

## Anaerobic Microbial Diversity and Abundance in Xinjiang Low-Temperature Water-Flooding Petroleum Reservoir

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### Abstract

The important role of anaerobic microorganisms in oil recovery has attracted attention, but anaerobic microbial diversity and abundance in low-temperature water-flooding oil reservoir has not been studied. To investigate anaerobic microorganisms with diverse physiological and metabolic abilities in a low-temperature water-flooding petroleum reservoir in Xinjiang, China, four types of anaerobic microorganisms were enriched on the selective cultures, and microbial communities in four anaerobic enrichments and production water (PW) were analyzed based on construction of a 16S rRNA gene clone library. Analysis of 16S rRNA gene sequencing and phylogenetic diversity reveals that the reservoir harbours large amounts of anaerobic microorganisms. *Arcobacter*, *Thalassolituus*, *Azoarcus*, and *Marinobacterium* are predominant groups in the PW clone library. *Acidaminobacter*, *Spirochaete*, *Spaerochaeta*, and *Geovibrio* are dominant and have the potential to grow using hydrocarbons as carbon sources. Fermentative bacteria mainly belong to *Spirochaete*; the nitrate-reducing bacteria are *Pseudomonas*, *Acidaminobacter*, *Acetobacterium*, *Spirochaete*, and *Spaerochaeta*; and the sulfate-reducing bacteria are closely related to *Desulfovibrio*, *Desulfuromonas*, *Sulfurospirillum*, and *Spirochaete*. *Spirochaetes* are present in the five samples indicating diverse metabolic abilities and indigenous presence in oil reservoirs. Knowledge of the predominant microbial communities in the reservoir conditions was proposed as a reference in formulating a strategy to enhance oil recovery through injection of selected nutrients or inhibitors to stimulate selective microbes.

**Keywords:** Microbial community, 16S rRNA, Clone library, Microbial enhanced oil recovery

### 1. Introduction

Petroleum reservoirs are populated mainly by anaerobic microorganisms because of low redox potential [1-3]. Since the first sulfate-reducing bacteria (SRB) were isolated from production water (PW) in 1926, culture-dependent and -independent methodologies, particularly 16S rRNA-based molecular identification methods, have revealed diverse microorganisms inhabiting petroleum reservoirs [4, 5]. Anaerobic enrichments from oil reservoirs have shown that isolating hydrocarbon-degrading bacteria (HDB), fermentative bacteria (FB), nitrate-reducing bacteria (NRB), and SRB are the important populations in reservoir ecosystems, and have critical roles in the microbial enhancement of the oil recovery process [6].

Anaerobic microbial activity reported in oil reservoirs has diverse physiological and metabolic abilities that could be beneficial or detrimental to oil recovery. For example, HDB possess the ability to selectively hydrolyze oil components to methane and CO<sub>2</sub> [7], resulting in increase of pressure in reservoirs, which enhances oil production. Anaerobic fermentative bacterial activity also produces

gases, acids, and solvents that are believed to enhance oil recovery in many field trials of microbial enhancement of oil recovery (MEOR) [8]. In comparison, widespread presence of SRB in oil reservoirs is believed to be responsible for reducing oil quality and increasing refining costs of oil and gas due to the production of H<sub>2</sub>S leading to the souring of oil [9]. A strategy of stimulating NRB by adding nitrate had been applied to reduce the activity of SRB based on the mechanism of competition for electrons or hydrogen [10].

The effective application of MEOR is generally believed to involve a combination of multiple microbial mechanisms utilizing groups of beneficial bacteria with different physiological and metabolic abilities or through the addition of selected chemicals, nutrients or manipulation of the environment [11]. Microorganisms are important participants for MEOR; therefore, a comprehensive insight into the microbial communities of oil reservoirs is essential in planning the field trial of MEOR [12]. Recently, the technology of 16S rRNA gene clone library has provided significant insights into microbial diverse physiological and metabolic abilities in petroleum reservoirs. Woodruff [13] suggested that fermentative bacteria, sulfide oxidizers and SRB could be potential bacterial syntrophs present in 25°C water-flooding oil reservoir. Grabowski et al. [14] investigated the microbial diversity in a non-water flooded 20 °C oil reservoir and observed that many anaerobic microorganisms were involved in the food chain in this oil

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reservoir. However, anaerobic microbial diversity at low-temperature water-flooding oil reservoirs remains scarce.

In this study, 16S rDNA clone libraries were performed to investigate the distribution and abundance of anaerobic microbial community of HDB, FB, NRB, and SRB and the PW from the low-temperature water-flooding petroleum reservoir in Xinjiang, China. This reservoir would improve oil recovery by stimulating reservoir microorganisms because of lower water-flooding efficiency. Therefore, the primary objective of the study is to provide ecological information on microbial populations and the biological control potential for SRB. This study also aims to understand the significance of these anaerobic bacteria in oil recovery processes and to utilize beneficial microbes in future applications of MEOR in such oil reservoirs.

The rest of this paper is organized as follows. Section 2 presents the materials and methods in detail. Section 3 describes the results and discussions of the study, and finally, the conclusions are summarized in Section 4.

## 2. Materials and methods

### 2.1 Sample collection

The PW was collected directly from the wellhead of production well T6073 in Xinjiang No. 6 oilfield on July 20, 2016. Oil-water samples were placed in sterile 500 ml serum bottles, and then sealed with rubber stoppers to maintain anoxic condition; and then, the samples were transported to the laboratory immediately for further analysis. The *in situ* temperature was 20.6 °C with approximately 800 m depth, and crude oil viscosity was 80 mPa·s. The formation water salinity is approximately 5000 mg/L.

### 2.2 Enrichment of anaerobic microorganisms

Four types of anaerobic enrichment cultures were conducted using selective media inoculated with PW from the Xinjiang No. 6 oilfield. Each 100 ml medium was placed in a 250 ml serum bottle and flushed with a mixture of N<sub>2</sub> and CO<sub>2</sub> (4:1, under atmospheric pressure) for 30 min to eliminate oxygen from the medium. Resazurin (0.5 mg/L) served as a redox indicator. The media were inoculated with 10% (v/v) PW. Petroleum from the same oil field was sterilized in hermetically sealed ampules for 30 min at 0.5 atm to use as carbon sources in some of these enrichments.

Anaerobic hydrocarbon-degrading bacteria (HDB) were enriched using R medium containing sterilized oil (2%) [15]. The fermentative bacteria (FB) group was enriched for use of the medium supplemented with peptone (5.0 g/L) and glucose (10.0 g/L) [16]. Nitrate-reducing bacteria (NRB) were enriched in Adkins medium with sodium citrate (8.5 g/L) as the energy and sodium nitrate as the electron acceptor [17]. Sulfate-reducing bacteria (SRB) were obtained using Postgate's B medium containing sodium thiosulfate (10.0 g/L), sodium lactate (4.0 g/L), and sodium ascorbate (4.0 g/L) and then supplemented with microelements and reductant of Na<sub>2</sub>S · 9H<sub>2</sub>O (200 mg/L) [18]. All media were incubated at 20 °C for 14 days.

### 2.3 Genomic DNA extraction

After the preceding steps, 100 ml PW sample and 5 ml anaerobic enrichments were centrifuged to pellet cells. Following the manufacturer's instruction for TIANamp Micro DNA Kit (DP316) (Tian Gen Biotech (Beijing) Co.

Ltd., China), genomic DNA was extracted in triplicate to avoid bias and the DNAs were mixed.

### 2.4 Construction of 16S rRNA gene libraries

The 16S rRNA gene was amplified by PCR using universal bacteria primers 27F/1492R. Bacteria 16S rRNA gene amplifications were conducted with template DNA following the previous PCR reacting system and thermal cycle programme [19].

Amplicons were cloned with a TA cloning vector kit (Promega, Madison, WI, USA) according to the manufacturer's instruction. Five clone libraries were constructed for the DNAs extracted from PW and the four anaerobic enrichment cultures. Then, 200 clones in each clone library were obtained to select positive clones through PCR with the sets of vector-specific primers T7/SP6. PCR products of positive clones were subjected to amplified ribosomal DNA restriction analysis (ARDRA) with *Hinf*I and *Hae*III (Fermentas, Lithuania) [20].

### 2.5 Data analysis

Clones were classified into different operational taxonomic units (OTU) based on ARDRA profiles. Representative clones, belonging to different OTUs, were selected for 16S rDNA sequencing. Clone libraries were statistically evaluated by rarefaction analysis using Analytic Rarefaction 1.3 in which the expected numbers of different ARDRA groups versus the numbers of positive clones in each library were calculated. Shannon indices characterize the 16S rDNA clone libraries [21].

Sequencing was conducted using an ABI PRISM 3730 DNA sequencer (SinoGenoMax Co. Ltd., Beijing, China). The obtained sequences were manually checked and edited using DNAMAN version 5.2.2.0. Using a BLAST search tool, a representative sequence from each OTU was compared with sequences in the GenBank database to find the most closely related sequences and organisms. Sequences with more than 97% similarity were considered to be the same phylotype. Neighbour-joining tree was constructed by using ClustalX version 1.83 and MEGA version 4.1 [22, 23]. Bootstrap analysis with 1000 resamplings was conducted to assign confidence levels to the nodes in the distance and parsimony trees.

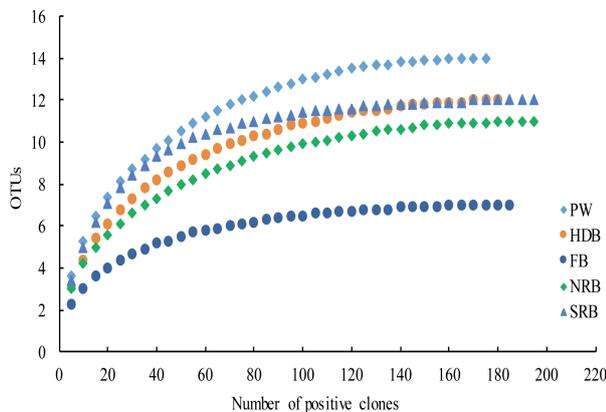
## 3 Results and discussions

### 3.1 Statistical analysis of 16S rRNA gene clone libraries for four anaerobic enrichments and production water

To investigate the main anaerobic microbial communities within a low-temperature oil reservoir subjected to long-term water flooding, the PW retrieved directly from one production well was used in anaerobic enrichments designed to obtain four clone libraries, namely, HDB for hydrocarbon-degrading bacteria, FB for fermentative bacteria, NRB for nitrate-reducing bacteria, and SRB for sulfate-reducing bacteria. Meanwhile, microorganisms in PW are also analyzed using a 16S rDNA clone library. Based on sets of vector-specific primers T7/SP6, 948 positive clones out of a total of 1000 clones in the five clone libraries are identified.

According to ARDRA profiles, the number of positive clones in the clone libraries of PW, FB, HDB, NRB, and SRB are identified as 14, 7, 12, 11, and 12 OTUs, respectively. The richness of OTUs observed in the rarefaction curves is higher in the PW than in the four

anaerobic cultures. Moreover, rarefaction curves of the five clone libraries tend to approach the saturation plateau, indicating that the positive clones in the clone library could effectively cover the diversity of microorganisms (Fig. 1).



**Fig. 1.** Rarefaction analysis of 16S rRNA gene positive clones for clone libraries of PW, FB, HDB, SRB, and NRB using software of Analytic Rarefaction

The Shannon index of bacterial diversity was calculated to assess the evenness of the OTU distribution. NRB has the highest Shannon index (Tab. 1), indicating that the highest bacterial diversity was present in NRB; in contrast, FB has the lowest Shannon's index (1.32).

**Table 1.** Statistical analysis of five clone libraries

Library	PW	FB	HDB	NRB	SRB
Number of Positive clones	176	190	186	198	198
OTUs	14	7	12	11	12
Shannon	2.05	1.32	2.06	2.32	1.98
Simpson	0.63	0.48	0.66	0.76	0.69

### 3.2 Anaerobic microbial communities in the low-temperature water-flooding oil reservoir

The diversity and relative abundance of microbial communities in the four anaerobic enrichments and the production water are shown in Fig. 2. The predominant group in the clone library of PW is *Arcobacter*, which accounts for 58%, while the clone libraries of HDB, FB, NRB, and SRB are predominated with microbial groups affiliated with *Acidaminobacter*, *Sphaerochaeta*, *Pseudomonas*, and *Desulfovibrio*, which accounts for 54%, 24%, 39%, and 48%, respectively. The remaining bacterial sequences of the PW clone library are mainly of the genera *Thalassolituus*, *Azoarcus*, *Marinobacterium*, *Sulfurimonas*, *Azovibrio*, and *Desulfomicrobium*. In HDB, many microorganisms, such as *Acidaminobacter*, *Spirochaete*, *Sphaerochaeta*, *Geovibrio*, *Arcobacter*, and *Acetobacterium*, have the potential to use hydrocarbons. Unclassified bacteria accounted for 68%, are the most frequently detected in the FB. The remaining genera of NRB are mainly *Acidaminobacter*, *Acetobacterium*, *Spirochaete*, and *Sphaerochaeta*, while the remaining genera of SRB are closely related to *Desulfuromonas*, *Sulfurospirillum*, *Brachymonas*, and *Spirochaete*.

Numerous microorganisms are detected in the PW sample and not observed in the anaerobic enrichment cultures (Fig. 2). Aerobic and anaerobic microorganisms *Azovibrio*, *Azoarcus*, and *Arcobacter* are identified in the PW. Moreover, *Spirochaeta* appeared in all of the five samples, which indicates that the genus has diverse metabolic abilities in an oil-bearing environment. Additionally, *Sphaerochaeta* is also detected in HDB and NRB. *Desulfovibrio* is present in HDB and FB, while *Denitrovibrio* is identified in FB and NRB. Unclassified bacteria are detected in all samples, especially the dominant group in the FB library.

### 3.3 Phylogenetic diversity analysis of Anaerobic microbial populations in the oil reservoir

To further analyze the microbial diversity of the low-temperature water-flooding oil reservoir, the sequences of five clone libraries were used to construct a phylogenetic tree with closely related bacterial sequences. The phylogenetic diversity is displayed in a phylogenetic tree (Fig. 3).

Microbial communities are significantly different between the production water and the four anaerobic enrichments. For example, microaerophilic bacteria *Azovibrio restrictus*, *Azoarcus communis*, and *Arcobacter defluvi* are detected in PW [24]. Water flooding is always regarded as a significant factor that influences oxygen content [25], which affects native microbial communities in oil reservoirs. The sample was collected from the oil reservoir subjected to long-time water flooding since 1974. Moreover, microbial communities show that the dominant microbes among the five clone libraries are different at phylum level. *ε-proteobacteria* is the dominant group in the PW library, while the predominant groups of known cultivated bacteria are closely related to *Firmicutes*, *α-proteobacteria*, *γ-proteobacteria*, and *δ-proteobacteria* in the HDB, FB, NRB, and SRB enrichment cultures, respectively.

Notably, however, *Firmicutes* is not detected in PW and *Defferribacteres* is identified in HDB. The reasons for these differences are as follows. First, the four anaerobic enrichment cultures adopt selective media to enrich culture-specific species, while the PW retrieved from the reservoir directly is used to extract genomic DNA predominating under the reservoir conditions. Second, we investigate only the microbial communities in the water sample in this study without considering other parameters such as strata pressure, crude oil, and formation water, which are significant environmental factors for anaerobic enrichment. Third, various biases occur in the extraction of genomic DNA and TA clones as well as in PCR amplification [19].

Limited knowledge is available on microbial diversity in complex oil field environments and thus, only a few species have been reported [12]. New species may exist in the low-temperature water-flooding oil reservoir. Sequences related to *Brachymonas petroleovorans* (94% identity) and *Desulfuromonas michiganensis* (93%–96% identity) in the SRB sample, and to *Pseudobutyrvibrio ruminis* (94%) in the FB sample have been detected and had less than 97% similarity to the species reported in the previous papers. Unclassified bacteria are obtained in all samples, indicating which the oil reservoir harbours a unique community of novel bacterial species or genera.

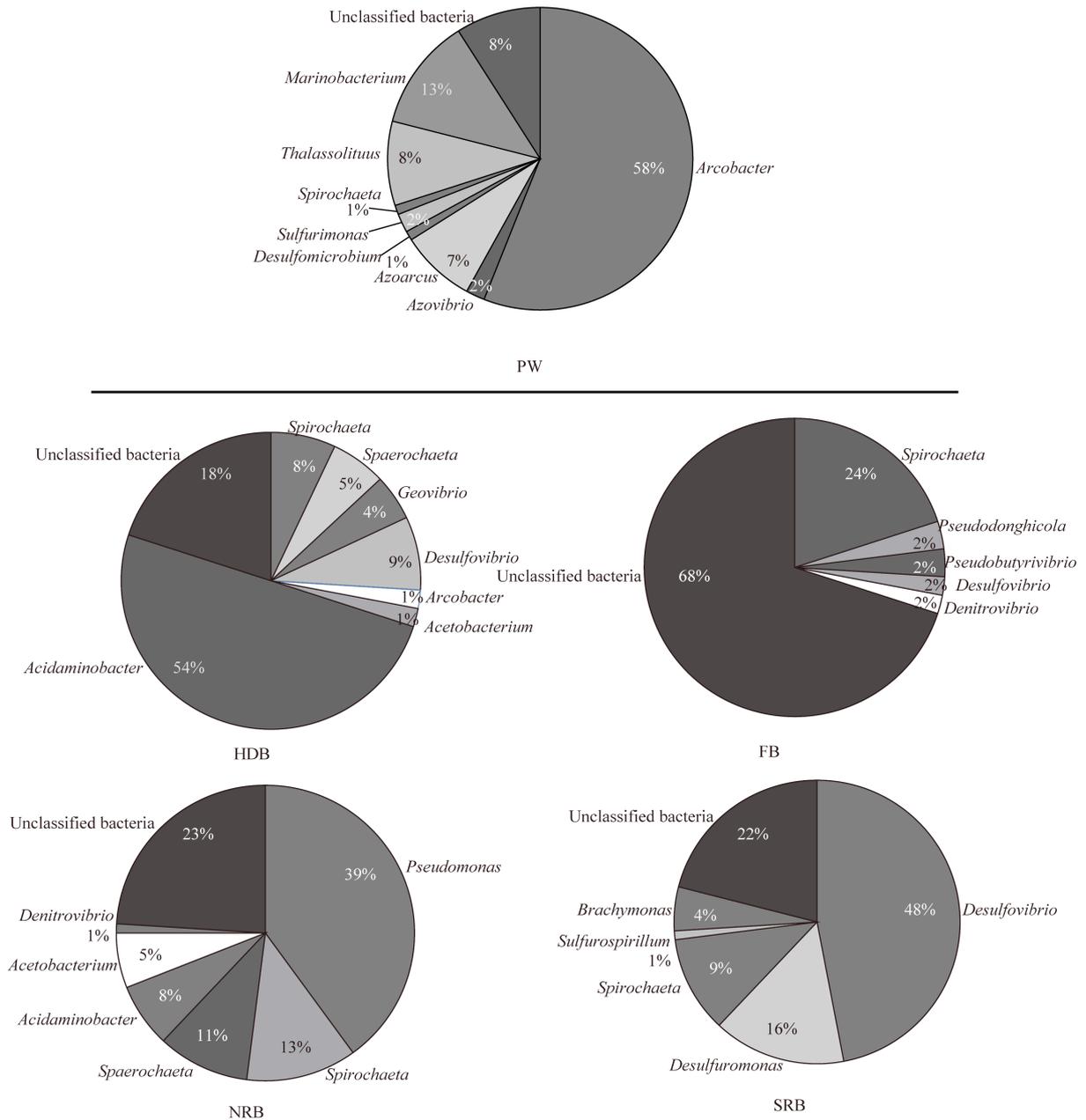


Fig. 2. Relative abundances and diversity of microbial community in production water (PW) and anaerobic enrichments clone libraries (FB, HDB, SRB, and NRB)

### 3.4 Potential microorganisms to microbial enhanced oil recovery

In the study, the four selective anaerobic media were used to enrich FB, HDB, NRB, and SRB, and then microorganisms that are both beneficial and detrimental to MEOR appear in the four anaerobic enrichment cultures and the PW. *Thalassolituus oleivorans* and *Brachymonas petroleovorans* could grow using hydrocarbons as carbon sources [26, 27]. *Acidaminobacter hydrogeniformans* and *pseudobutyrvibrio ruminis* are also identified and could be selectively activated for their metabolites [28, 29], thereby reducing oil viscosity or interfacial tension and leading to increased production of biomass production to improve oil recovery.

Denitrifying bacteria, such as *Pseudomonas stutzeri* and *Denitrovibrio acetiphilus* [30-32], are present in the NRB. However, bacterial sequences of SRB are assigned to

*Desulfovibrio caledoniensis*, *Desulfovibrio dechloracetivorans*, and *Geovibrio thiophilus*. Therefore, enhancing or stimulating the activity of such strains could be useful when SRBs are detrimental or become a problem for MEOR due to the production of H<sub>2</sub>S [6].

### 3.5 Indigenous Spirochaetes in oil reservoirs

*Spirochaetes* are detected in the four anaerobic enrichments and the PW. The study reveals that *Spirochaetes* could exhibit diverse metabolic abilities in the low-temperature water-flooding oil reservoir. Additionally, mesophilic fermentative halophilic *Spirochaeta smaragdinae* was isolated from an African oil field [33]. Moreover, *Spirochaetes* were obtained in a low-salinity non-flooded 20 °C oil reservoir in water-flooding Enermark oil fields and in a non-flooded high-temperature oil reservoir [14, 34, 35].

Thus, *Spirochaetes* could be considered as indigenous bacteria in oil reservoirs.

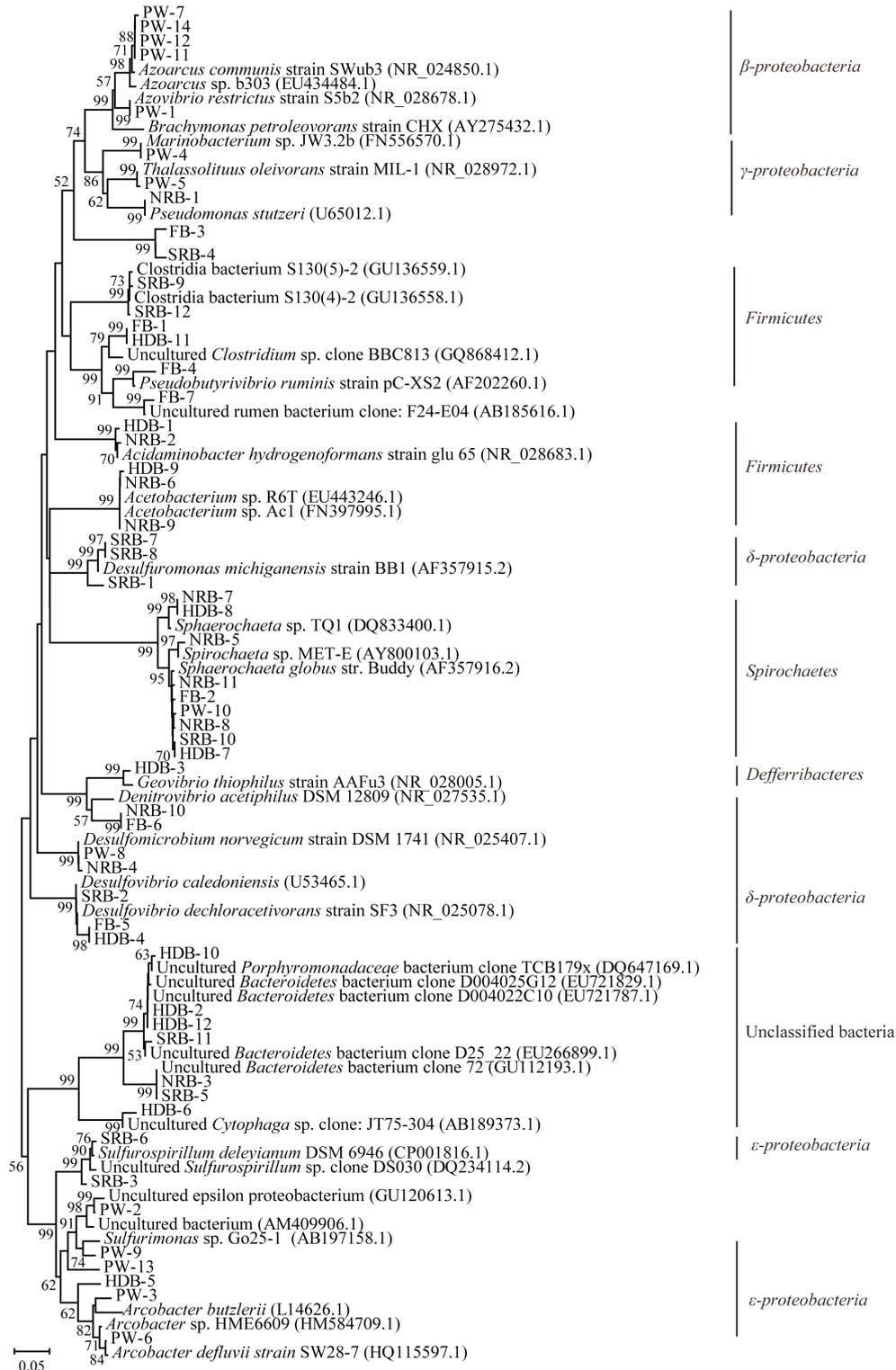


Fig. 3. Phylogenetic tree of 16S rDNA phylotypes of four anaerobic enrichments and production water clone libraries

#### 4. Conclusions

To provide ecological information on oil recovery and biological control potential for SRB in the Xinjiang low-temperature water-flooding petroleum reservoir, this study set up four types of anaerobic enrichment cultures using four selective media for HDB, FB, NRB, and SRB. Then, 16S rDNA clone library, gene sequencing, and phylum diversity analysis were conducted to reveal the distribution and

abundance of anaerobic microbial populations in the oil reservoir. The following conclusions could be drawn:

(1) Beneficial bacteria to microbial enhanced oil recovery are identified. Hydrocarbon-degrading bacteria, fermentative bacteria, and nitrate-reducing bacteria are detected in the petroleum reservoir. Beneficial bacterial communities could be enhanced through injection of chemicals such as surface-active molecules or selected nutrients or through manipulation of culture conditions.

Furthermore, beneficial microorganisms could be used to enhance oil recovery in the low-temperature water-flooding oil reservoir.

(2) SRB bacteria are most numerous in the oil reservoir and cause substantial damage to oil recovery. Knowledge of the presence of detrimental SRB bacteria can be mitigated by using selected inhibitors or simulating NRB activity.

(3) The bacterial sequences of the four anaerobic enrichments and the PW closely related to the genus *Spirochaetes*, which has diverse metabolic abilities in the low-temperature water-flooding oil reservoir, could be considered as indigenous in petroleum reservoirs.

Thus, the study was applied to MEOR in the oil reservoir by injecting proper nutrients, activating selectively beneficial microorganisms, and inhibiting the detrimental

ones in the reservoir. The role of anaerobic microorganisms in the reservoir conditions remains ambiguous because of their diverse metabolic abilities and therefore could be further investigated.

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