rightarrow 10C_2H_3O_2^- + 2N_2 + 2H_2O + H^+$	(7)

[a] *Calculated values for $\Delta G^{0'}$ (kJ per mol of sulfate or nitrate reduced) based on data by Thauer et al. (1977) were: (1) -50, (2) -56, (3) -495, (4) -496, (5) -496, (6) -497, and (7) -500.*

multichannel pump (Minipuls-3, 8-channel head, Gilson Inc.) was used to deliver water into the columns. PVC extension tubing (ID = 0.76 mm, Gilson, F117956) was used with PVC calibrated tubing (ID = 0.76 mm, Gilson, F117936) being used in the pump. SRB enrichment was inoculated from the bottom three-way Luer-Lock valve, while samples were taken from the effluent valve. The pore volume (PV) of the packed columns was determined by the weight difference between the column saturated with sterilized water and the dry column. Porosity was calculated as the fraction of PV over the total volume of the column. Anaerobic, sterile CSBA-S medium was then pumped from the medium container into the columns with effluent being collected in stoppered serum bottle effluent containers. The medium containers were fitted with 60 mL plastic syringes with piston, which were filled with N_2-CO_2, whereas the effluent containers were fitted with initially empty 60 mL plastic syringes with piston. This allowed continuous balancing of pressure (**Figure 1**). SRB enrichment (0.5 PV) was then inoculated into the bioreactor columns through the bottom inlet three-way valve (**Figure 1**). The columns were then incubated at room temperature (~23°C) for 2 weeks to establish SRB activity without injection of medium (Callbeck et al., 2011). Subsequently, CSBA-S medium containing 2 mM sulfate and 3 mM VFA was continuously injected into columns at a low flow rate, which was gradually increased to the values indicated in **Table 3**. The bioreactors were then eluted at this constant flow rate until 1.8–2 mM sulfide were produced in the effluent. The bioreactors were then ready for treatment with biocides and/or nitrate.

Biocide and Nitrate Injection

The biocides used were Glut, BAC and cocodiamine, as well as THPS and Glut_BAC (a mixture containing 42.5% w/w Glut and 7.5% w/w BAC). Glut and BAC were purchased from Sigma-Aldrich and ICN, respectively, whereas Glut_BAC, THPS and cocodiamine were provided by collaborator companies, indicated in the Acknowledgments. All biocides were liquid concentrates except BAC, which was a white solid of which a 50 mM (18,000 ppm) stock solution was made. Biocides were

TABLE 3 | Dimensions, characteristics and operating conditions of the bioreactors used.

Columns	BV#	Column size L*D (cm)[a]	Pore volume (PV, mL)	Total volume V (mL)[b]	Porosity (%)	Flow rate (mL/h)	Retention time (h)[c]	Velocity (cm/h)[d]
Plastic	0, 1, 2, 3	12.2*2.7	25.7	69.8	36.8	3.7	6.9	1.8
Glass	4, 5, 6, 7, 8	9.8*2	10.9	30.8	35.4	2.7	4.0	2.45

[a] *L is the length and D is the diameter of the bioreactor columns.*
[b] $V = \pi\ (D/2)^2 * L$.
[c] *Retention time is pore volume divided by the flow rate.*
[d] *velocity is L divided by retention time.*

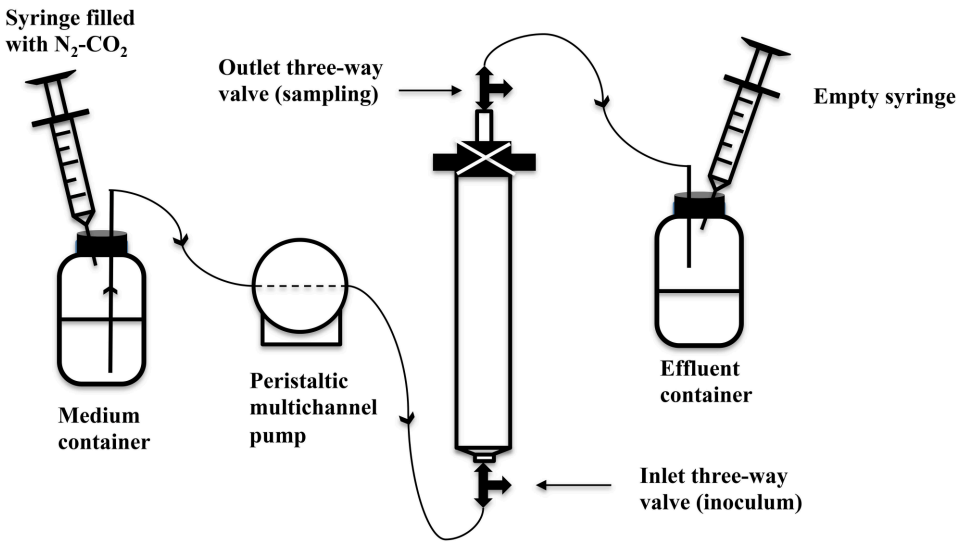


FIGURE 1 | Schematic of up-flow sand-packed bioreactor system modeling a souring oil field subjected to injection with nitrate and pulses of biocide. Note that the actual flow direction in the field may be horizontal between the injection well and the production well. In an upflow bioreactor the inlet is equivalent to the injector and the outlet is equivalent to the producer.

added into the medium directly from concentrates. Biocide concentrations are indicated as ppm of the active ingredient. The bioreactor columns were continuously injected with CSBA-S (2 mM sulfate) or CSBA-SN (2 mM sulfate and 2 mM nitrate) medium. Biocide treatment was initiated by injecting medium with biocide and was stopped by switching back to medium without biocide. The sulfide recovery time (RT), the time needed for recovery of the sulfide concentration to 0.8 to 1 mM (from 1.7 to 2 mM initial sulfide), was determined to represent the inhibition/kill efficacy of biocides (Gardner and Stewart, 2002). The next pulse of biocide treatment was initiated after the sulfide concentration had recovered to 1.7–2 mM for at least 4 days to ensure the complete recovery of SRB activity. Medium with a defined concentration of biocide was injected into 60 ml plastic bioreactor columns for 5 days or in 30 ml glass bioreactor columns for 1 h.

Chemical Analysis

Samples of 0.5 mL were taken from the effluent three-way valve. The concentration of aqueous sulfide was determined immediately using the methylene blue method (Trüper and Schlegel, 1964). High-performance liquid chromatography (HPLC, Waters 600) with an IC-PAK anion column (4.6 × 150 mm, Waters) eluted with 24% v/v acetonitrile, 2% v/v butanol, and 2% v/v borate/gluconate concentrate at a flow rate of 2.0 mL/min was used to detect sulfate with a Waters 423 conductivity detector and nitrite and nitrate with a UV/VIS-2489, Waters detector at 220 nm (Mand et al., 2014).

Analysis of Duplicates

Bioreactors were repeatedly injected with the same biocide (e.g., BV1 and BV4 with Glut only). Injections of a given concentration was usually done only once, i.e., multiple injections were mostly at different concentration. Repeated injections of the same concentration were done as indicated in Table S1. The RTs derived from these differed on average by about 33%. Hence, we will regard differences of two-fold or more as significant, when these are observed at multiple biocide concentrations.

RESULTS

Souring Control by Pulses of Biocide in the Absence of Nitrate

Five-day pulses of the biocides Glut, BAC, and cocodiamine were injected into bioreactors BV1, BV2, and BV3, respectively.

Injection of 50, 100, or 200 ppm of Glut had no effect on sulfide production in BV1, meaning that RT was 0 h. However, injection of 400, 600, or 1000 ppm gave inhibition of sulfide production with RTs of 73.2, 130.8, and 245.9 h, respectively (**Figure 2A**, Table S2). Although continuously increasing doses of biocide were applied in the case of Glut, this was not done routinely to avoid adapting the bioreactor community to ever increasing doses of biocide. Bioreactor BV2 was injected with 5-day pulses of 36, 180, 360, 1080, 100, 1440, and 800 ppm of BAC giving RTs of 0, 209.8, 223, 314.8, 183.3, 472.1, and 249.2 h, respectively (**Figure 2B**). Likewise for bioreactor BV3, application of 50, 25, 12.5, 100, and 150 ppm of cocodiamine gave RTs of 300.9, 168.5, 0, 228.7, and 264.8 h (**Figure 2C**). A survey of all sulfide RTs obtained for injection of 5-day biocide pulses is given in Figure S1A and Table S2. In the absence of biocide injection, no significant fluctuations in the eluted sulfide concentrations were observed. Bioreactor BV0 injected with CSBA-S medium containing 2 mM sulfate and 3 mM VFA, without biocide, continuously produced 1.78 ± 0.11 mM sulfide ($N = 70$) over 350 days (results not shown).

Bioreactors BV4, BV5, and BV8 were injected with 1 h pulses of Glut, BAC and cocodiamine, respectively, after which the sulfide RT was recorded as indicated in Figure S2A. Injection of 4000, 2000, 1000, 500, 300, and 5000 ppm of Glut gave RTs

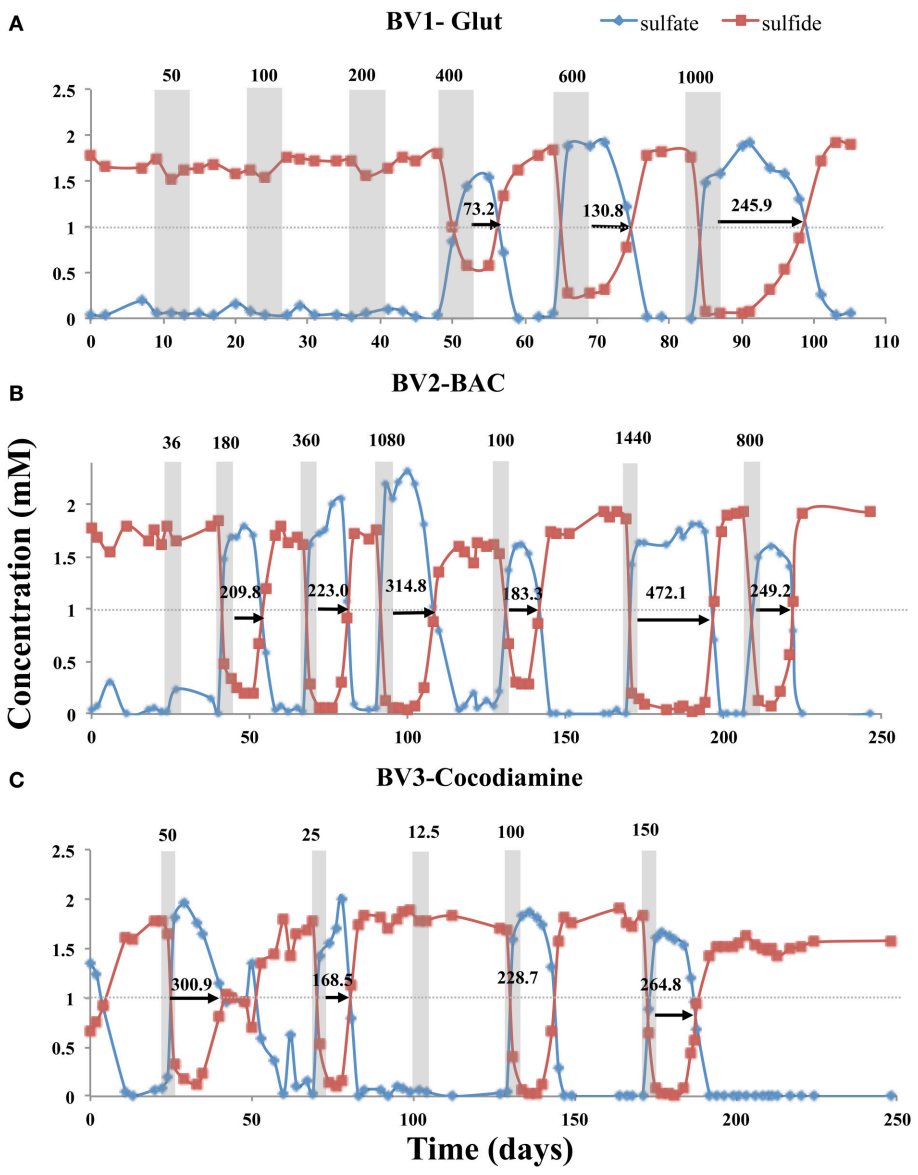


FIGURE 2 | Effect of 5-day biocide treatment on sulfide production in the absence of nitrate. Bioreactors BV1, BV2, and BV3 were treated with **(A)** Glut, **(B)** BAC, and **(C)** cocodiamine, respectively. The concentrations of sulfate and sulfide are shown, as indicated. Shaded rectangles indicate the 5-day periods for pulsing biocides. The biocide concentrations (ppm) are indicated above the shaded rectangles. The sulfide recovery times are indicated by the arrows and numbers (h).

of 214.6, 135.4, 84.7, 0, 0 and 234.9 h, respectively (Figure S2A, Table S3). This indicated a similar threshold for action of Glut under short-high injection conditions, as compared to long-low injection conditions of 400-500 ppm. The similar threshold may be caused by rapid chemical reaction of Glut with the ammonium in the medium (4.7 mM), inactivating the biocide. Assuming reaction of 1 ammonium/Glut, the calculated threshold is 4.7 mM (470 ppm).

For BAC injection of 500, 1000, 2000, 3000, 3500, and 2500 ppm gave RTs of 0, 0, 0, 250.4, 381.2, and 159.5 h (Figure S2B, Table S3), whereas for cocodiamine injection of 100, 200, 400, 800, 1000, 2000, 3000, and 4000 ppm gave RTs of 0, 0, 0, 0, 0, 40.4, 58.4, and 109.1 h, respectively (Figure S2C, Table S3). Hence, these two biocides appeared to have thresholds in the range of 2000–2500 and 1000–2000 ppm, respectively (Figure S1B, Table S3), whereas under long-low injection conditions these were in the range of 36–100 and 12.5–25 ppm, respectively (Table S2). The effectiveness of the three tested biocides under short-high injection conditions depended on the concentration range used. For concentrations up to 2000 ppm the effectiveness of Glut exceeded that of cocodiamine and BAC, whereas for concentrations of 3000 ppm or higher BAC appeared most effective (Figure S1B).

In order to more appropriately compare the effectiveness of long-low vs. short-high applications of biocides to inhibit sulfide production from bioreactors, the measured RTs should be plotted against the total amount (mg) of biocide dosed. Because bioreactors with two different PVs were used (**Table 3**) we divided the total injected amount by the PV, as indicated in **Figure 3**. The results indicated that the short-high strategy worked best for the fast-acting Glut (**Figure 3A**). The long-low strategy worked best for the more slowly acting cocodiamine (**Figure 3C**).

Souring Control by Pulses of Biocide in the Presence of Continuous Nitrate

Continuous injection of bioreactors BV1, BV2, and BV3 with CSBA-SN medium, containing 2 mM nitrate and 2 mM sulfate gave complete reduction of both electron acceptors. When the nitrate concentration was increased to 4, 8, or 13.3 mM partial souring control was observed with 8 and 13.3 mM nitrate (results not shown). Following return to injection of CSBA-SN with 2 mM nitrate, 5 day pulses of Glut, BAC or cocodiamine were injected in bioreactors BV1, BV2, and BV3. In addition to concentrations of sulfate and sulfide, those of nitrate and nitrite were also measured. Injection of 400, 600, 1000, 300, and 200 ppm of Glut gave RTs for production of sulfide of 68.8, 244.5, 588.7, 147.2, and 0 h, respectively. This was associated with breakthrough of maximum nitrate concentrations of 1.21, 2.0, 1.82, 0.81, and 0 mM. Only trace nitrite was observed (**Figure 4A**, Table S2).

When injecting 5-day pulses of 100, 800, 1080, and 360 ppm BAC, RTs of 0, 622.8, 370.5, and 608.2 h were found with peak nitrate and nitrite concentrations of 0.22 and 0.08, 1.63, and 0.53, 1.79, and 0.64, and 1.58 and 0.21 mM, respectively (**Figure 4B**, Table S2). Injection of 25, 50, 100, 150, and 12.5 ppm of cocodiamine gave RTs of 85.7, 285.1, 231.7, 259.0, and 0 h with peak nitrate and nitrite concentrations of 0 and 0.21, 0.1, and 0.2, 1.2, and 0.4, 0.8, and 0.3, and 0 and 0 mM, respectively (**Figure 4C**, Table S2).

A comparison of sulfide RTs for 5-day biocide injections in the absence and presence of nitrate is provided in **Figure 5**. No difference was observed in the case of cocodiamine (**Figure 5C**). Higher RTs were observed in the presence of nitrate for 360 and 800 ppm BAC (**Figure 5B**) and for 600 and 1000 ppm of Glut (**Figure 5A**). In the case of BAC increased production of nitrite could contribute to these increased RTs.

Results for pulsing Glut, BAC and cocodiamine for 1 h under continuous injection of 2 mM nitrate are shown in Figures S2, S3. The derived RT values as well as peak nitrate and nitrite concentrations are summarized in Table S3. Injection of 2000, 3000, or 4000 ppm Glut gave breakthrough of 0.21, 0.25, and 0.88 mM of nitrite, respectively (Table S3). Hence, nitrite was observed with Glut (0–0.9 mM), BAC (0–0.43 mM) and cocodiamine (0–0.64 mM). A comparison of derived RT values as a function of biocide concentration in the absence or presence of nitrate is shown in **Figure 6**. RT values in the presence of nitrate were smaller than in its absence for injections of Glut and BAC, but not for injections of cocodiamine (**Figures 6A,B,E**).

Use of Other Biocides: Glut_BAC and THPS

The effects of injection of Glut_BAC or THPS were only studied for 1-h pulses in the absence or presence of nitrate. The results are summarized in Table S3. Injection of up to 4000 ppm of

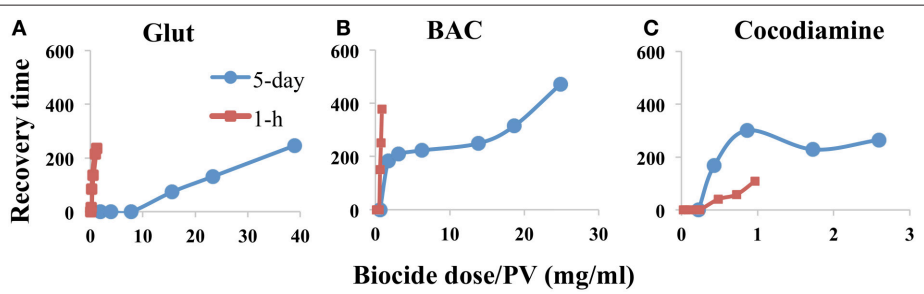


FIGURE 3 | Sulfide recovery time (h) as a function of biocide dose divided by bioreactor pore volume (mg/ml). Data are compared for 5-day and for 1-h pulses of biocide, as indicated, for bioreactors without continuous nitrate injection. Bioreactors were treated with **(A)** Glut, **(B)** BAC, and **(C)** cocodiamine.

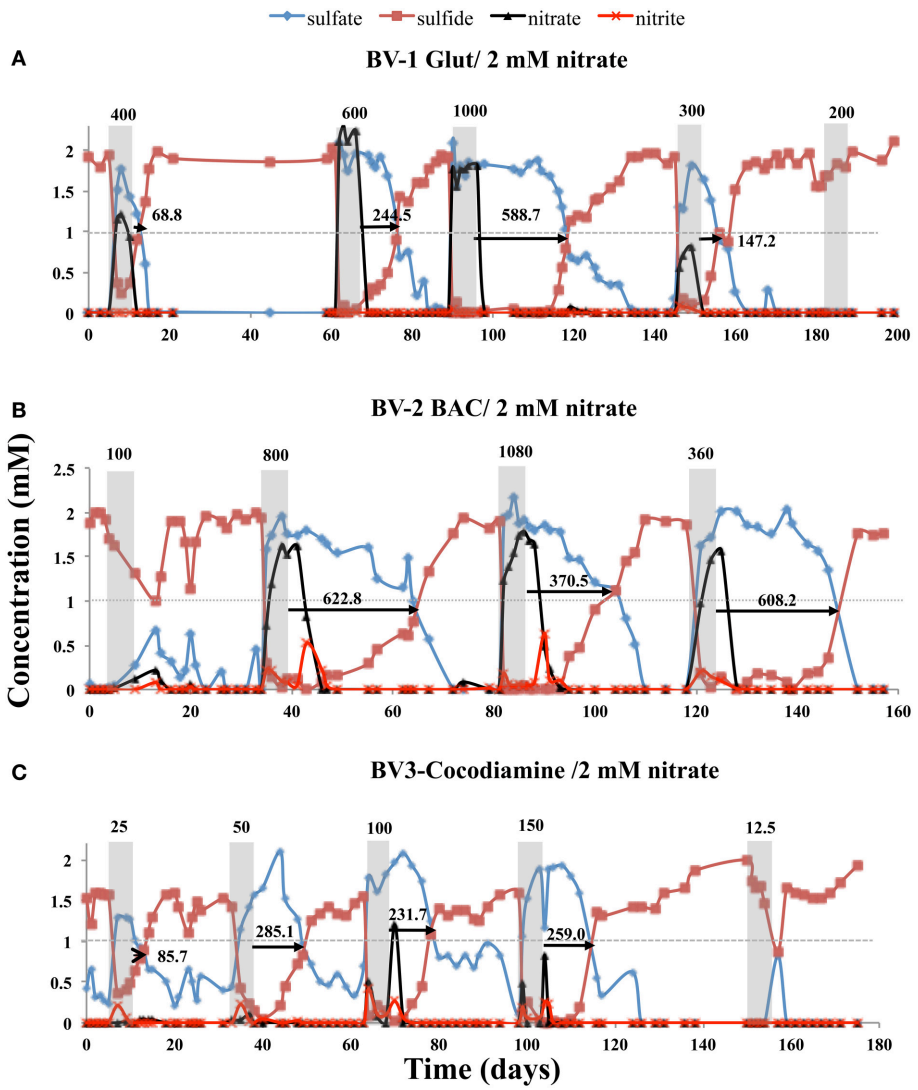


FIGURE 4 | Effect of 5-day biocide treatment on sulfide production in the presence of nitrate. Bioreactors BV1, BV2, and BV3 were treated with **(A)** Glut, **(B)** BAC, and **(C)** cocodiamine, respectively. The concentrations of sulfate, sulfide, nitrate and nitrite are shown, as indicated. Shaded rectangles indicate the 5-day periods for pulsing biocides. The biocide concentrations (ppm) are indicated above the shaded rectangles. The sulfide recovery times are indicated by the arrows and numbers (h).

Glut_BAC and of up to 3000 ppm of THPS increased RT to similar values of 300–400 h, as observed for Glut and BAC (**Figure 6**). For bioreactors with nitrate up to 0.87 mM of nitrite was produced during injections with Glut_BAC, but less (up to 0.24 mM) during injections with THPS. RT values for injections with these biocides were similar or lower for bioreactors with nitrate, than for bioreactors without nitrate (**Figures 6C,D**).

DISCUSSION

The concentrations of biocides needed to control SRB activity in bioreactors in this study were high with hundreds of ppm needed for 5-day and thousands of ppm needed for 1-h pulses (**Figures 5**, **6**), which is higher than for previous studies (Reinsel et al., 1996; Baudrion et al., 2000; Bartlett and Kramer, 2011; Moore and Cripps, 2012). The likely explanation is that due to the continuous injection of medium highly active SRB populations grew in the bioreactor columns as biofilms, which have higher resistance to biocides than planktonic cells (Baudrion et al., 2000; Gardner and Stewart, 2002). The tightly packed sand grains within the bioreactor columns provided a much larger surface area than coupon surfaces in a Robbins device, which is often used to study biocide resistance of biofilms (Grobe and Stewart, 2000). This may also explain the high biocide concentrations needed. When comparing the doses used for the two treatment strategies in the absence of nitrate, it appeared that the fast-acting Glut performed better during 1-h injections at high concentration, whereas the more slowly acting cocodiamine performed best during 5-day injections at lower concentration (**Figure 3**).

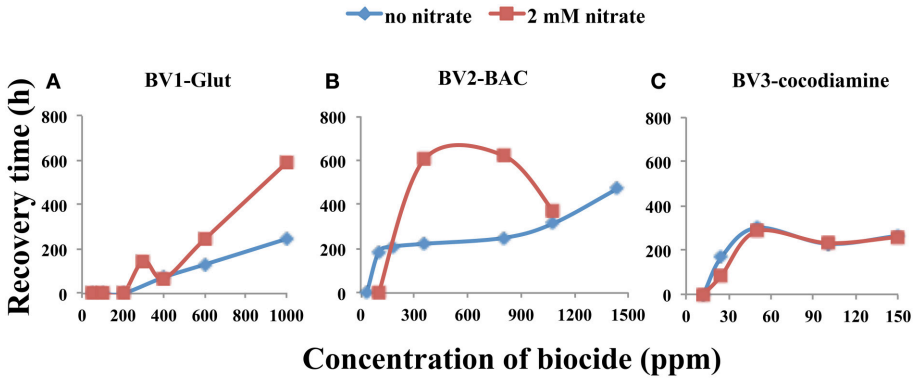


FIGURE 5 | Relation between sulfide recovery time (RT, h) and biocide concentration for 5-day pulses in the absence and presence of nitrate, as indicated. Data are for **(A)**, Glut, **(B)**, BAC, and **(C)** cocodiamine.

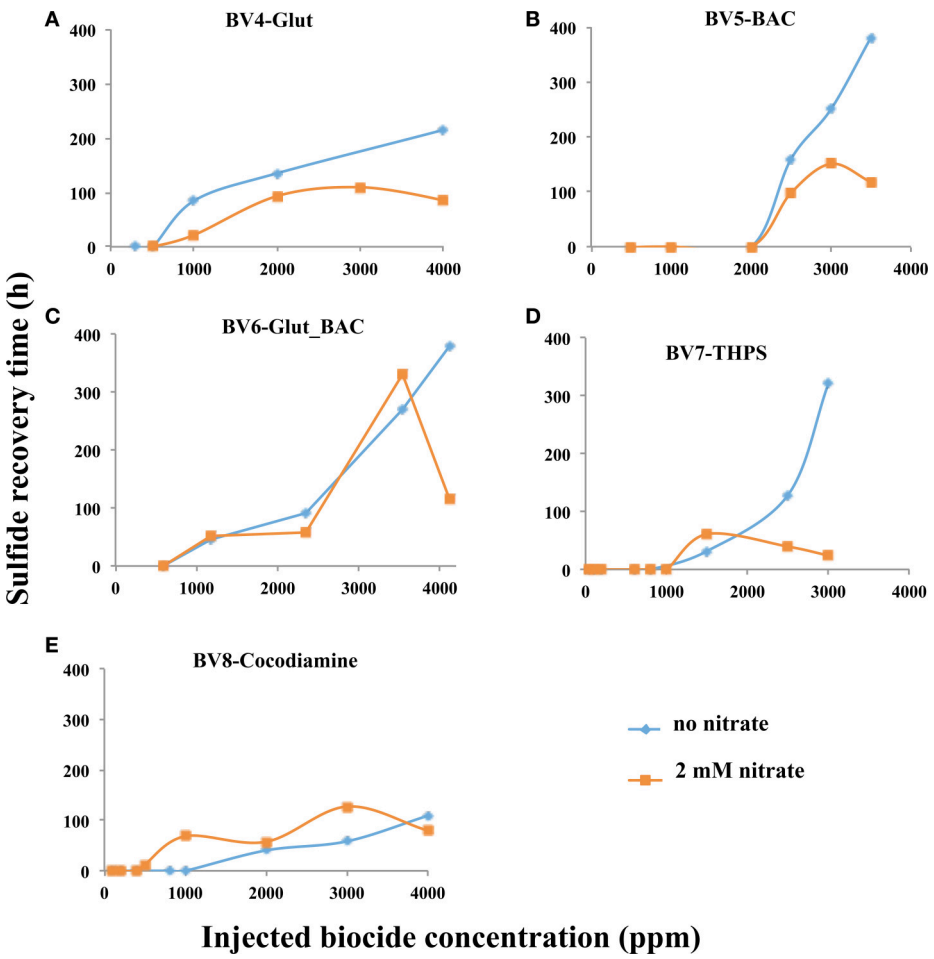


FIGURE 6 | Relation between sulfide recovery time (RT, h) and biocide concentration for 1-h pulses in the absence and presence of nitrate, as indicated. Data are for **(A)**, Glut, **(B)**, BAC, **(C)** Glut_BAC, **(D)** THPS, and **(E)** cocodiamine.

When nitrate is reduced by NRB it inhibits SRB by competitive exclusion. NRB grow faster, to a higher cell density and at a higher redox potential than SRB. Moreover they produce nitrite, which is a strong SRB inhibitor (Reinsel et al., 1996; Sturman et al., 1999; Haveman et al., 2004). Hence if electron donor (e.g., VFA) is limiting, we expect partial reduction of nitrate

with accumulation of nitrite and permanent inhibition of SRB. However, if electron donor is in excess, as in the present study, the outcome will be different. When a consortium of NRB and SRB was inoculated into a serum bottle, containing medium with nitrate, sulfate and heavy MHGC oil as excess electron donor, then nitrate was reduced first, followed by reduction of sulfate, which in turn was followed by methanogenesis (Agrawal et al., 2012). In bioreactors injected with limiting nitrate and sulfate and excess VFA, or containing excess heavy oil, these temporal zones are also spatially separated causing a zone of nitrate reduction near the bioreactor inlet to be followed by a zone of sulfate reduction further downstream (Callbeck et al., 2011, 2013). The NRB biomass near the inlet may exceed the SRB biomass further downstream by an order of magnitude, because nitrate reduction yields much more energy than sulfate reduction (**Table 2**).

The use of multiple agents to control sulfidogenesis has indicated synergy between two types of biocides (Al-Hashem et al., 1998; Greene et al., 2006; McGinley and Van Der Kraan, 2013), a biocide and a metabolic inhibitor (nitrite or molybdate) (Greene et al., 2006), and two types of metabolic inhibitors (Mustafa and Shahinoor Islam Dulal, 1996; Nemati et al., 2001b; AI-Refaie et al., 2009). However, the combined effect of biocide and nitrate on souring control has not been studied, likely because nitrate only becomes inhibitory to SRB following its reduction to nitrite. The inhibitory effect of nitrite on SRB has been extensively studied (Reinsel et al., 1996; Sturman et al., 1999; Greene et al., 2003; Haveman et al., 2004). As an analog of sulfite, nitrite binds to dissimilatory sulfite reductase (Dsr), preventing sulfide production. Additionally, nitrite can chemically react with sulfide forming N_2 and elemental sulfur (Reinsel et al., 1996).

When a biocide is pulse-injected into a zoned bioreactor the NRB biomass may protect the SRB biomass from killing by the biocide, depending on the mechanism of action of the biocide (**Table 1**). Glut is a cross-linking agent that irreversibly reacts with amino groups of proteins and nucleic acids, while BAC and cocodiamine are quaternary cationic surfactants that form micelles and can physically interact with cell membranes, causing rupture of the cells (Greene et al., 2006). Because NRB are located closer to the biocide injection point, NRB biomass may protect SRB biomass from chemical attack by Glut. However, in the case of physically interacting biocides it is less clear whether such protection is possible. If, in a zoned system, BAC or cocodiamine bind to and kill NRB biomass these could subsequently interact with and kill SRB biomass. On the other hand SRB biomass may become more sensitive to biocide, if its action on NRB leads to accumulation of nitrite. Nitrite accumulation may occur if the activity of nitrate reductase, reducing nitrate to nitrite, is less affected by the biocide than the activity of enzymes acting in the reduction of nitrite to N_2. Such differential action could be caused, for instance, by the fact that nitrate reductase is often cytoplasmic-membrane bound, whereas nitrite-, NO- and N_2O-reductase are periplasmic-membrane bound (Zumft, 1997). The latter may thus be more easily accessed by biocides. Indeed breakthrough of nitrite (up to 0.8 mM, 40% of injected nitrate) during biocide treatment was observed with all biocides (Tables S2, S3) with lower values being observed for THPS (Table S3: up to 0.24 mM nitrite). Greene et al. (2006) investigated the effect of combined addition of nitrite and biocide and found that sulfide production by SRB was synergistically inhibited by nitrite and Glut, BAC, cocodiamine or bronopol, but not by nitrite and THPS, which was thought to chemically react with nitrite.

Hence, synergy between continuously injected nitrate and pulsed biocide is possible but it is hard to predict. It was observed for short-high concentration pulses of cocodiamine (**Figure 6E**) and for long-low concentration pulses of Glut and BAC (**Figures 5A,B**). Synergy is expected for a compound, which strongly inhibits the reduction of nitrite without affecting the reduction of nitrate. All biocides tested had this property to some extent. Further work should, therefore, concentrate on finding agents, which are better at this than those tested so far.

AUTHOR CONTRIBUTIONS

YX contributed to setting up the experiments as designed, collecting all data and interpretation of the data and also contributed to the writing of the manuscript during all stages. GV advised on experimental design and operation, interpretation of the data and writing of the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01387

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