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Microbial diversity in long-term water-flooded oil reservoirs with different *in situ* temperatures in China

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Water-flooded oil reservoirs have specific ecological environments due to continual water injection and oil production and water recycling. Using 16S rRNA gene clone library analysis, the microbial communities present in injected waters and produced waters from four typical water-flooded oil reservoirs with different *in situ* temperatures of 25°C, 40°C, 55°C and 70°C were examined. The results obtained showed that the higher the *in situ* temperatures of the oil reservoirs is, the less the effects of microorganisms in the injected waters on microbial community compositions in the produced waters is. In addition, microbes inhabiting in the produced waters of the four water-flooded oil reservoirs were varied but all dominated by Proteobacteria. Moreover, most of the detected microbes were not identified as indigenous. The objective of this study was to expand the pictures of the microbial ecosystem of water-flooded oil reservoirs.

S ince 1859, the first oil well drilled near Titusville, Pennsylvania, oil reservoirs have been subjected to exploitation due to the demand of energy requirement saw a worldwide expansion. Multiple groups of microorganisms with diverse physiological characteristics and metabolic abilities and phylogenetic affiliations have gradually been recovered from oil reservoirs[1–3]. Magot[3] identified sulfate reduction, fermentation, acetogenesis and methanogenesis as the major metabolic processes carried out by indigenous microbes in oil reservoirs. However, determining whether a microorganism is indigenous to an oil reservoir became increasingly difficult due to the introduction of allochthonous microorganisms and other sources of contaminations by enhanced oil recovery (EOR) processes. The major methods of improved oil recovery are water-flooding, gas injection, chemical flooding, and thermal recovery. All these techniques inevitably cause the change of physical and chemical characteristics and microbial compositions of oil reservoir ecosystems on short or long term bases[2,4]. Water-flooding, the least expensive and most widely used method for secondary oil recovery, is a technique where freshwater, seawater or recycled reservoir produced water after separation is reinjected into the reservoir formation of wells that have reduced production to displace/push some of the remaining oil toward producing wells in the same reservoir.

Recently, much attention has been paid to the potential applications of microbial enhanced oil recovery (MEOR) techniques, resulting in an increased interest in knowledge of microbial communities in subsurface oil reservoirs[1,2,4]. Most studies of microbial communities in oil reservoirs have for practical and economical reasons normally been carried out on samples obtained from the oil well production heads, and most of oil reservoirs under study are subjected to water-flooding[5–8]. Although the results of these studies, especially those based on culture-independent methods, broaden our knowledge of microbial diversity in oil reservoir's ecosystem and the detection of organisms however, they did not provide sufficient evidence whether these organisms were indigenous to the reservoirs. Water-flooding is believed to be the main contaminating factor to result in the continued reinoculation of reservoir with surface microorganisms as well as the alteration of the geochemistry of the formations[9]. Some microbes in injected water are thought to possess exceptional survival abilities and could be detected by various culture-dependent and culture-independent techniques long after being introduced to oil reservoirs[2]. However, detailed studies about the effects of the microbial communities present in injected water on the community in the oil reservoirs are limited. The main reason could be that most oil reservoirs, especially those
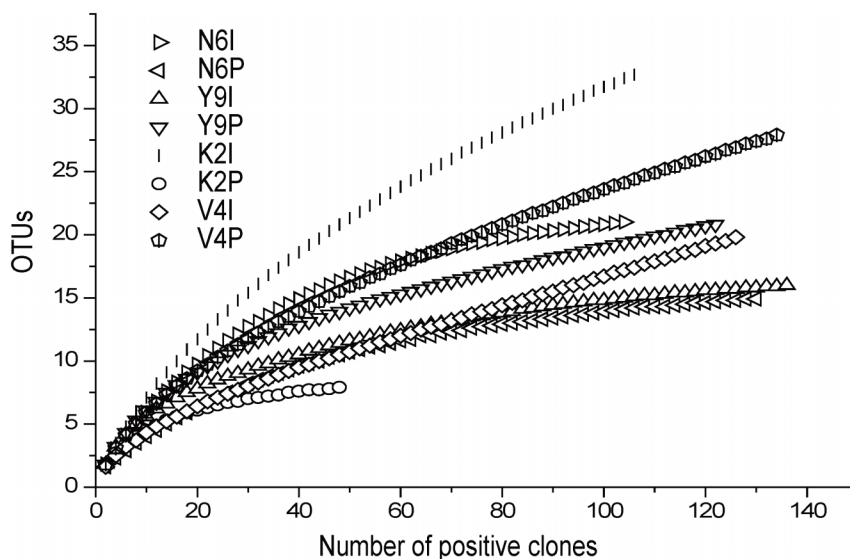
Figure 1 | Rarefaction analysis of 16S rRNA gene positive clones for clone libraries of N6I, N6P, Y9I, Y9P, K2I, K2P, V4I and V4P using Analytic Refraction software.

in China, have been subjected to water-flooding for decades while no data about the microbial communities in these oil reservoirs before water-flooding were available. In order to discover the effects of microbes in injected water on microbial community in oil reservoirs, an alternative investigating strategy of comparison of microbial community compositions in injection and production well samples was applied in the study.

In general, the physical characteristics and chemical composition of oil reservoir environments are the main factors that determine the profile of the microbial communities in the ecosystems. Temperature appears to be the most important limiting factor for microbial growth in oil reservoirs[1,3]. For oil reservoirs, temperature increases generally with depth at a mean rate of $3°C$ per 100 m, therefore, different oil reservoirs have different *in situ* temperatures, which could have great effects on the possibility for the organisms in injected water to survive or thrive in oil reservoirs. In order to gain convictive investigating data of effects of microbes in injected water on microbial communities in oil reservoirs, samples from four water-flooded oil reservoirs with different *in situ* temperatures were selected in this study.

Microbial diversity assessment based on 16S rRNA gene clone library technique has yielded amazing insights into the community composition in a variety of environments. In this study, after comparing microbial communities in samples of injection and production wells from the four oil reservoirs with different *in situ* temperatures, microbial communities detected in samples of produced waters were also analyzed. We aim to expand knowledge of microbial ecosystem in oil reservoirs subjected to water-flooding and more importantly provide a significant referencing value for the performance of microbial enhanced oil recovery (MEOR) in theses oil fields.

Results

Microbial community compositions in samples of the injection and production wells from the oil reservoir with an *in suit* temperature of 25°C. No. 6 oil reservoir, a typical representative of low-temperature oil reservoir with an *in suit* temperature of $25°C$ in Xinjiang oil field, was used in the study. Two 16S rRNA gene clone libraries, N6I and N6P, were constructed from wellhead samples of injection well of T6186 and production well of T6073. 21 and 15 OTUs were identified in each clone library according to ARDAR of 104 and 131 positive clones in N6I and N6P, respectively.

Rarefaction curves of the two libraries tended to approach the saturation plateau indicating that positive clones in each library could well cover the diversity of bacteria (Fig. 1).

In N6I, 51% of the sequences were identified as unclassified bacteria, the remaining sequences were clustered within 4 phyla: Proteobacteria (32%), detailed in δ- (18%), γ- (12%), and ε- (2%), followed by Cytophaga-Flavobacter-Bacteroidetes (CFB) group (12%), Spirochetes (4%) and Deferribacteres (1%) (Fig. 2). Unlike N6I, only 2.4% sequences in N6P were unclassified, the predominant sequences were affiliated with δ-Proteobacteria (74%) which is followed by CFB (9.3%), Spirochetes (3.9%), Deferribacteres (2.4%) (Fig. 2). According to the results of phylogenetic affiliations obtained from algorithm of BLAST of nucleotide, 81% sequences in N6I and
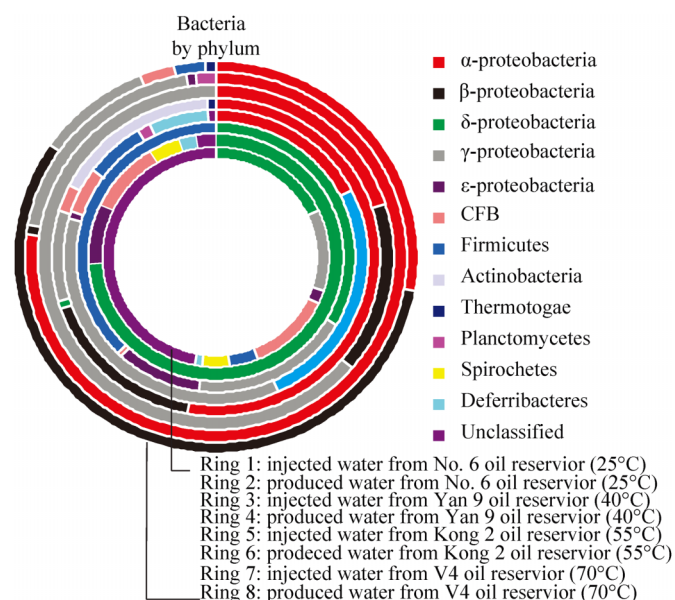

Figure 2 | Bacterial population dynamics of the injected waters and produced waters collected from the four water-flooded oil reservoirs as revealed by 16S rDNA clone libraries. Samples from inner to outer of the ring chart were injected water and produced water from the oil reservoir with high temperature (70°C) to the oil reservoir with low temperature (25°C).

94.6% sequences in N6P had a relatively low similarity (< 96%) to the sequences in GenBank database, which indicated that the oil reservoir harbours unique microbial communities comprising several unknown genera or species. In N6I (Fig. 3), the predominant group were the unknown bacterium (46%), and the other bacterial groups were uncultured CFB group (12%), *Sphinqobium* sp. (5%), *Syntrophus* sp. (5%), *Desulfuromusa* sp. (4%), *Azovibrio* sp. (4%), *Geobacter* sp. (4%), uncultured Desulfobacterales bacterium (3%), *Pseudomonas* sp. (3%), *Desulfuromonas* sp. (2%), *Acrobacter* sp. (2%), *Denitrovibrio* sp. (1%). In N6P, uncultured Desulfobacterales bacterium was predominant at 66.4%, followed by uncultured CFB group (9.3%), *Syntrophus* sp. (3.9%), *Spirochaetes* sp. (3%), *Acrobacter* sp. (2.4%) and *Denitrovibrio* sp. (1.5%) (Fig. 3). Obviously, the microbial communities in N6I and N6P appeared different, but even so, there were 5 common phyla, 5 common families, and 7 common existing OTUs in N6I and N6P (Table 1).
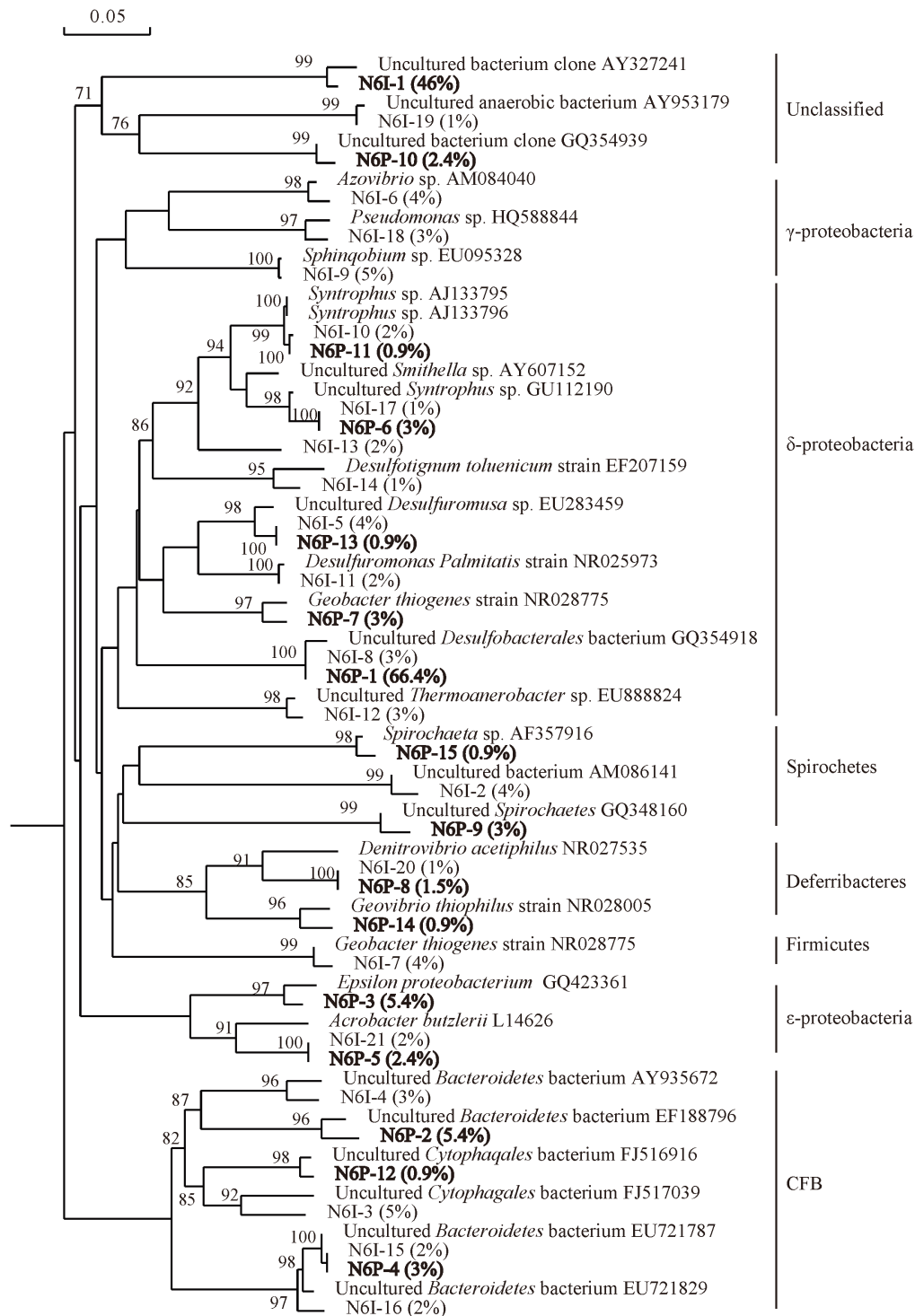

Figure 3 | Phylogenetic tree of bacteria 16S rDNA phylotypes of N6I and N6P (25°C). The tree constructed was based on approximately 800bp 16S rRNA genes and their nearest clones retrieved from the GenBank database. The percentages behind the OTUs represent the relative abundance of each OTU. Bootstrap values are indicated at branch points. The scale bar represents 5% estimated sequence divergence.

Table 1 | Co-existing families and the relative abundance of each family in the clone libraries created using the samples of injected water and produced water from each oil reservoir and the number of co-existing OTUs

Co-existing Plylum	Co-existing Family	Relative abundance (%)		Co-existing OUT
		IW (OTUS)	PW (OTUS)	
N6 (25°C)				
Delta	Syntrophaceae	5 (3)	3.9 (2)	2
	Desulfuromonadaceae	6 (2)	0.9 (1)	1
	Uncultured Desulfobacterales	3 (1)	66.4 (1)	1
Epsilon	Campylobacteraceae	2 (1)	2.4 (1)	1
CFB	Cytophaqales	5 (1)	0.9 (1)	
	Bacteroidetes	7 (3)	8.4 (2)	1
Spirochetes		4 (1)	3.9 (2)	
Deferribacteres	Deferribacteraceae	2.4 (2)	1 (1)	1
Unclassified		47 (2)	2.4 (1)	
Total relative abundance (%)		81.4	90.2	7
Y9 (40°C)				
Delta	Desulfuromonadaceae	28.9 (1)	0.9 (1)	
Gamma	Pseudomonadaceae	15.5 (2)	32.5 (1)	
Epsilon				
CFB				
Firmicutes				
Total relative abundance (%)		44.5	33.4	
K2 (55°C)				
Alpha	Rhizobiaceae	2.8 (1)	6 (1)	
Beta				
Gamma	Xanthomonadaceae	0.9 (1)	2 (1)	
	Pseudomonadaceae	6.4 (5)	62 (4)	
Total relative abundance (%)		10.1	70	
V4 (70°C)				
Alpha	Rhodobacteraceae	0.8 (1)	0.8 (1)	
Beta				
Gamma	Pseudomonadaceae	0.8 (1)	8.5 (7)	
Total relative abundance (%)		1.6	9.3	

Microbial community compositions in samples of the injection and production wells from the oil reservoir with an *in suit* temperature of 40°C. Water-oil samples from injection well of L28-45 and production well of L28-46 in Yan 9 oil reservoir with an *in suit* temperature of 40°C were used in the study. Two 16S rRNA gene clone libraries, Y9I and Y9P, were constructed. According to ARDAR of 137 and 124 positive clones, 15 and 21 OTUs were identified in Y9I and Y9P, respectively. Rarefaction curves of the two libraries showed that the positive clones in each library could well cover the diversity of bacteria (Fig. 1).

In Y9I, detected bacteria were clustered mainly within two phyla: Proteobacteria (61.7%) and Firmicutes (37.4%). Among Proteobacteria, δ-, γ-, and ε- Proteobacteria represented 33.3%, 18.4% and 10%, respectively. In Y9P, Proteobacteria predominated at 79.9%, including α- (17.6%), δ- (25.7%), γ- (35.7%), and ε- (0.9%), followed by Firmicutes (6.5%), Deferribacteres (6.3%), CFB (4.9%) and Planctomycetes (1.6%) (Fig. 4).

55.6% sequences in Y9I and 50.8% sequences in Y9P were identified as unknown bacteria due to their low similarity (<96%) with reference sequences in GenBank database. The dominant bacteria groups in Y9I were affiliated with *Desulfuromonas* sp. (29.8%), *Clostridium* sp. (20%), *Fusibacter* sp. (14.4%) and *Pseudomonas* sp. (13.3%). However in Y9P, dominant bacteria were *Pseudomonas aeruginosa* (32.5%), *Desulfovibrio* sp. (15.7%), *Methylopila* sp. (11.1%), *Flexistipes* sp. (6.3%) (Figure 4). Although there were 5 co-existing phyla, microbial communities in wellhead samples of the injection well and the production well appeared divergent widely with only 2 common families, (Desulfuromonadaceae and Pseudomonadaceae) and no common OTU (Table 1).

Microbial community compositions in samples of the injection and production wells from the oil reservoir with an *in suit* temperature of 55°C. Water-oil samples from injection well of 1098-8 and production well of 1017-7 in Kong 2 oil reservoir with an in suit temperature of 55°C were used in the study. According to ARDAR of 108 and 50 positive clones in clone libraries of K2I and K2P, 33 and 8 OTUs were identified in each clone library. Rarefaction curves of the two libraries indicated that the positive clones in each library could well cover the diversity of bacteria (Fig. 1). The predominant phylum in K2I was Proteobacteria predominated at 79.8%, encompassing α- (52.9%), β- (16.8%), δ- (0.9%), γ- (9.2%). The other phyla in K2I were Actinobacteria (16.7%), CFB (2.7%) and Thermotogae (0.9%). In K2P there was only one phylum of Proteobacteria, including α- (20%), β- (16%), and γ- (64%) (Fig. 2).

92% and 91.8% sequences in K2I and K2P were not affiliated with specific genera. The dominant populations in K2I were *Rhodobacter* sp. (47.3%), followed by Candidatus sp. (16.7%), *Alcaligenes* sp. (5.6%), *Pseudomonas* sp. (6.4%), *Pusillimonas* sp. (4.6%). In K2P, *Pseudomonas* sp. covered a major percentage of 62%, which was followed by *Stappia* sp. (14%), *Thauera* sp. (12%), *Sinorhizobium* sp. (6%), *Azovibrio* sp. (4%) and *Stenotrophomonas* sp. (2%) (Fig. 5). The results revealed that the microbial communities in well-head samples from the injection well and the production well were different. In addition, the co-existing common phyla were α-, β- and γ- Proteobacteria, and the co-existing common families were Rhizobiaceae, Xanthomonadaceae and Pseudomonadaceae. There were no co-existing common OTU (Table 1).

Microbial community compositions in samples of the injection and production wells from the oil reservoir with an *in suit*
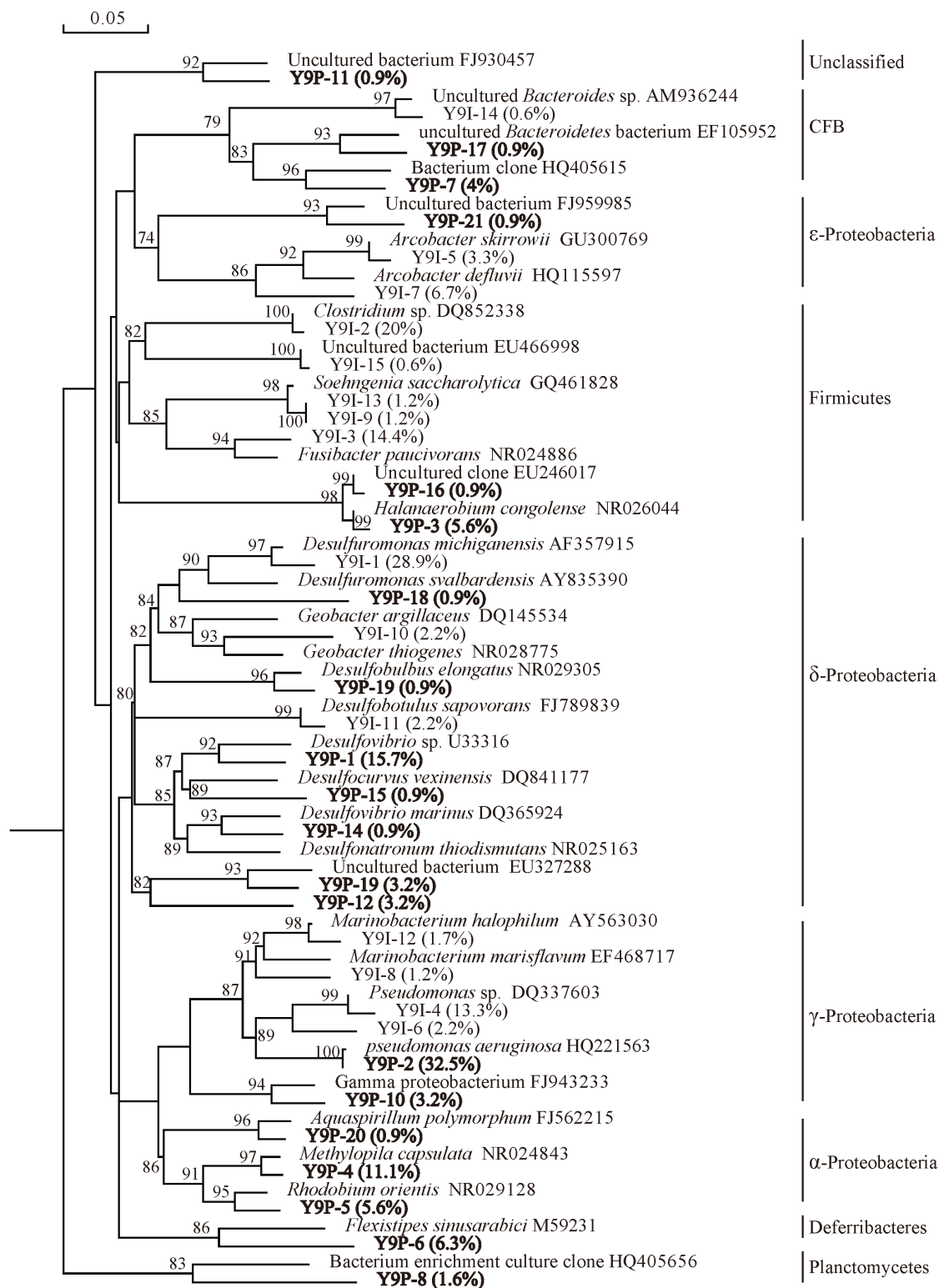
Figure 4 | Phylogenetic tree of bacteria 16S rDNA phylotypes of Y9I and Y9P (40°C). The tree constructed was based on approximately 800bp 16S rRNA genes and their nearest clones retrieved from the GenBank database. The percentages behind the OTUs represent the ralative abundance of each OTU. Bootstrap values are indicated at branch points. The scale bar represents 5% estimated sequence divergence.

temperature of 70°C. Water-oil samples from injection well of V187 and production well of V149 in V4 oil reservoir with an *in suit* temperature of 70°C were used in the study. According to ARDAR of 128 and 135 positive clones, two 16S rRNA gene clone libraries, V4I and V4P were constructed with 20 and 28 OTUs in each clone library. Rarefaction curves of the two libraries also indicated

that the positive clones in each library could well cover the diversity of bacteria (Fig. 1). The predominant phylum in V4I was Proteobacteria representing 98.7% including α- (77.5%), β- (0.8%), γ- (19.6%) and ε- (0.8%). Another phylum in V4I was Planctomycetes (1.6%). In V4P, Proteobacteria also were predominant at 94.8% including α- (27.6%), β- (57.1%), γ- (9.3%). The other
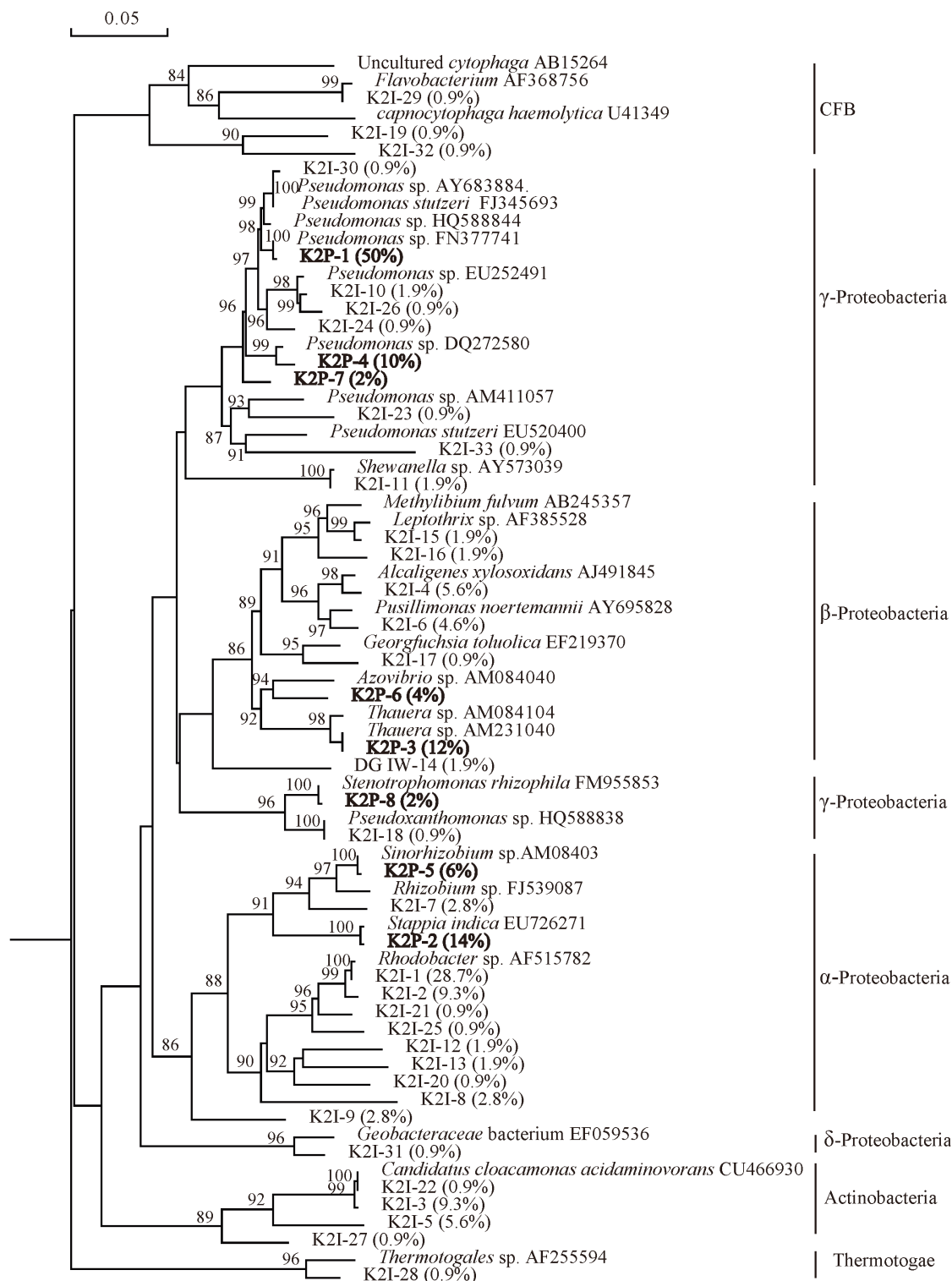
Figure 5 | Phylogenetic tree of bacteria 16S rDNA phylotypes of K2I and K2 (55°C). The tree constructed was based on approximately 800bp 16S rRNA genes and their nearest clones retrieved from the GenBank database. The percentages behind the OTUs represent the ralative abundance of each OTU. Bootstrap values are indicated at branch points. The scale bar represents 5% estimated sequence divergence.

phyla detected were CFB (3.1%), Firmicutes (2.3%) and Thermo-togae (0.8%) (Fig. 2).

The microbial communities in the samples of the injection well and production well of the oil reservoir were also different. In the sample of the injection well, *Erythrobacter* sp. predomenated at 60.2%, which was followed by *Lysobacter* sp. (18.8%), *Roseovarius* sp. (8.6%). In the sample of the production well, the dominant bac-terial populations included *Petrobacter* sp. (57.1%), *Hyphomonas* sp. (18.5%), *Pseudomonas* sp. (8.5%), *Rhizobium* sp. (5.9%) (Fig. 6). In V4I and V4P, 96.8% and 90% sequences were not affiliated with a specific genera, the common co-existing phyla were α-, β- and γ-Proteobacteria while the common co-existing families were Rhodocyclaceae and Pseudomonadaceae and no common co-exist-ing OTU were detected (Table 1).

Discussion

Water-flooding procedure is always regarded as an important source affecting native-microbial communities in oil reservoirs[2,10]. However, surveys investigating how microbial communities in production wells are affected by microorganisms in injected water are limited. In this study, microbial communities in samples of the injection wells and their adjacent production wells in four typical oil reservoirs with different *in situ* temperatures were investigated to explore this topic
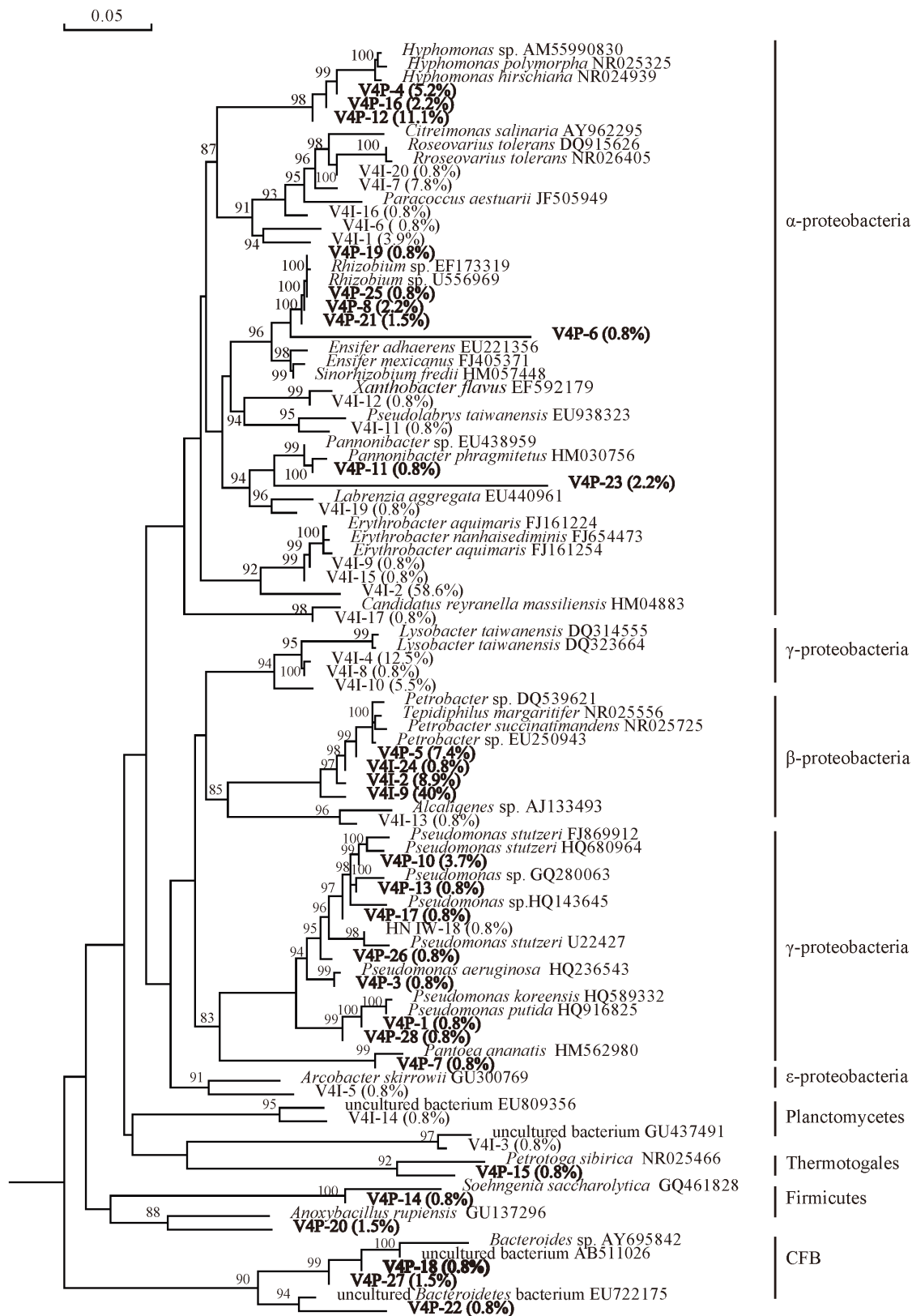

Figure 6 | Phylogenetic tree of bacteria 16S rDNA phylotypes of V4I and V4P (70°C). The tree constructed was based on approximately 800bp 16S rRNA genes and their nearest clones retrieved from the GenBank database. The percentages behind the OTUs represent the relative abundance of each OTU. Bootstrap values are indicated at branch points. The scale bar represents 5% estimated sequence divergence.

systematically. The results showed that microbial communities in samples of the injection well and the production well in each oil reservoir, under study, were different. The increase of *in situ* temperature of the oil reservoir exacerbated the difference in microbial communities in samples of the injection and production wells. A reduction in the number of common co-existing families and OTUs in the samples collected from the injection and production wells of the oil reservoirs with higher temperatures was observed.

Several previous studies on microbial communities of oil reservoirs also pointed out significant difference between the microbial community structure in samples of injection and production wells. Ren *et al.*[11] reported that only 2 co-existing OTUs appeared in the samples of the injection and production wells of a high-temperature oil reservoir (69°C). In our study, analogously, there were no co-existing OTU in samples of injection and production wells of oil reservoirs (K2 and V4) with high temperatures; and at family level, only 9.3% and 1.6% sequences in clone libraries of the injection wells appeared in the clone libraries of the production wells in oil reservoirs of K2 (55°C) and V4 (70°C), respectively (Table 1). In the low-temperature oil reservoir (25°C) although microbial communities were different, there were 5 co-existing families, and 7 co-existing OTUs in the samples of the injection well and the production well. At family level, 81.4% sequences in the sample of the injection well appeared in the sample of the production well (Table 1). She *et al.*[12] reported similarities between an injection well and two production wells in a low-temperature oil reservoir of 30% and 20%, respectively, using Unweighted Pair Group Method Clustering (UPGMC).

For the mesothermal Yan 9 oil reservoir (40°C) in our study, there was no co-existing OTU in samples of the injection and production well, but 44.5% sequences, at family level, in the sample of the injection well appeared in the sample of the production well (Table 1). Therefore, although microbial communities in injection wells appeared different to those in the production wells, the effects of the microorganisms in injected water on microbial community compositions in oil reservoirs remained ; and the *in situ* temperatures of oil reservoirs was a crucial factor for the degrees of effect, the higher the *in situ* temperature, the less such effects were.

The results show that bacterial communities in the samples of produced water were different to those detected in samples of the injection wells in the four oil reservoirs. However, great care has to be taken when drawing inferences concerning *in situ* microbes of oil reservoirs in produced water. An investigation carried out by Dahle *et al.*[10] indicated that microorganisms in produced water might only to a small extent reflect the indigenous microorganisms in the respective oil formations, even when the formations are not water-flooded. Considering the difficulty of judging the indigenous nature of encountered bacteria and the specific environments of water-flooded oil reservoirs, we classified the detected bacteria in the produced water samples of the study and compared them with those previously reported in samples from oil reservoirs to expose microbial communities inhabiting oil reservoirs subjected to water-flooding in China.

Alphaproteobacteria appeared in samples of production wells from Yan 9 (40°C), Kong 2 (55°C) and V4 (70°C) oil reservoirs at a predominance level of 17.6%, 20% and 27.8%, respectively (Fig. 2). Among all the encountered members belonging to Alphaproteobacteria, sequences of one OTU detected in the sample from the Kong 2 oil reservoir were affiliated with the genus of *Stappia*. The *Stappia*-related clones were reported to be detected in oil phase separated from produced oil-water sample from a mesothermic oil field[13], while the remaining Alphaproteobacteria were never reported to be isolated or detected in oil reservoirs. Collectively, members belonging to the order of Rhizobiales (*Methylopila* sp., *Rhodobium* sp., *Sinorhizobium* sp. and *Rhizobium* sp.) appeared in the three produced water samples and indicating that Rhizobiales might be a common Alphaproteobacteria inhabiting water-flooded oil

reservoirs. The second dominant microbial population affiliated with the *Hyphomonas* sp., in the produced water sample from V4 oil reservoir was often reported in marine environments[14,15] and seldom detected in oil reservoirs.

Betaproteobacteria appeared in samples of production wells from Kong 2 (55°C) and V4 (70°C) oil reservoirs. All the detected groups of Betaproteobacteria were members belonging to the family of Rhodocyclaceae genera of *Thauera* and *Azovibrio* in the sample from Kong 2 oil reservoir and members related to *Petrobacter* sp. in the sample from V4 oil reservoir. *Thauera* sp. was seldom found in samples from oil reservoirs, recently, *Thauera* –related phylotype was only detected in oil fields injected with nitrate[16,17]. *Petrobacter*-affiliated populations were predominant at 57.1% in the produced water from V4 oil reservoir. This genus was isolated and detected in samples from higher temperature oil reservoirs[18,19], suggesting that they may belong to the natural community in higher temperature oil-bearing formations.

Microbial groups belonging to Deltaproteobacteria covered an overwhelming percentage of 74.2% in the lower temperature oil reservoir No. 6, and 25.7% in the mesothermic oil reservoir of Yan 9. The detected sequence affiliated with uncultured Desulfobacterales bacterium, that is a common order detected in oil reservoirs[2], were predominant at 66.4% abundance in the produced water from oil reservoir of No. 6. Moreover, a co-existing OTU of the uncultured Desulfobacterales bacterium appeared in the sample of the injected water. In addition, bacteria related to *Syntrophus* sp. were also found in the in the produced water from oil reservoir of No. 6. The genus of *Syntrophus* was recently demonstrated to be important in processes that are happening in oil reservoirs[20,21] and members of this genus were also identified in enrichments derived from an oil reservoir in Canada[22]. However they could not be considered indigenous because of a co-existing OTU detected in the sample of the injected water.

Members of the genus of *Desulfovibrio* appeared in produced water samples from mesothermic oil reservoir of Yang 9 (40°C). *Desulfovibrio* was reported to be a major mesophilic genus that is recovered frequently from oil fields[23–25]. Although no clone affiliated to the genus was found in the injected water sample, the indigenous nature of the detected *Desulfovibrio*-related bacteria remained unidentified without further culture-dependent assessment.

Members represented by *Geobacter*-related and *Desulfuromonas*-related phylotypes, belonging to the order of Desulfuromonadales, were found in both produced and injected water samples in both oil reservoirs. Notably, in the samples of the produced water, these bacteria were prevailing, suggesting that bacteria affiliated with *Desulfuromoas* sp. and *Geobacter* sp. were exogenous in the both oil reservoirs No. 6 and Yan 9.

Bacteria affiliated with *Pseudomonas* sp. phylum Gammaproteobacteria were often detected in oil reservoirs[7,8,26]. We detected these bacteria in all reservoirs, except for the lower temperature of No. 6 oil reservoir. . In samples of produced water from oil reservoirs of Yan 9 and Kong 2, *Pseudomonas* sp. was at 32.5% and 62%. *Pseudomonas*, however were not believed to originate from oil reservoirs. The fact that these mesophiles were dominant at high temperature oil reservoir Kong 2 and the higher temperature oil reservoir of V4 implied that water flooded oil reservoirs were open and recycling systems and some microbial communities detected in produced water might come from the cooler portions near wellbores[7,8].

Two OTUs, accounting for 7.8% of the clone library constructed from the produced water from low temperature oil reservoir No. 6 (25°C), were clustered within the Epsilonproteobacteria. Sequences of one OTU were related to uncultivated species and sequences of the other OTU similar to *Arcobacter* sp. which have been previously reported in clone libraries from oil field produced water[27], and to be the dominant microbial group in non-water-flooded low-temperature oil reservoir[5]. However, with the appearance of the

Table 2 | The locations and characteristics of injection and production wells from the four oil fields in China used in this study

Oil Field	Location	Oil Reservoir	Wells	Waterfloodig Date	Sampling Date	Depth (m)	*In situ* Temperature
Xinjiang	Junggar basin	No. 6	T6186 T6073	1974	10/09/2009	800	25°C
Changqing	Ordos Basin	Yan 9	L28-45 L28-46	1999	03/05/2010	1289	40°C
Dagang	Bohai Sea coast	Kong 2	1098-8 1017-7	1979	06/08/2004	1400	55°C
Henan	Henan Province	V4	V187 V149	1977	05/09/2010	1355	70°C

Arcobacter-related sequences in injected water samples, the bacteria involved *in situ* were still unknown.

CFB-affiliated sequences, detected in the produced sample from low temperature oil reservoir No. 6, were generally distantly related to known cultivated species, the results were similar to that reported by Grabowski *et al.*[5]. However, most of the sequences were close to sequences that were also retrieved from a mesothermic petroleum reservoir[28]. In the water samples of the mesothermic oil reservoir Kong 2 and the higher temperature oil reservoir V4, bacteria belonging to CFB group, accounted to 2.7% and 3.1%, respectively. Similarity, most of them were uncultured but related to environmental sequences associated with oil reservoirs[26,28,29]. Although members of CFB group were detected widely in samples collected from oil reservoirs, their ecological significance and indigenous nature are not clear due to their uncultured characteristics, which underlined the need to isolate and characterize pure cultures of these microorganisms.

Bacteria affiliated with *Halanaerobium* sp. detected in produced water sample of Yan 9 oil reservoir were closely related to strains isolated from an African oil field[30]. Interestingly, although non *Halanaerobium*-related sequence was found in the injected water sample of Yan 9 oil reservoir, sequences belonging to other genera of Firmicutes (*Clostridium*, *Fusibacter* and *Soehngenia*) occupied a dominant ralative abundance (37.4%) in the injected sample, which indicated that the subsurface strata might have an anaerobic high-salt condition.

Sequences of the genera of *Denitrovibrio* and *Geovibrio*, covered limited percentages of 1.5% and 0.9% the clone library of produced water from the low temperature oil reservoir No. 6. *Denitrovibrio*-related bacteria isolated from reservoirs appeared the ability to reduce nitrate by denitrification[17,31]. In the produced water sample from the mesochermic oil reservoir Yan 9, harboured *Flexistipes*-related bacteria that were closely similar with the isolate originated from a multiply extreme environment with high temperature, high salinity, and high concentrations of heavy metals[32], which further confirmed that the Yan 9 oil reservoir might be anaerobic and high insalt content.

Sequences affiliated with the phylum of Spirochetes were only detected at low temperature oil reservoir of No. 6. One OTU was closely similar to the phylotype present in the production waters of a low-temperature biodegraded oil reservoir[5].

Among the 28 OTUs in clone library of the sample from the higher-temperature V4 oil reservoir, only one OTU (V4P-15), occupying only 0.8% ralative abundance, is relatively distantly related to *Petrotoga sibirica* (92%). *Petrotoga* species are considered as typically indigenous since it has been isolated only from oil reservoirs so far[33,34].

In conclusion, bacteria inhabiting in the produced waters of the four water-flooded oil reservoirs in China were dominated by Proteobacteria. At low-temperature oil reservoir No 6, δ-Proteobacteria was prevailing while at high-temperature oil reservoirs Kong 2 and V 4, α-, β-, γ-Proteobacteria were dominant. In mesothermic oil reservoir Yan 9, α-, δ- and γ-Proteobacteria were foremost. Most detected bacteria could not be identified as indigenous. In the study, we just focused on bacteria inhabiting in oil reservoirs without

involving archaea that will be investigated in our following study. Apparently, water-flooded oil reservoirs are changing environments, harboring groups of surviving bacteria indigenous and exogenous that form new ecological systems different to the original ones. The identification of microbes present in produced waters within water-flooded oil reservoirs reflects the inhabiting microbial communities which are of great importance to ecological study of the specific environments of water-flooded oil reservoirs.

Methods

Sample collection. We obtained permission from the Institute of Oil Recovery Research of Xinjiang, Changqing, Dagang and Hennan Oil Field Ltd. for data acquisition and field studies which did not involve endangered and protected species. Eight wellhead samples of injection wells and production wells from the four oil reservoirs were collected. All the oil reservoirs under study are subjected to water-flooding. The detail information about the oil reservoirs and sampling is shown in Table 2. The water-oil samples were retrieved from wellheads and stored in sterile plastic bottles and transported to the laboratory as soon as possible for cultural and molecular analyses.

16S rRNA gene amplification, library construction and sequencing. For water-oil samples retrieved from wellheads, in the study, 100 ml water sample is an optimal amount for cell collection (10,000 g, 10 min). DNA in the pellet cells were extracted following the manufacturer's protocol for the FastDNA Spin Kit for Soil (Qbiogen, Carlsbad, CA. USA). Obtained DNA was detected by agarose gel electrophoresis.

The bacterial 16S rDNA in the bulk DNA was amplified using the universal bacteria-specific primers, 27F and 1492R[35]. The 25μl PCR reacting system contains a 2.5μl of 10×PCR buffer (Mg^{2+} plus), 10 nmol of deoxynucleotide triphosphates, 1 U Taq DNA polymerase (TakaRa), 10 pmol of each primer, and 1μl of template DNA. The thermal cycling conditions were as follows: an initial denaturation at 94°C for 5 min, 40 cycles of 94°C for 30 sec, 56°C for 60 sec, 72°C for 90 sec, and a final extension step of 72°C for 10 min[26,36]. Amplified fragments were approximately 1450 bp.

After purified with an Agarose Gel DNA Purification Kit, amplicons of 16S rRNA genes were (TianGen Biotech, Beijing, China) cloned into Trans1-T1 competent cells (TransGen Biotech, Beijing, China) using PGEM-T Easy Vector (Promega, Madison, WI, USA) as instructed by the suppliers. Randomly chosen one hundred and fifty putative clones (white) from each plate were transferred to another labelled LB plate with ampicillin (100μg/ml) and cultured at 37°C overnight. A re-amplification, with sets of vector-specific primers T7/SP6, was taken to determine positive clones. During the re-amplification procedure, a small quantity of cells of putative clones was used as template DNA in the reaction mixtures. PCR products of positive clones were classified into different operational taxonomic units (OTUs) using amplified ribosomal DNA restriction analysis (ARDRA) with *Hinf*I and *Hae*III (TaKaRa)[26,36]. Representative clones were selected for sequencing which was performed using an ABI PRISM 3730 DNA sequencer (SinoGenoMax Co., Ltd., Beijing, China). Clone libraries were statistically evaluated by rarefaction analysis using Analytic Rarefaction 1.3 (http://www.uga.edu/strata/software/Software.html) software.

Taxonomic classification and phylogenetic analysis of 16S rRNA gene sequences. The obtained sequences were manually trimmed and edited using DNAMAN version 5.2.2.0. The obtained sequences were submitted to the GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) employing the Basic Local Alignment Search Tool (BLAST) algorithm of nucleotide to determine their phylogenetic affiliations. The sequences being the nearest neighbours of the submitted sequences, included cultured and uncultured sequences, were cited from the GenBank. Sequences, sequenced and cited, were aligned using DNAMAN software to construct phylogenetic trees with maximum likelihood.

AY770937 (clone library of K2I), AY770933-AY770940 (clone library of K2P), JQ519768-JQ519786 (clone library of V4I) and JQ519699-JQ519726 (clone library of V4P).

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Author contributions

Experiments were conceived and designed by Y.H.S., F.Z. and D.J.H. and performed by F.Z., L.J.C. and X.T.Z. Data were analyzed by F.Z., L.J.C., I.M.B., X.T.Z., F.C.S., Z.L.W. and L.J.Y. The manuscript was written by F.Z.

Additional information