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Molecular analysis of the microbial community structures in water-flooding petroleum reservoirs with different temperatures

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Abstract

Temperature is one of the most important environmental factors regulating the activity and determining the composition of the microbial community. Analysis of microbial communities from six water-flooding petroleum reservoirs at temperatures from 20 to 63 °C by 16S rRNA gene clone libraries indicates the presence of physiologically diverse and temperature-dependent microorganisms in these subterrestrial ecosystems. In high-temperature petroleum reservoirs, most of the archaeal sequences belong to the thermophilic archaea including the genera *Thermococcus*, *Methanothermobacter* and *Thermoplasmatales*, most of the bacterial sequences belong to the phyla *Firmicutes*, *Thermotogae* and *Thermodesulfobacteria*; in low-temperature petroleum reservoirs, most of the archaeal sequences are affiliated with the genera *Methanobacterium*, *Methanoculleus* and *Methanocalculus*, most of the bacterial sequences to the phyla *Proteobacteria*, *Bacteroidetes* and *Actinobacteria*. Canonical correspondence analysis (CCA) revealed that temperature, mineralization, ionic type as well as volatile fatty acids showed correlation with the microbial community structures. These organisms may be adapted to the environmental conditions of these petroleum reservoirs over geologic time by metabolizing buried organic matter from the original deep subsurface environment and became the common inhabitants in subsurface environments.

1 Introduction

Petroleum reservoirs represent extreme anaerobic environments because of the temperature, pressure and salinity with multiphase fluids of oil, gas and water. Microorganisms in such subterranean play an important role in energy flow and nutrients cycling. The microbial activity, in particular sulfate-reducing bacteria (SRBs) not only causes issues like reservoir souring and corrosion of drilling equipment with H_2S, but potentially can also be used to our advantage, e.g., microbial-enhanced oil recovery (MEOR) applications. Extraction of currently usable energy from marginal petroleum reservoirs by

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microbial conversion of residual oil to methane (natural gas) has received renewed attention in the past decade (Parkes, 1999; Suflita et al., 2004; Gieg et al., 2008; Jones et al., 2008; Wang et al., 2010, 2011; Mbadinga et al., 2011, 2012). Microorganisms with diverse physiological and metabolic abilities and phylogenetic affiliations have been recovered from oil reservoirs by culture-dependent and culture-independent approaches since the first sulfate-reducing bacteria (SRB) was isolated from production water (Bastin et al., 1926). Though isolation efforts have identified numerous bacterial and archaeal species that are capable of mediating various metabolic processes occurring in oil fields, culture-independent 16S rRNA gene- and functional genes-based investigations have provided new information on the microbial community composition in such deep-subsurface ecosystem (Li et al., 2010, 2011).

Culture-independent surveys of high-temperature oil reservoirs have been conducted in oil fields in continental and offshore California (Orphan et al., 2000, 2003), an offshore oil field in Qinghuang, China (Li et al., 2007b), Huabei oil field in continental China (Li et al., 2006, 2007a), Dagang oil field in China (Nazina et al., 2006), and in Troll oil formation in the North Sea (Dahle et al., 2008). Bacterial sequences affiliated with *Firmiucutes* are the most frequency detected in these high-temperature oil reservoirs. In addition, the discovery of moderately thermophilic members of the *Bacteroidetes* (genus *Anaerophaga*) in Troll oil formation in the North Sea and sulfate reducing *Nitrospira* (genus *Thermodesulfovibrio*) in the Huabei and Qinghuang oil fields in China have not been previously isolated from oil reservoirs. Most of Archaeal belong to methanogens including methyltrophic, acetoclastic as well as CO_2-reducing methanogens and CO_2-reducing methanogens are the most common members in high-temperature oil reservoirs. Moreover, methanogenesis from acetate driven by syntrophic acetate oxidation has been documented in high-temperature reservoir (Nazina et al., 2006).

Compared with high-temperature oil reservoirs, few 16S-based analysis of the microbial community in low-temperature oil reservoirs have been reported. The bacterial diversity in a low-temperature, low-salinity, non-water flooded oil reservoir (Pelikan

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lake oil field) in Western Canada was extremely low with only one phylotype related to the genus *Arcobacter* (*ε-Proteobacteria*) (Grabowski et al., 2005). Several potentially metabolic active fermentative and/or acetogenic microorganisms, sulfide-oxidizers and sulfate-reducers were identified from a low-temperature oil reservoir in Western Canada by 16S rRNA gene clones library analysis (Voordouw et al., 1996).

The ecological processes of these microorganisms play considerable important roles in energy flow and nutrients cycling in subsurface ecosystem. The distribution of different microbial community structures in petroleum reservoirs depends entirely on their adaption to the in situ physical and chemical variables, including temperature, pH, and salinity. Despite of the available studies on microbial population both in high- or low-petroleum reservoirs, microbial populations in reservoirs from low to high temperatures has not been studied in a single investigation. In this report, the distribution of microbial community in petroleum reservoirs from production water of several petroleum reservoirs at temperature of 20, 32, 37, 45, 58 and 63 °C was investigated by 16S rRNA gene library analysis. Microbial community data were also correlated with environmental factors using canonical correspondence analysis (CCA).

2 Materials and methods

2.1 Collection of sample and nucleic acid extraction

The production water samples of six production oil wells (S1–S6) from six Block affiliated with 3 oil fields of China: Well Z3-26 belongs to Zhan 3 of Shengli oil field; Well B18-43 to Ba 19, Well B51-45 to Ba 51 and Well M17-10 to Menggulin of Huabei oil field; Well 7222 to No. 7, Well 6190 to No. 6 of Kelamayi oil field. The temperature and the mineralization of these six petroleum reservoirs sampled range from 20 to 63 °C and 1301 to 11196 $mg\,l^{-1}$, respectively and the pH of these production waters were neutral or slightly alkaline. The characterization of the petroleum reservoirs water sampled are listed in Table 1. In order to characterize the microbial community from

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the different temperature petroleum reservoir, these samples were grouped into two classes: high-temperature (45 ~ 63 °C) and low-temperature (20 ~ 37 °C).

Ten liters of production water at wellhead were taken from each of the six production oil wells from six petroleum reservoirs. Samples were collected into sterile bottles to full capacity after discarding the initial oil/water mixture. The bottles were tightly sealed to avoid oxygen intrusion and immediately transported to the laboratory and filtered directly to minimize the chance of community changes. During filtration procedures, the residual oil was removed by heating the sample to 50 °C for 15 min and by phase separation in 2 l sterilized separatory funnel. The water samples were filtered through 0.22 µm polycarbonate membranes (25 mm diameter; Millipore, Bedford, USA). The polycarbonate membranes containing the cells was placed in a sterile centrifuge tube containing sterile silica beads for beating to break the cells. Genomic DNA was extracted by a method developed previously in this laboratory (Li et al., 2007b).

2.2 16S rRNA gene amplification and cloning

16S rRNA genes were amplified by PCR using the primers B27F [5′-AGAGTTTGATCCTGGCTCAG-3′] and B1492R [5′-TACGGYTACCTTGTTACGACTT-3′] (Nazina et al., 2006) for bacteria, and the primers A21F [5′-TTCCGGTTGATCCYGCCGGA-3′] (DeLong, 1992) and A1041R[5′-GGCCATGCACCWCCTCTC-3′] (Kolganova et al., 2002) for archaea. The final 50 µl reaction mixture volume contained 2 µl of template DNA, 0.5 µM of each primer, 25 µl of 2 × master mix (Promega, USA), 21 µl of nuclease-free water. Polymerase chain reaction cycles were performed on a Peltier thermal cycler (Bio-Rad, USA) as follows: after 5 min of initial denaturation at 95 °C, nucleic acids were amplified for 30 cycles (45 s of denaturation at 95 °C, 45 s of annealing at 50 °C and 1 min of elongation at 72 °C) followed by a final extension step at 72 °C for 20 min. PCR products were separated on 0.8 % (w/v) agarose gel and stained with ethidium bromide. The amplicons were cloned into a pMD®19-T Simple vector (Takara, Japan) according to the manufacturer's instructions.

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2.3 Sequencing and phylogenetic analysis

Inserts of selected clones were amplified by PCR with forward M13F (5′-GTTTTCC CAGTCACGA-3′) and the reverse M13R (5′-CAGGAAACAGCTATGAC-3′) plasmid specific primer set. The sequencing was performed on an ABI 377 sequencer (Dye Terminator Cycle Sequencing Ready Reaction FS Kit; PE Applied Biosystems) using M13 universal sequencing primers. Obtained DNA sequences were checked for vector by VecScreen Widget 1.0 software before further analysis. Sequence data were aligned using the the NAST alignment algorithm (DeSantis et al., 2006a) on the Greengenes website (http://greengenes.lbl.gov), with clones having similarities of 98 % or above grouped into operational taxonomic units (OTUs). The clones were homology-searched using Ribosomal Database Project II (Wang et al., 2007). The nearest relatives of each OTU were identified using the BLASTN network service (Altschul et al., 1997). Chimeras were detected using Bellerophon, version 3 (Huber et al., 2004; DeSantis et al., 2006) and removed from further examination. Phylogenetic trees were constructed based on the neighbor-joining algorithm (Saitou and Nei, 1987) using the MEGA5 software (Tamura et al., 2011). Bootstrap analysis with 1000 replicates was applied to assign confidence levels to the nodes in the trees.

2.4 Statistical analysis

The coverage of each clone library was calculated by the equation $C = [1 - n_1/N] \times 100$, where n_1 is the number of OTUs represented by only one clone and N is the total number of clones examined (Good, 1953). To examine the temperature distribution of microbial community in production water of petroleum reservoir, 16S rRNA gene sequences were analyzed with the online software UniFrac (http://bmf2.colorado.edu/unifrac/) using the principal coordinates analysis (PCoA) as suggested previously (Lozupone and Knight, 2005). Correlations between the microbial communities and environmental factors were determined by the canonical correspondence analysis (CCA) using the

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software CANOCO (ver. 4.5, Microcomputer Power, Ithaca, NY, USA) (ter Braak and Šmilauer, 2002).

2.5 Nucleotide sequence accession numbers

Partial 16S rRNA gene sequences for Bacteria and Archaea obtained in this study were deposited in GenBank databases under accession numbers JQ433723-JQ433816 and JF754550-JF754565.

3 Results

3.1 Diversity of microbial community in water-flooding petroleum reservoirs

Six production water samples from six water-flooding petroleum reservoirs with different temperatures were analyzed by PCR amplification with bacterial and archaeal specific primer sets. For the members in the domain bacteria, 93, 226, 254, 80, 185 and 142 clones were randomly selected from the libraries of production water samples S1, S2, S3, S4, S5 and S6, respectively. Of the total sequences screened by MOTHUR software version 1.6, 5, 16, 10, 13, 30 and 31 operational taxonomy units (OTUs) were obtained from S1, S2, S3, S4, S5 and S6, respectively and classified into thirteen different phylogenetic groups (phylum level) (Figs. 1–3). The coverage of the clone library was 100 % for S1, S2, S3 and S5, and 96 % for S4, as well as 99 % for S6 from rarefaction analysis.

For the members in the domain archaea, 125, 56, 79, 60, 24 and 33 clones were randomly selected from the libraries of production water samples S1, S2, S3, S4, S5 and S6, respectively. Of the total sequences screened by MOTHUR software version 1.6, 5, 1, 4, 2, 2 and 2 OTUs were obtained from S1, S2, S3, S4, S5 and S6, respectively and classified into eleven different phylogenetic groups (genus level) (Fig. 4). The coverage of the clone library was all 100 % except 98 % for S1 from rarefaction analysis.

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Bacterial and archaeal DNA sequences based on the percentage representation of major phylum or genus in clone libraries from the six different temperature reservoirs are shown in Fig. 5. The bacterial sequences were clustered within thirteen phyla: *Proteobacteria* (*α*-, *β*-, *γ*-, *δ*-, *ε*-), *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Chloroflexi*, *Thermotogae*, *Thermodesulfobacteria*, *Deinococcus-Thermus* and TM7. Compared with low temperature reservoir, bacterial sequences affiliated with the phylum *Firmicutes* account for the highest percentage in S2 (65.9 %) and S3 (29.9 %) from high temperature petroleum reservoir. In contrast, *Proteobacteria* (*α*-, *β*-, *γ*-, *δ*-, *ε*-) account for the highest percentage in the S4, S5 and S6 from low temperature petroleum reservoirs. *α-Proteobacteria* increased with the decrease of petroleum reservoir temperature, but the percentage of *β-Proteobacteria* decreased with the reduction of petroleum reservoir temperature. *γ-Proteobacteria* shared similar high percentage (30 ∼ 40 %) in the S4, S5 and S6 as well as in S2 (31.5 %). However, it is inetresting that all the bacterial sequences affiliated with *γ-Proteobacteria* are found in S1. *ε-Proteobacteria* was encountered in S2 and S5 accounting for 2.7 % and 5.1 %, respectively. *γ-Proteobacteria* was detected in S3 and S6 with 7.1 % and 0.7 %, respectively. *Bacteroidetes* was another frequently encountered phylum in low temperature petroleum reservoir of S5 and S6 for 9.0 % and 17.6 %, respectively. *Thermotogae* with higher percentage accounted for 19.5 % in a high temperature petroleum reservoir. The remaining bacterial phyla account for relative low abundance in the six petroleum reservoirs.

The archaeal sequences were clustered within eleven phylogenetic groups (genus level): *Methanocella*, *Methanosaeta*, *Methanomethylovorans*, *Methanolinea*, *Methanocalculus*, *Methanoculleus*, *Methanothermobacter*, *Methanobacterium*, *Thermococcus*, *Halogeometricum* and *Thermogymnomonas*. In the high-temperature oil reservoirs, most of the genera belong to the thermophilic archaea. The sequences affiliated with the genus *Thermococcus* and *Methanothermobacter* were account for high percentage, 88.8 % of total archaeal clones with the genus *Thermococcus* in S1 and all the archaeal clones affiliated with the genus *Methanothermobacter* in S2. The

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sequences affiliated with the genus *Methanomethylovorans* was only found in S3 accounting for 54.4 %. The sequences affiliated with the genus *Methanolinea* were detected in S4 with a high percentage (70 %). The sequences affiliated with the genus *Methanobacterium* were the most abundance in low temperature petroleum reservoir, accounting for 79.2 % in S5 and 66.7 % in S6.

3.2 Microbial community classification of water-flooding petroleum reservoirs

PCoA of bacterial and archaeal community structures were carried out by Unifrac based on the phylogenetic tree of 16S rRNA gene sequences in the six investigated petroleum reservoirs indicates that bacteria and archaea display high niche specificity (Fig. 6). S5 and S6 were collected from low temperature petroleum reservoirs grouped together, sharing similar bacterial and archaeal community structures. Although S1 and S2 represented high temperature petroleum reservoirs, both bacteria and archaea community structure did not grouped together because of a great difference in mineralization and the concentration of Cl^-. In addition, S2 and S3 grouped together, sharing similar bacterial community structures in PCoA of bacterial classification while S3 and S4 grouped together, sharing similar archaeal community structures in PCoA of archaeal classification.

3.3 Correlations of microbial communities with environmental factors

To find out the relationships between the distribution of microbial community and the environmental variables of the petroleum reservoirs, canonical correspondence analysis was conducted based on the bacterial and archaeal 16S rRNA gene sequences and the major physiochemical parameters of petroleum reservoirs (Table 1). The first two axes of the CCA analysis explained 69.9 % and 55.3 % of the total variance for the bacterial and archaeal communities, respectively (Fig. 7). The physiochemical parameters of petroleum reservoirs were divided to three groups to better analyze the relationships. In the first group, the differences in the bacterial and archaeal community structures

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(Fig. 7A, a) were related to temperature, mineralization, average permeability, oil viscosity, effective porosity, effective thickness and water flooding operation years. The bacterial phylogenic group of *Firmicutes* and *Thermotogae* had positive correlation with the temperature, *γ-Proteobacteria* and *Chloroflexi* with the effective porosity and oil viscosity, others with the effective thickness and water flooding operation years; most of the archaeal phylogenic group (*Methanocella*, *Methanosaeta*, *Methanomethylovorans*, *Methanolinea*, *Methanothermobacter*, *Thermococcus*, *Halogeometricum* and *Thermogymnomonas*) correlated positively with the temperature, others with the water flooding operation years, mineralization and effective thickness. In the second group, the differences in the bacterial and archaeal community structure (Fig. 7B, b) were related to differences in the concentration of Cl^-, SO_4^{2-}, PO_4^{3-}, NO_3^-, Na^+, K^+, Ca^{2+}, Mg^{2+}, and Mn^{2+}. The bacterial phylogenic group of *Bacteroidetes*, *α-Proteobacteria*, *Actinobacteria*, and *Deinococcus-Thermus* showed positive correlation with the concentration of NO_3^-, *β-Proteobacteria* with the concentration of Cl^-, *γ-Proteobacteria* and *Chloroflexi* with the concentration of SO_4^{2-} and PO_4^{3-}; the archaeal phylogenic group of *Methanosaeta*, *Thermococcus*, *Halogeometricum* and *Thermogymnomonas* correlated positively with the concentration of SO_4^{2-}, *Methanocalculus*, *Methanobacterium* and *Methanoculleus* with the concentration of NO_3^-. In the third group, the differences in the bacterial and archaeal community structure (Fig. 7C, c) were related to differences in the concentration of volatile fatty acid including acetate, propionate, isobutyrate and butyrate. The bacterial phylogenic group of *Thermotogae* showed positive correlation with the concentration of propionate, *Firmicutes* with the concentration of acetate, except for *γ-Proteobacteria* and *Chloroflexi* others were with the concentration of isobutyrate; the archaeal phylogenic group of *Methanomethylovorans*, *Methanothermobacter*, *Methanolinea* and *Methanocella* had positive correlation with the concentration of butyrate, *Methanoculleus* with the concentration of isobutyrate.

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4 Discussion

It has been widely accepted that the combination of temperature, salinity and pressure in subsurface petroleum reservoirs drastically reduces microbial populations and metabolic activity. The microbial population differs from one oil reservoir to another because of the variations in their physical, chemical, and geochemical entities. The physiologically diverse microbial assemblage of microorgnisms are distributed in such ecosystems. From the about 1000 bacterial and 400 archaeal 16S rRNA gene sequences retrieved from production water samples of six different oil reservoirs with temperature from 20 to 63 °C and salinity from 1.12 to 15.73 g L^{-1}, the bacterial sequences affiliated with the phylum *Firmicutes* had the greatest proportion in high temperature reservoirs. Dominant groups within the *Firmicutes* were the family *Peptococcaceae*, *Thermoanaerobacteraceae*, *Syntrophomonadaceae*, *Lachnospiraceae*, *Erysipelotrichaceae* and Incertae Sedis XII. Members of these groups are obligate anaerobic and thermophilic microorganisms. The sequence type (S2 B clone: 94) retrieved from oil reservoirs with temperature of 58 °C in this study is most closely related to *Pelotomaculum thermopropionicum*, a member of *Clostridiales*, and a thermophilic propionate-oxidizing anaerobic bacterium, isolated from anaerobic sludge blanket reactor in Niigata, Japan (Imachi et al., 2002). Such microorganism was once postulated to be associated with hydrocarbon-degradation (Abu Laban et al., 2009) (Gieg et al., 2008). The sequence type (S2 B clone: 127) is most closely related to *Thermacetogenium phaeum*, is a strictly anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium, which can oxidize acetate in co-culture with a thermophilic hydrogenotrophic methanogen (Hattori et al., 2000). The sequence types (S2 B clone: 210 and S3 B clone: 14) are most closely related to *Syntrophothermus lipocalidus*, a novel thermophilic, syntrophic isobutyrate-oxidizing bacterium (Sekiguchi et al., 2000). According to the physicochemical data from production water samples and CCA analysis, the bacterial group *Firmicutes* showed higher values for acetate, propionate, isobutyrate and butyrate (Fig. 7C), suggesting that VFA components within the production water serve

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as their major carbon sources, and demonstrated a higher support between the phylogenetic data and the physicochemical measurements. Other sequence type within the phylum *Firmicutes* also had a greater proportion in high temperature reservoirs, such as sequence type (S3B clone: 98), most closely related to *Fusibacter* spp., an anaerobic, thiosulfate-reducing bacterium isolated from an oil-producing well (Ravot et al., 1999). It is not a typical thermophilic microorganism with optimal grew temperature at 37 °C and such type sequences were also found in low temperature petroleum reservoir represented as S6 B clone 70 with petroleum temperature at 20 °C. CCA analysis showed that the phylum *Thermotogae* is another bacterial group correlated positively with the temperature. The sequence types (S2 B clone: 210 and S3 B clone: 14) are most closely related to *Thermotoga hypogeal*, a xylanolytic, thermophilic, strictly anaerobic bacterium isolated from an oil-producing well (Fardeau et al., 1997). Many thermophilic bacteria with optimum growth temperatures from 45 to 80 °C and have been isolated from oil fields (Beeder et al., 1995; Cayll et al., 1995; Jeanthon et al., 1995; Ravot et al., 1995; Rees et al., 1995; Fardeau et al., 1996, 1997; Nilsen et al., 1996). In this study, although S1, S2, S3 represent thermophilic temperature, not all of the bacterial sequences belong to thermophilic microorganisms, especially the bacterial sequences affiliated with *Pseudomonas* spp. within the phylum *γ-Proteobacteria* in S1 with temperature at 63 °C. It may be that the sample was contaminated during handling judging by the single type of bacterial diversity and non-temperature dependent of physiological characteristics. However, it is a general trend that microbial populations are greatly reduced in petroleum reservoirs with the combination of high-temperature, high-mineralization and high concentration of SO_4^{2-}. High-temperature oil reservoirs contained bacterial sequences affiliated with the *α*-, *β*-, *γ*-, *δ*-, and *ε-Proteobacteria*, but low-temperature oil reservoirs by the *γ- Proteobacteria*. This is best illustrated by the no correlation between physicochemistry and phylogeny, where *γ-Proteobacterial* sequences related to strict aerobes and facultative anaerobes.

In addition, CCA analysis indicated that temperature exhibited the greatest influence on the archaeal community. The archaea identified from the petroleum reservoirs

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are overwhelmingly methanogens including methyltrophic (*Methanomethylovorans*), acetoclastic (*Methanosaeta*) and CO_2-reducing methanogens (*Methanothermobacter*, *Methanoculleus*, *Methanobacteria*, *Methanocalculus Methanocella* and *Methanolinea*), possibly being mesophilic or thermophilic. In contrast, in sample S1 with the highest temperature (63 °C), besides *Methanosaeta* (0.8 % of total clones), majority of the clones were phylogenetically related to the genus *Thermococcus* (88.8 % of total clones) and to the species *T. sibiricus* (99 % sequence similarity) in these reservoirs (Fig. 4). Clones pertaining to *Thermogymnomonas* (9.6 % of total clones) and *Halogeotricum* (0.8 % of total clones) were also detected, but at a less extent in sample S1. In this respect, the ecological significance of methanogenic archaea in petroleum reservoirs is to serve as terminal electron acceptor, e.g., hydrogenotrophic ones in the complete oxidation of hydrocarbons. Most of the sequences assigned to CO_2-reducing methanogens in present researchis in line with the view that CO_2-reducing methanogens being the most commonly encountered in both cultivation and culture-independent studies of oilfield archaea (Head et al., 2010). Moreover, methanogenesis from acetate driven via syntrophic acetate oxidation has been documented in high-temperature oil reservoirs and is responsible by *Methanothermobacter–Thermoanaerobacter* co-culture (Shestakova et al., 2011) as well as in methanogenic alkanes degradation enrichment derived from production water of high-temperature oil reservoir (Mbadinga et al., 2012). Furthermore, the high apparent abundance of thermophilic, syntrophic acetate, propionate, isobutyrate and butyrate-oxidizing bacterium *Firmicutes* (*Thermoanaerobacter*) as well as thermophilic CO_2-reducing methanogens *Methanothermobacter*, coupled with the transiently high levels of corresponding substrate metabolites detected in high-temperature oil reservoir (Table 1), further demonstrating that syntrophic acetate oxidation is the main methanogenic pathway in a high-temperature petroleum reservoirs in situ. In addition to the well-known CO_2-reduction pathways, the type of methyl/methanol-utilizing methanogens as the second most common group in the present research contribute significantly in subsurface biogas formation (Strapoc et al., 2011). Although obligate acetate utilizers are represented by small

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populations of *Methanosaeta*, the potential contribution of acetate pathways cannot be ignored. In addition, metabolic types of the active methanogens vary substantially between reservoirs and appear to be controlled by local geochemical conditions within the reservoirs.

5 Conclusions

Taken together, the presence of physiologically diverse and temperature-dependent microbial community inhabiting in petroleum reservoirs. The high abundance thermophilic archaea including the genus *Thermococcus*, *Methanothermobacter* and *Thermoplasmatales*, and the bacterial sequences belong to the phylum *Firmicutes*, *Thermotogae* and *Thermodesulfobacteria* in high-temperature petroleum reservoirs relative to low-temperature petroleum reservoirs. The high abundance of the archaeal sequences belong to the genus *Methanobacterium*, *Methanoculleus* and *Methanocalculus*, and bacterial sequences belong to the phylum *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* in low-temperature petroleum reservoirs relative to high-temperature petroleum reservoirs. The results of canonical correspondence analysis (CCA) and principal coordinates analysis (PCoA) showed a consistency between the phylogenetic data and the physicochemical measurements for the sampled environment and further demonstrated that syntrophic acetate oxidation is the main methanogenic pathway in a high-temperature petroleum reservoirs.

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Table 1. Characterization of the water samples collected from different petroleum reservoirs.

	S1 Z3-26	S2 B18-43	S3 B51-45	S4 M17-10	S5 7222	S6 6190
Depth (m)	~ 1300	~ 1490	~ 1101	~ 802	~ 1088	~ 480
Temp (°C)	63	58	45	37	32	21
pH	7.1	7.2	7.2	7.2	7.1	7.0
Effective thickness (m)	4.2	5.0	5.2	14.4	15.7	18.4
Effective porosity (%)	30	17.3	22.2	24.7	17.4	20.5
Average permeability ($\times 10^{-3}$ μm^2)	800	691	12.6	675.3	274	466
Oil viscosity (mPa s)	1720	13.7	402	179.1	44.8	417
Water flooding operation (yr)	22	10	4	22	46	38
Mineralization (mg l^{-1})	8425	2891	4091	1121	15728	4212
Cl^- (mg l^{-1})	3850	361	819	447	2000	3864
SO_4^{2-} (mg l^{-1})	2244	12.1	32.4	6.8	7.7	124.8
PO_4^{3-} (mg l^{-1})	0.1	Nd	Nd	0.08	Nd	Nd
NO_3^- (mg l^{-1})	Nd	Nd	Nd	Nd	1.4	34.1
Na^+ (mg l^{-1})	3313	1629	1064	618.3	5399	4196
K^+ (mg l^{-1})	94.2	28.1	22.3	4.2	45.6	35.1
Ca^{2+} (mg l^{-1})	195.6	3.6	53.0	19.2	128.2	103.3
Mg^{2+} (mg l^{-1})	46.1	1.4	17.6	0.15	64.0	44.7
Mn^{2+} (mg l^{-1})	0.3	Nd	0.1	Nd	0.4	0.3
Acetate (mg l^{-1})	32	856	57.9	5.3	6.97	344
Propionate (mg l^{-1})	1.2	8.0	Nd	Nd	Nd	Nd
Isobutyrate (mg l^{-1})	Nd	13.8	Nd	9.8	Nd	32.7
Butyrate (mg l^{-1})	0.2	2.3	0.5	2.3	Nd	Nd

(Nd: not detected)

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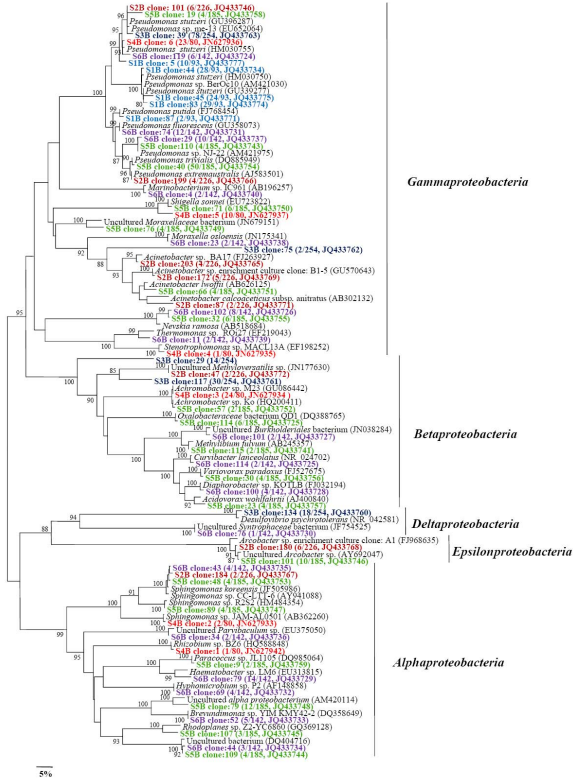

Fig. 1. Phylogenetic tree of the *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria* and *Epsilonproteobacteria* 16S rRNA gene phylotypes retrieved from six different petroleum reservoirs (shown in bold) and closely related sequences from GenBank database. Alignments 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) of ≥ 75 % are reported. Scale bar represents nucleotide changes per site. Sampling locations are as named in Table 1.

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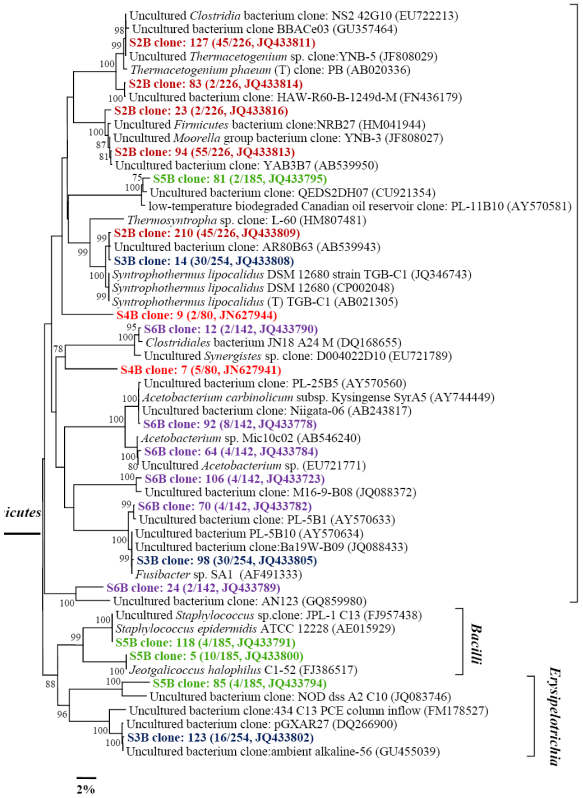

Fig. 2. Phylogenetic tree of the *Firmicutes* 16S rRNA gene phylotypes retrieved from six different petroleum reservoirs (shown in bold) and closely related sequences from GenBank database. Alignments to related sequences (shown with accession number) were performed with MEGA 5 software. The topology of the tree was obtained with the neighbor-joining method. Bootstrap values (n = 1000 replicates) of ≥ 75 % are reported. Scale bar represents nucleotide changes per site. Sampling locations are as named in Table 1.

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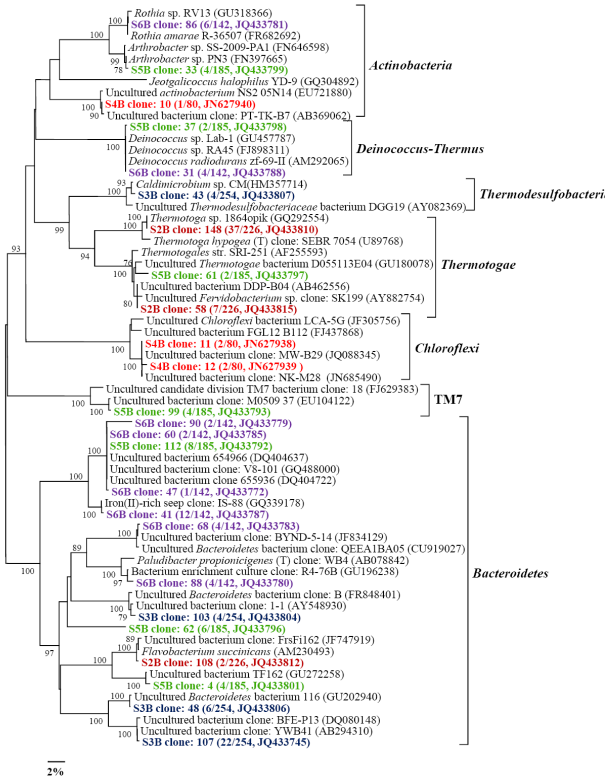

Fig. 3. Phylogenetic tree of the *Actinobacteria*, *Thermodesulfobacteria*, *Thermotogae*, *Deinococcus-Thermus*, *Chloroflexi*, *Bacteroidetes* and TM7 16S rRNA gene phylotypes retrieved from six different petroleum reservoirs (shown in bold) and closely related sequences from GenBank database. Alignments 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) of ≥ 75 % are reported. Scale bar represents nucleotide changes per site. Sampling locations are as named in Table 1.

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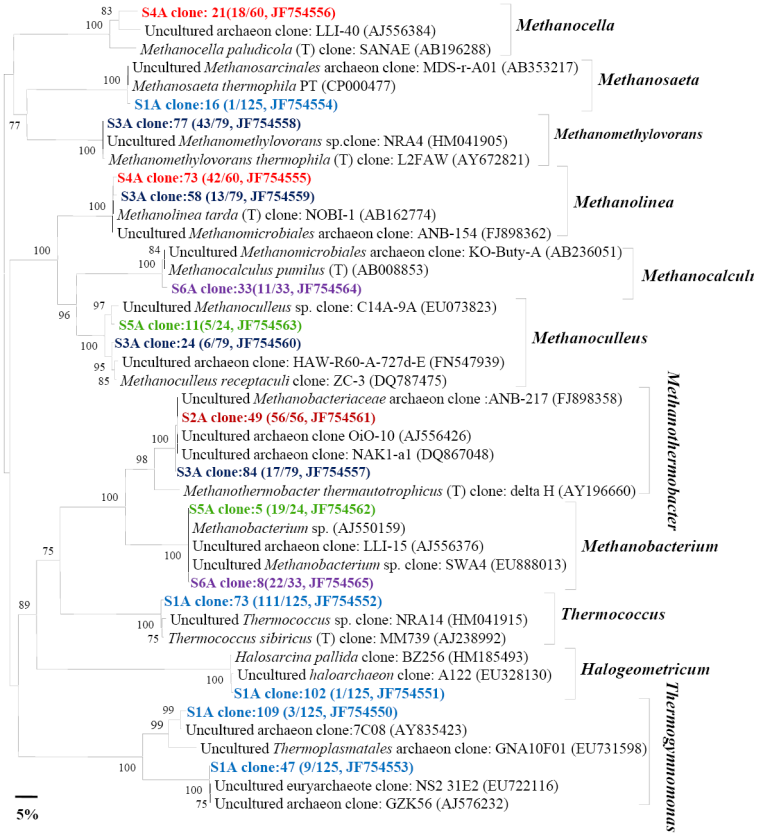

Fig. 4. Phylogenetic tree of the archaeal 16S rRNA gene phylotypes retrieved from six different petroleum reservoirs (shown in bold) and closely related sequences from GenBank database. Alignments to related sequences (shown with accession number) were performed with MEGA 4 software. The topology of the tree was obtained with the neighbor-joining method. Bootstrap values (n = 1000 replicates) of ≥ 75 % are reported. Scale bar represents nucleotide changes per site. Sampling locations are as named in Table 1.

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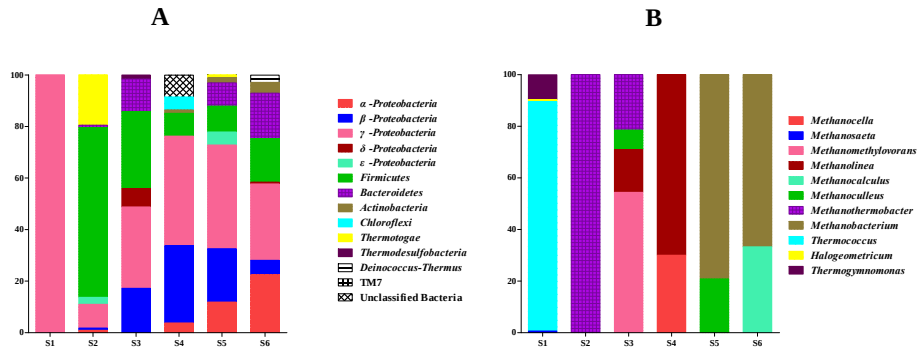

Fig. 5. Relative proportion of bacterial **(A)** and archaeal **(B)** taxa from 16S rRNA gene sequence clone libraries constructed from DNA extracted from production waters collected from oil reservoir with temperature 63, 58, 45, 37, 32 and 20 °C. Sampling locations are as named in Table 1.

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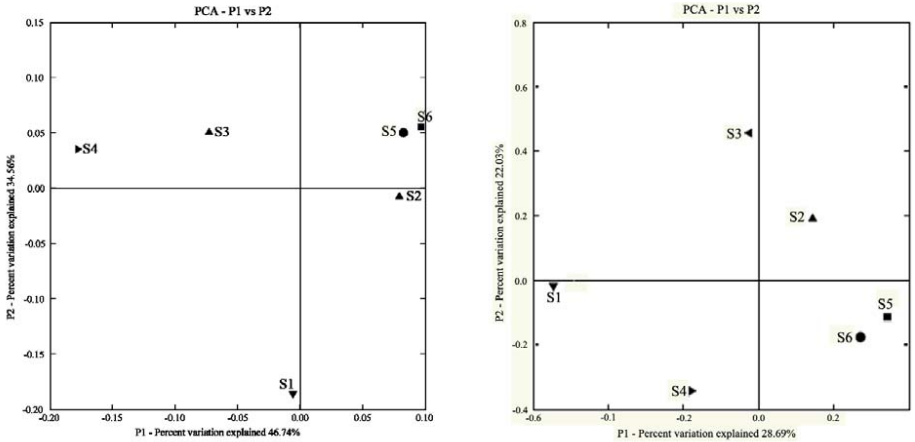

A B

Fig. 6. PCoA ordination diagrams of the bacterial **(A)** and archaeal **(B)** assemblages calculated with 16S rRNA gene sequences from production water of petroleum reservoirs. Shown are the plots of the first two principal coordinate axes (P1 and P2) for PCoA and the distributions of the bacterial and archaeal assemblages (designated with the sampling wells) in response to these axes.

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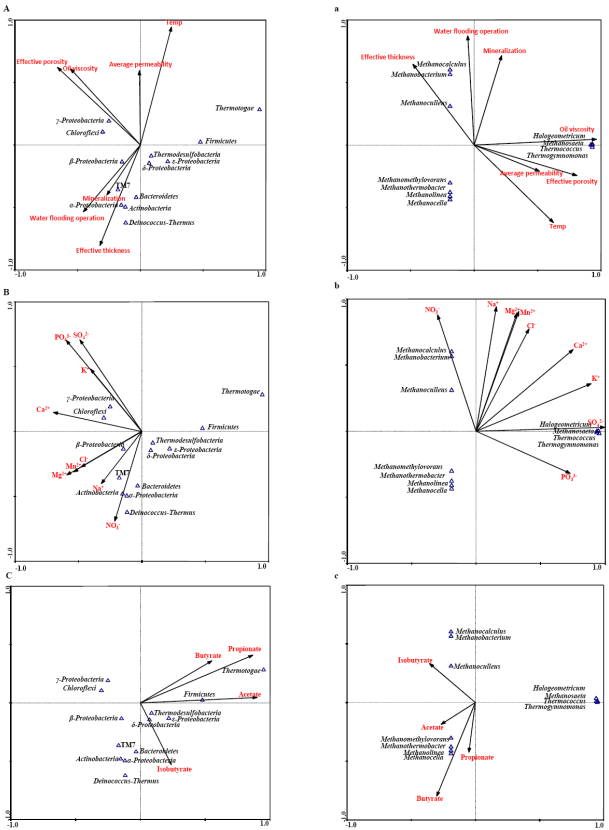

Fig. 7. CCA ordination plots for the first two dimensions to show the relationship between the bacterial and archaeal diversity with environmental factors analyzed using a 16S rRNA gene sequence in the production water of petroleum reservoirs. Correlations between environmental variables and CCA axes are represented by the length and angle of arrows (environmental factor vectors), designated with the bacterial group was showed in (**A**, **B** and **C**) and designated with the archaeal group was showed in (**a**, **b** and **c**).

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