African Journal of Biotechnology Vol. 10(53), pp. 11004-11010, 14 September, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1188
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Relationship of microbial diversity from recycled injection water and high-temperature petroleum reservoirs analyzed by polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE)

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Accepted 7 July, 2011

The microbial communities and diversities in production water from three high-temperature, water-flooded petroleum reservoirs in the same injection area of the Chenghai area of Dagang oil field in China and their recycled injection water sample were characterized by polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) analysis. Results show that microbial sequences mainly belonged to the divisions *Proteobacteri*a, *Bacteroidetes*, *Spirochaetes*, *Firmicutes* and *Euryarchaeota*. Many mesophilic and thermophilic microorganisms were found in samples of produced water and injection water. *Thermoanaerobcter*, *Desulfotomaculum, Methanolinea, Methanomethylovorans* and *Geoglobus* were detected in all samples. Mesophilic microorganisms found in the samples of produced water, mainly belonged to *Brevundimonas, Agrobacterium*, *Pseudomonas, Sphingomonas, Acinetobacte, Dechloromonas, Clostridium* and *Methanobacterium*. All of them were detected in the injection water samples, except *Brevundimonas* and *Methanobacterium*, which indicates that injection exploration may be the main reason why mesophilic bacteria was detected in high-temperature petroleum reservoirs of Dagang. Results suggest that reinjection of water not only introduces enormous mesophilic microbe into high-temperature petroleum reservoirs, but also cause thermophilic microorganisms cross-contamination among wells in the same injection area.

Key words: Petroleum reservoir, recycled injection water, DGGE, microbial community, cross-contamination.

INTRODUCTION

The existence of microorganisms in the subsurface of petroleum reservoir has been known for many years (Bastin et al., 1926), and a great variety of microorganisms have been isolated or detected. To study the characteristics of the oil microbial communities, a large number of culture-dependent and culture-independent techniques have been applied in the research on petroleum microbiology. Using traditional approaches, many physiological types of microorganisms have been isolated from different geographic oil reservoirs. These microorganisms include sulfate reducers (Rueter et al., 1994), sulfidogens (Haridon et al., 1995), fermentative bacteria (Grassia et al., 1996), manganese and iron reducers (Greene et al., 1997; Slobodkin et al., 1999), methanogens (Nilsen and Torsvik, 1996), and acetogens (Davydova-Charakhch et al., 1993). Other studies using molecular techniques found that oil reservoirs contain complex micro-ecosystems with thermophilic and mesophilic microbe coexisting (Orphan et al., 2000; Li et al., 2007; Wang et al., 2008; Kaster et al., 2009). The general characteristics of the environment in deep subsurface petroleum reservoirs are high temperature, high salinity, high

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Table 1. Physico-chemical characteristics of each petroleum reservoir and the injection water.

Physico-chemical characteristics	Injection water	ZH-1	ZH-3	Z4-7
Depths (below the sea floor)	-	1071 m	1065 m	1566 m
Geological age		Neogene	Neogene	Paleogene
Temperature (℃)	-	57.9	57.5	75
Density (g/cm^3 20℃)	-	0.9543	0.9336	0.9266
Viscosity (50℃mPa·s)	-	599.0	534.4	48.80
Water (%)	-	32	25	36
Sulfate (mg/l)	11	10	6	8
Acetate (mg/l)	10.1	6.2	5.4	9.5
H_2S (mg/l)	0.0	0.0	0.0	7
Salinity (mg/l)	6100	5900	6340	4780
pH	8.23	8.10	8.06	8.38

pressure and lackness of dissolved oxygen. Based on the physicochemical characteristics of the reservoir rock, thermophilic microorganisms are usually regarded as indigenous microbes, while mesophilic microorganisms are known as exogenous pollutants (Magot et al., 2000).

Water-flooding is an important technique of oil exploitation used by many oil-fields, and flooded water is usually recycled from production water. Tons of unsterilized formation water is separated from the total production fluid from different oil fields and injected into the same injection area, to maintain the formation pressure. With the injection water, large numbers of microorganisms are introduced into the petroleum reservoirs, which may cause microbial cross-contamination among different oil wells in the same injection area. Then, the microbial communities and diversities will be changed, making it difficult to research the indigenous microbes in these fields. However, very few studies had been carried out to address this issue.

This study mainly focuses on analyzing of microbial community and diversity of three production wells and their recycled–injection water by using polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE). Results indicate that reinjection water not only introduce enormous mesophilic bacteria into high-temperature petroleum reservoirs, but also causes thermophilic anaerobic bacteria cross-contamination among wells in the same injection area.

MATERIALS AND METHODS

Characteristics of Dagang oil fields

The Chenghai area of Dagang oilfield is located at Hebei province, China. In March 2008, there were 19 oil producing wells. The geological age of the reservoirs belongs to the Neogene and Paleogene. The depth and temperature of different wells varies greatly. The depth varies from underground 1000 to 1600 m, and reservoirs temperature varies from 55 to 83℃. The oil fields are exploited by recycled water-flooding. After collection and water-oil separation in sewage treatment plant, the coproduced water is recycled and reinjection into reservoirs. The water samples from three producing wells and the injection water were examined in this study. Detailed physical-chemical characteristics of each petroleum reservoir and the injection water are shown in Table 1.

Sample collection and preparation

In May 2008, the samples of production fluid (oil-water mixture) and injection water were collected directly into sterile carboys from production wellheads and filtered-water reservoir, respectively. All samples were stored at 4℃ and analyzed within 48 h.

DNA extraction

Phase separation of the oil-water mixture was accomplished by heating samples to 70℃ for 10 min with Teflon separatory funnels (Orphan et al., 2000). Microbial biomass from the water phase (0.5 L) was collected by filtration with 0.22 µm Sterivex filters (Millipore). The Sterivex filters were then placed into sterilized Eppendorf and genomic DNA was extracted by Soil DNA Fast Extraction Kit (Bioteke Corporation). Nucleic acids were stored at -20℃.

Amplification of bacterial and archaeal 16S rDNA high variable region

The variable region V3 of the bacterial 16S rDNA was amplified by PCR using the primers described by Muyzer et al. (1993). The samples were amplified within two steps. First, the modified Touch-down PCR program was employed (Wang et al., 2008) with primers 341F-GC and 534R. After the initial amplification, the reaction mixture was diluted 10-fold as templates, then a “reconditioning PCR” was employed (Thompson et al., 2002) with primers 341F and 534R. The variable region V3 of archael 16S rDNA was amplified within two steps too. In the first round, primers ARCH46f (Ovreas et al., 1997) and ARCH1017r (Barns et al., 1994) were used to amplify archeal 16S rDNA sequences. In the second round, primers ARCH344f-GC (Raskin et al., 1994) and UNIV522 (Amann et al., 1995) were used to amplify archeal 16S rDNA variable V3, with 1:1000 dilution of the first-round PCR product as templates (Roling et al., 2004).

Denaturing gradient gel electrophoresis analysis

DGGE was performed using a Bio-Rad Dcode system (Bio-Rad,

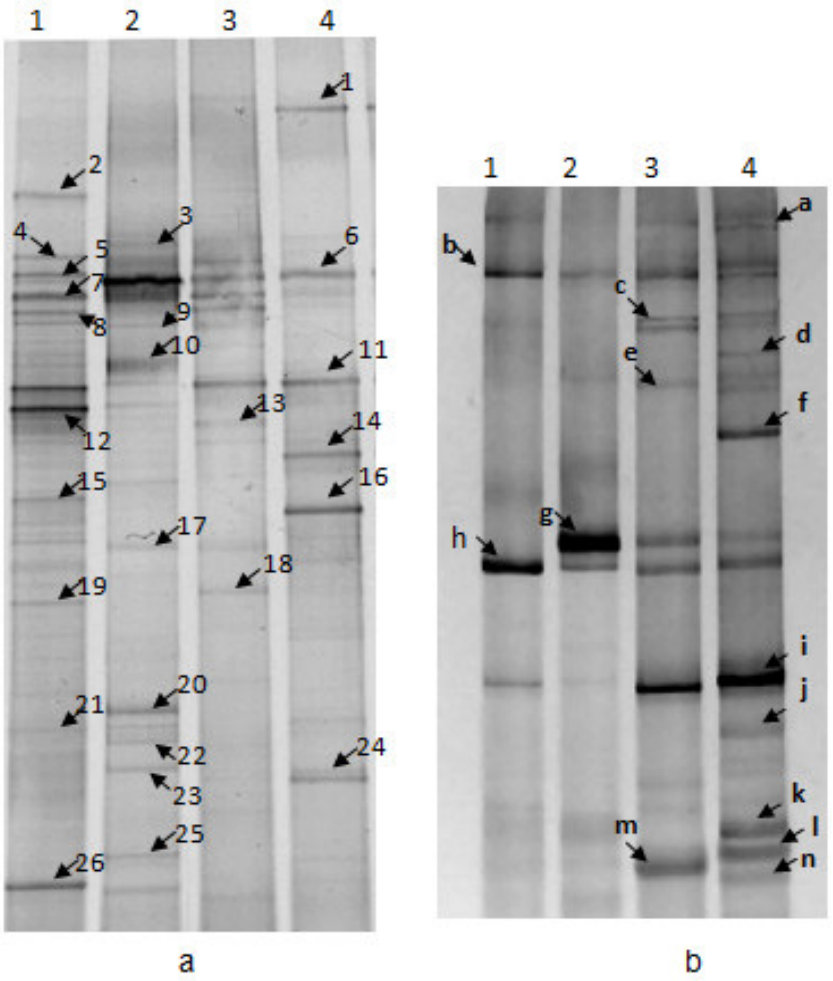

Figure 1. DGGE profiles of 16S rRNA gene fragments amplified from Dagang produced water samples and recycle water with bacterial (a) or archeal (b) primer pairs. Sample of recycle water is shown in lane 1, sample from well ZH-1 is shown in lane 2, sample from well ZH-3 is shown in lane 3, and sample from Z4-7 is shown in lane 4. Letters on the sides of the gels indicate the clone bands corresponding to bacteria and species shown in Tables 2 and 3.

Mississauga, Ont., Canada), as described by the manufacturer. The PCR products were separated using 8% polyacrylamide gels. Thirty five to sixty five percent of denaturant gradients was used to separate the variable region V3 of bacterial 16S rDNA, and 25 to 50% of denaturant gradients was used to separate the variable region V3 of archaeal 16S rDNA (100% denaturant contained 7 M urea and 40% deionized formamide). After electrophoresis at constant voltage of 200 V at 60℃ for 4 h, the gels were stained with 1000×SYBR Green I for 45 min and visualized by UV transillumination.

DNA sequences and phylogenetic analysis

For sequencing, all dominated bands in DGGE gels were excised and placed in a sterile Eppendorf containing 50 µl of sterile water at 4℃ overnight. 5 µl of the solution was used as template for PCR. The productions were recovered by a DNA Recovery Kit (Takara), ligated to pMD-19T cloning Vector (Takara) and transformed into *Escherichia coli* JM109 (Takara). Three positive clones from one DGGE band were selected randomly for sequencing analysis. Sequence analysis was done using Clustalx and MEGA 4.1 software.

A total number of 40 partial 16S rDNA sequences were submitted to GenBank with the accession numbers HM153443 to HM153482.

RESULTS

Phylogenetic affiliation of dominant bacterial phylotypes

The DGGE profiles of bacteria communities are shown in Figure 1. Twenty-six bacterial DGGE bands were unambiguously excised and sequenced, and the bacteria diversities are summarized in Table 2. Bands at the same position in the gel had identical sequences. They were affiliated to *Proteobacteri*a, *Bacteroidetes*, *Spirochaetes*,

Table 2. Bacterial diversity of each production well and injection well.

Phylum	Clone	Relative species and clone	Similarity (%)	Injection water	Z-H1	Z-H3	Z4-7
Proteobacteria	2 (HM153444)	*Pseudomonas nitroreducens* (AM088473)	100	+			
	3 (HM153445)	*Pseudomonas stutzeri* (AF094748)	100		+		
	4 (HM153446)	*Sphingomonas koreensis* (AF131296)	97.5	+		+	
	5 (HM153447)	*Acinetobacter johnsonii* (Z93440)	99.3	+			
	6 (HM153448)	*Pseudomonas nitroreducens* (AM088473)	98.7	+	+	+	+
	7 (HM153449)	*Dechloromonas denitrificans* (AJ318917)	96.1	+	+	+	+
	8 (HM153450)	*Dechloromonas denitrificans* (AJ318917)	95.4	+		+	
	9 (HM153451)	*Phenylobacterium lituiforme* (AY534887)	100	+	+	+	
	12 (HM153454)	*Brevundimonas mediterranea* (AJ227801)	100	+	+		
	13 (HM153455)	*Agrobacterium vitis* (U45329)	98.8			+	
	20 (HM153462)	Brevundimonas aurantiaca (AJ227787)	100		+		
	26 (HM153468)	*Agrobacterium vitis* (U45329)	100	+	+		
Bacteroidetes	1 (HM153443)	*Paludibacter propionicigenes* (AB078842)	96.8				+
Spirochaetes	21 (HM153463)	Uncultured Spirochaetes bacterium (CU925019)	99.3	+	+		+
Bacteria	23 (HM153465)	Uncultured bacterium clone ZB_P14_C08 (GQ328684)	100		+		
	25 (HM153467)	Uncultured bacterium clone UWL_CL-090621_OTU-13 (EU809296)	99		+		
Firmicutes	10 (HM153452)	*Clostridium sordellii* (AB075771)	100	+	+		
	11 (HM153453)	*Desulfotomaculum thermosubterraneum* (QD208688)	99.4	+	+	+	+
	14 (HM153456)	*Desulfotomaculum geothermicum* (AJ294428)	96.1				+
	15 (HM153457)	*Coprothermobacter proteolyticus* (X69335)	97.9	+			
	16 (HM153458)	*Thermoanaerobacter brockii* (L09165)	98.8			+	+
	17 (HM153459)	*Thermoanaerobacter brockii* (L09165)	99.4		+	+	
	18 (HM153460)	*Thermacetogenium phaeum* (AB020336)	99.1				
	19 (HM153461)	*Thermoanaerobcter subterraneus* (AF195797)	96.5	+			
	22 (HM153464)	*Thermoanaerobacter brockii* (L09165)	98.8		+		
	24 (HM153466)	*Desulfotomaculum geothermicum* (AJ294428)	96.7				+

The presence of a bacterial band on the gel is represented by + which corresponds to those of the bands on DGGE gels in Figure 1a.

Table 3. Archeal diversity of each sample of production wells and re-injection water.

Phylum	Clone	Relative species and clone	Similarity (%)	Injection water	Z-H1	Z-H3	Z4-7
Euryarchaeota	a (HM153469)	Uncultured methanogenic clone: H08 (AB186293)	100	+		+	+
	b (HM153470)	*Methanolinea tarda* (AB162774)	99.3	+	+	+	+

Table 3 Contd.

	c (HM153471)	*Methanoculleus thermophilus* (AB065297)	99.5			+	+
	d (HM153472)	*Methanoculleus thermophilus* (AB065297)	98.5				+
	e (HM153473)	*Methanoculleus thermophilus* (AB065297)	99.3			+	+
	f (HM153474)	M*ethanobacterium formicicum* (AF169245)	98.5				+
	g (HM153475)	Uncultured archaeaclone:TCC18 (AB178483)	98.5		+	+	+
	h (HM153476)	*Methanomethylovorans thermophila* (AY672821)	97.1	+	+	+	+
	i (HM153477)	*Geoglobus ahangari* (AF220165)	98.5	+	+	+	+
	j (HM153478)	*Geoglobus ahangari* (AF220165)	95.6				+
	k (HM153479)	Uncultured *Methanobacteriaceae* clone ANB-2(FJ898359)	98.5		+		+
	l (HM153480)	Uncultured archaeaclone C1Ar1A04 (FJ874780)	97				+
	m (HM153481)	*Methanoculleus thermophilus* (AB065297)	98.6			+	
	n (HM153482).	Uncultured Methanobacteria archaeron clone NRA12 16S ribosomal (HM041913)	99				+

The presence of an archaeal band on the gel is represented by + which corresponds to those of the bands on DGGE gels in Figure 1b.

Firmicutes and uncultured bacterial clones. Approximately, 88% of these bacterial bands showed similarities of more than 95% to cultured organism as given in Table 2. Twelve bacterial bands were placed into *Proteobacteria*. One bacterial sequence was affiliated with *Bacteroidetes*. Eleven bacterial bands were placed in *Firmicutes*. And two bands belonged to unidentified bacteria; there was no bacterium closely relative to them.

Phylogenetic affiliation of dominant archaeal phylotypes

As seen in Figure 1b, 14 bands were excised and sequenced after DGGE analysis of amplifications obtained by the application of Archaea-Specific primer. All of them yielded reliable bands most affiliated to *Euryarchaeota*, 10 bands belonged to methanogenic archaea. Bands at the same position in the gel had identical clones. Approximately 71% of these archaeal bands showed similarities of more than 95% to cultured organism as given in Table 3.

DISCUSSION

Results of DGGE profiles suggested that the microbial community of bacterial diversity was more diverse than archaeal diversity in injection water and produced well of ZH-1, and archaeal diversity was more diverse than bacterial diversity in ZH-3 and Z4-7. A total of 26 dominant bacterial bands and 14 dominant archaeal bands were determined. Thirteen bacterial bands and 1 archaeal band were related to mesophilic microorganisms, these bands were 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 13, 20, 26 and f. Ten bacterial bands and 8 archaeal bands belonged to thermophilic microorganisms, they were bands 9, 11, 14, 15, 16, 17, 18, 19, 22, 24 and c, d, e, m, b, h, l, j. The others were uncultured microorganisms. Some uncultured microorganisms detected suggested that large scale isolation and culture are needed to further analysis the microbial community of oil reservoirs. And some new microbes would be found.

The thermophilic bacteria and archaea found were present in the genera *Thermoanaerobacter*, *Phenylobacterium*, *Desulfotomaculum*, *Thermacetogenium*, *Methanoculleus*, *Methanomethylovorans* and *Geoglobus*. *Thermoanaerobacter* was the main thermophilic fermentative bacteria, which was widespread in high-temperature oil fields (Orphan et al., 2000), and the main products were low fatty acids and H_2+CO_2. It should be noted that all members of the order methanogens were hydrogenotrophic or methylotrophic *Methanomicrobiales*, which was consistent with the results of previous studies of methanogens from high-temperature oilfields (Nilsen and Torsvik, 1996; Li et al., 2007). Some fat acid oxidizing microorganisms, such as *Geoglobus*, *Desulfotomaculum* and *Thermacetogenium* were found in the oil fields. *Thermacetogenium phaeum* was found to produce well of ZH-3, which was an acetate-

oxidizing bacterium, capable of oxidizing acetate during syntrophic growth with hydrogen-utilizing methanogens (Hattori et al., 2000).

Desulfotomaculum thermosubterraneum was found in all oil fields, and *Desulfotomaculum geothermicum* was found in oil field of Z4-7. *Desulfotomaculum* are sulfate-reducing bacteria, which can use fatty acids with versatile electron acceptors other than sulfate for anaerobic respiration, with syntrophic grow and hydrogen-utilizing methanogens too, in low contraction of sulfate. *Geoglobus ahangari* a hyperthermophilic archaea, was found in all samples, which is capable of oxidizing acetate and long-chain fatty acids (Kashefi et al., 2002). The results suggest that methanogenesis process may be reservoir major metabolic pathways, and hydrogen-utilizing methanogens dominated in the Dagan oil fields and acetate must have been degraded by syntrophic associations with acetate-oxidizing components.

The microbial community of thermophilic microorganisms was very similar in each sample of water. *Desulfotomaculum, Thermoanaerobcter*, *Methanomethylovorans* and *Geoglobus* were found in all samples. This indicated that the microbial community of every production wells has been influenced by injection water greatly. Thermophiles found in recycled water showed that some thermophiles survived in the oil reservoirs, during recovering of the coproduce water. The injection recycled water was from 19 different oil production wells, and the temperature of oil-rich stratum changed very greatly from 55 to 83℃. The surviving microbes could be mixed from different production wells, and injected into different oil wells with tons of recycled water. For example, the *G. ahangari* (Kashefi et al., 2002) a hyperthermophilic archaea, grew at temperatures between 65 and 90℃, and just adapted to fit live in producing well Z4-7 (75℃), but also found in producing well ZH-1 (57℃) and ZH-3 (57.9℃). The result suggests that the community of thermophilic microbe is similar in same water injection area even with different geological conditions, because of the influence of recycled water.

Mesophilic microorganisms were usually considered as the exogenous microbes of high temperature oil reservoirs (Magot et al., 2000). A large number of mesophilic microbes were detected in sample of produced-water; the microbial sequences belonged to *Pseudomonas*, *Dechloromonas, Sphingomonas*, *Acinetobacter*, *Brevundimonas*, *Agrobacterium, Paludibacter, Clostridium, Methanobacterium* and *Methanolinea*. The mesophiles came from injection water, because all of them were detected in sample of injection water, except *Paludibacter* and *Methanobacterium*. These microbes were proved to be extensively distributed in water and soil contaminated by petroleum hydrocarbons (Adebusoye et al., 2007; Popp et al 2006; Pearson et al., 2008), and many of them could make use of hydrocarbon. The results suggest that injection exploration which may be the main reason by mesophilic bacteria was detected in high-temperature petroleum reservoirs of Dagang.

In summary, our data suggest that petroleum reservoir systems are far more complex than that previously estimated and remain largely unexplored. Our results also suggest that recycled water injection not only introduce enormous mesophilic bacteria into oil field, but also cause thermophilic anaerobic bacteria cross-contamination among wells in the same injection area, and the indigenous microbes may not be characterized in petroleum reservoirs because of the recycled water injection.

ACKNOWLEDGEMENTS

This work was supported by National Special Basic Research of China (no. SB2007FY400), and the National Basic Research Program of China (2009CB125910).

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