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Full Length Research Paper

Enrichment and diversity analysis of the thermophilic microbes in a high temperature petroleum reservoir

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Thermophilic microbial diversity in production water from a high temperature, water-flooded petroleum reservoir of an offshore oilfield in China was characterized by enrichment and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis. Six different function enrichment cultures were cultivated one year, at 75°C DGGE and sequence analyses of 16S rRNA gene fragments were used to assess the thermophilic microbial diversity. A total of 27 bacterial and 9 archaeal DGGE bands were excised and sequenced. Phylogenetic analysis of these sequences indicated that 21 bacterial and 7 archaeal phylotypes were affiliated with thermophilic microbe. The bacterial sequences were mainly bonged to the genera *Fervidobacterium*, *Thermotoga*, *Dictyoglomus*, *Symbiobacterium*, *Moorella*, *Thermoanaerobacter*, *Desulfotomaculum*, *Thermosyntropha*, *Coprothermobacter*, *Caloramator*, *Thermacetogenium*, and the archaeal phylogypes were represented in the genera *Geoglobus* and *Thermococcus*, *Methanomethylovorans*, *Methanothermobacter* *Methanoculleus* and *Methanosaeta*. So many thermophiles were detected suggesting that they might be common habitants in high-temperature petroleum reservoirs. The results of this work provide further insight into the thermophilic composition of microbial communities in high temperature petroleum reservoirs.

Key words: Thermophilic microbial diversity, petroleum reservoir, denaturing gradient gel electrophoresis.

INTRODUCTION

Attentions are increasingly highlighted to reveal the thermophilic microbial diversity in petroleum reservoirs ecosystem (Van Hamme et al., 2003). Culture-based and molecular techniques are usually used to reveal the microorganisms of high-temperature, petroleum-rich strata from a number of geographically distant oil reservoirs. Culture-based techniques are extremely useful to gain information and physiology of isolated organisms. However, they cannot give a clear picture of the complete microbial communities present in oil reservoirs, because most of microbes would not be isolated from enrichments (Suzuki et al., 1997; Ward et al., 1990).

Molecular techniques have been proven to be effective in investigating complex microbial assemblages in environmental samples and extensively used to investigate the microbial diversity in petroleum reservoirs, including reverse genome probing, oligonucleotide matrix array hybridization methods, restriction fragment length polymorphism (RFLP), 16S rRNA gene sequence analysis and denaturing gradient gel electrophoresis (DGGE). More complete characterization of microbial communities in subsurface oil reservoirs has been described with these methods (Orphan et al., 2000, 2003; Bonch-Osmolovskaya et al., 2003; Li et al., 2007a, b; Wang et al., 2008; Kaster et al., 2009). However, our current knowledge of the thermophilc microbial diversity in such subsurface ecosystems is still limited. All researches show a complex system consisted of a number of diverse thermophilic and mesophilic micro-organisms in high-temperature petroleum. For example, mesophilic bacteria account for 85% of the total clones detected in the long term water-flooded reservoir in

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China (Li et al., 2007a), 78% of the clones and 86% of the organisms detected are mesophilic bacterial clones and mesophilic methanogenic microoganisms in the Californian sulphur-rich reservoirs, respectively (Orphan et al., 2000). Mesophilic organisms are usually thought as exogenous to the high-temperature reservoir because the general characteristics in deep subsurface petroleum reservoirs are high temperature, high pressure and anaerobic environment. Many mesophilic organisms are detected in samples from high temperature oil wells, indicating that the microbial community has been contaminated very seriously, and more researches avoiding the impaction of mesophiles are needed.

Combined enrichment and molecular techniques can reveal the diversity of thermophilic microbes. Since mesophilies cannot survive for long in high-temperature, and molecular techniques may compare community composition in many different samples. In this study, enrichment culture techniques and DGGE of PCR-amplified 16S rDNA were used to investigate the microbial community structure in a high-temperature and water-flooded petroleum reservoir in the Chenghai 1 Unit of Dagang Oilfield located in Hebei Province, China. Our aim was to learn more about the thermophilic microbial diversity, which in turn will improve our understanding of the complex community that inhabited the subterranean petroleum- rich system.

MATERIALS AND METHODS

Petroleum reservoir characteristics and sample collection

Microorganisms of the Dagang oil field (well no Zhuanghai 4-7 of Chenghai 1 Unit) were studied. The oil field is exploited using water-flooding. The water for injection is separated from the oil produced fluid and recycled. The formations are situated 1965 to 1976 m below the sea floor and have a temperature of 75°C. The formation water of sodium hydrocarbonate type which has a low salinity (4780 mg/l) and a pH of 8.3 with low concentration sulfate (8 mg/l). In May, 2008, the samples of production water (oil-water mixture) were collected directly into sterile carboys from production wellheads. All samples were stored at 4°C and analyzed within 48 h.

Medium preparation and enrichment cultures

One basal medium with various carbon sources and electron acceptors were used in this study (Widdel et al., 2006). The following components (per liter distilled water): 0.5 g NaCl, 0.5 g $MgCl_2{\cdot}6H_2O$, 0.5 g KCl, 0.3 g NH_4Cl, 0.1 g $CaCl_2{\cdot}2H_2O$, 0.3 g KH_2PO4, 1.0 ml trace element solution, and 10 ml crude oil. Six enrichment medium were prepared based on basal medium for enriching different metabolic groups. No other carbon source and electron acceptor were added for hydrocarbon degrader, but glucose (0.2 g/l) was added for fermentative bacteria, Na-acetate (0.1 g) and 40 ml H_2+CO_2 (4:1) for methanogens, long-chain fatty-acid (0.1 g/l stearate and heptadecanoate) for long-chain fatty-acid degrader, Na_2SO_4 (3.0 g/l) for sulfate reducing bacteria (SRB) and $NaNO_3$ (1.0 g/l) for nitrate reducing bacteria (NRB).

The technique of modification Hungate (Miller and Wolin, 1974) was used throughout this study. 50 ml anaerobic media cultivation was carried out in 120 ml serum bottles. The pH was adjusted to 7.0 and the media dispensed to the serum bottles filled with N_2 for all media and with 2 ml CO_2/H_2 (80:20) for methanogens medium. 2.5 ml of production water was inoculated directly into selective media for enrichment. All of enrichment media were incubated without shaking at the reservoir temperature (75°C) for one year.

Nucleic acid extraction

Microbial biomass of enrichment samples was collected by filtration into 0.22 μm Sterivex filters. Sterivex filters were then placed into sterile Eppendorf and extracted by soil DNA fast extraction Kit (Bioteke Corporation). Nucleic acids were stored at -20°C.

PCR amplification

The bacterial 16S rDNA variable region V3 was amplified using primers described by Muyzer et al. (1993). The samples were amplified by two stops. At first, the modified Touch-down PCR program was employed (Wang et al., 2008). After the initial amplification, the reaction was diluted 10-fold as template, and a "reconditioning PCR" was employed (Thompson et al., 2002). The variable region V3 of archael 16S rDNA was amplified by two stops 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 a 1:1000 dilution of the first-round PCR product (Roling et al., 2004).

DGGE analysis

DGGE was performed using a Bio-Rad D-code system (Bio-Rad, Mississauga, Ont., Canada), as described by the manufacturer. The PCR products were separated using 8% polyacrylamide gels. In order to separate the products of bacteria and archaea, two denaturant gradients between 35 to 65% and 25 to 50% were used (100% denaturant contained 7 M urea and 40% deionized formamide). After electrophoresis at a constant voltage of 200 V for 4 h at 60°C, the gels were stained with 1000×SYBR Green I (Sigma) for 45 min and visualized by UV transillumination.

DNA sequences and phylogenetic analysis

For sequencing, all bands in DGGE gels were excised and placed in a sterile Eppendorf containing 50 μl of sterile water at 4°C overnight. 5 μl of the solution were used as template for PCR. The productions were recovered by a DNA Recovery Kit, and ligated to pMD-19T cloning vector and transformed into *Escherichia coli* JM109. 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 36 partial 16S rDNA sequences were deposited in the GenBank sequence database with accession numbers: HM153484 to HM153519.

RESULTS

DGGE analysis of prokaryotic community composition of different enrichment cultures

The DGGE profiles of bacterial and archaeal communities in all enrichment cultures were shown in

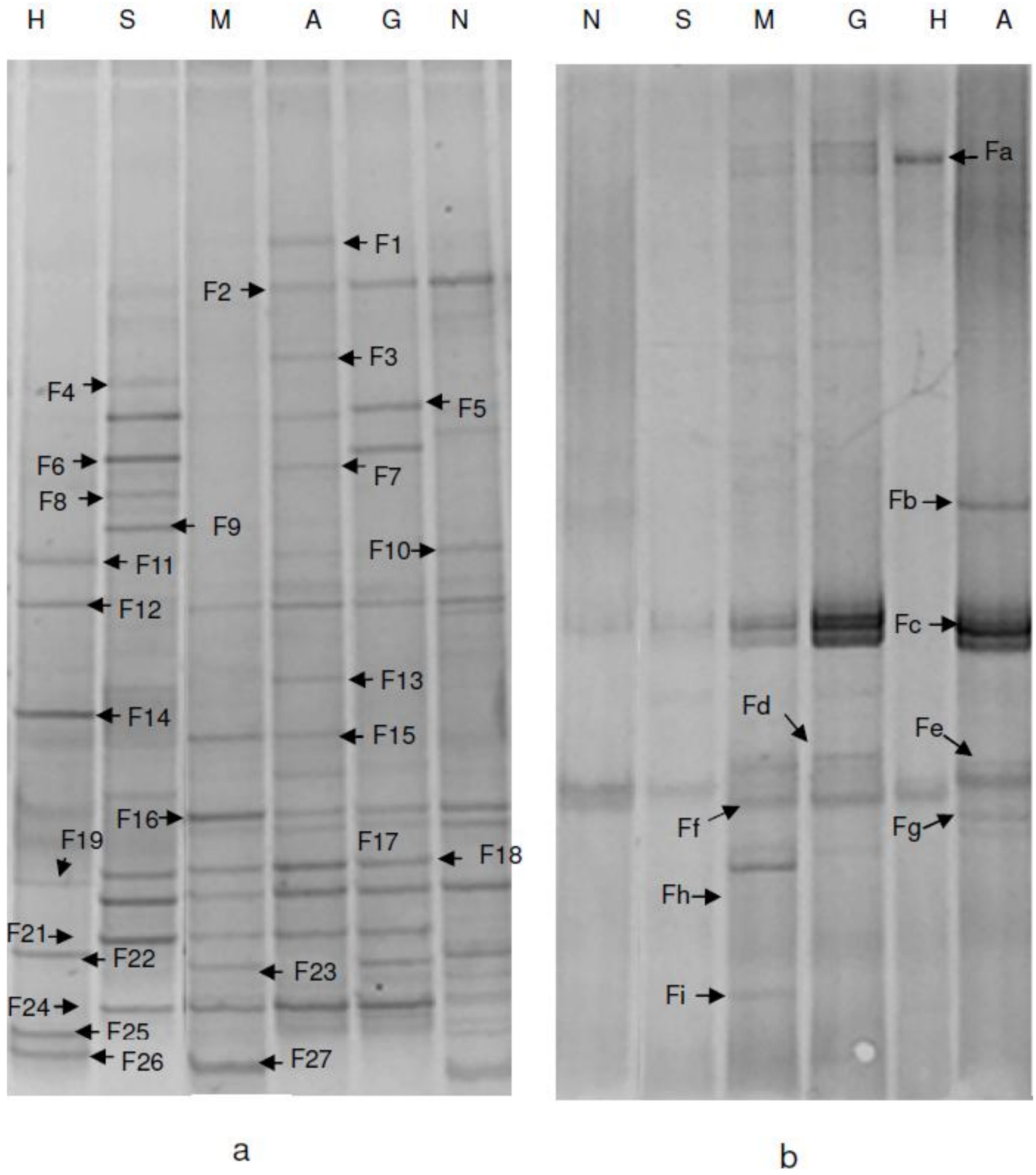

Figure 1. DGGE profiles of 16S rRNA gene fragments amplified from different enrichment cultures of produced water samples with bacterial (a) or archeal (b) primer pairs. Sample of hydrocarbon degrader enrichment is shown in lane H, sample of sulfate enrichment in lane S, sample of stearate and heptadecanoate enrichment in lane M, and sample of is shown in lane A, sample of Na-acetate (0.1 g) and 40 ml H_2+CO_2 (4:1) enrichment glucose enrichment in lane G, and sample of nitrate enrichment is shown in lane N. Letters on the sides of the gels indicate the clone bands corresponding to bacteria and species showed in Tables 1 and 2.

Figure 1. Results of DGGE analysis suggested that bacteria may be the dominant microbes, because more bacteria bands were found than archaeal. The microbial community was likely to vary throughout the enrichment culture depending on different functional enrichments cultures. The most diverse enrichment sample was the enrichment of H_2/CO_2 and Na-acetate, 15 different bacterial and 4 different archaeal bands were

determined. Furthermore, the least diverse sample was enrichment of hydrocarbon degrader, 6 bacterial and 2 archaeal bands were observed. Archaeal microorganisms might be inhibited by sulfate and nitrate, because only two dim archaeal bands were found in samples of SRB and NRB enrichments.

Phylogenetic affiliation of dominant bacterial

The DGGE profiles of bacterial communities in all enrichment cultures were shown in Figure 1a. The prominent DGGE bands were excised, re-amplified and sequenced. The sequences of a total of 27 DNA fragments were successfully determined, and no chimeric DNA fragments observed. They were affiliated to *Firmicutes*, *Thermotogae, Dictyoglomi*, *Actinobacteria, Proteobacteri*a and uncultured bacterial clones. The bacterial communities are summarized in Table 1.

Firmicutes

16 sequences were placed into *Firmicutes*. 6 bands were belonged to *Thermoanaerobacter*, F5, F14, F16, F17, F22 and F26 were closely affiliated with *Thermoanaerobacter brockii*, a hyperthermophilic, fermentative bacteria isolated from the Yellowstone Park hot springs (Lamed and Zeikus, 1981). Band F8 was most related to *Thermoanaerobacter yonsiensis*, an extremely thermophilic, xylose-utilizing bacterium, isolated from a geothermal hot stream at Sileri on Java island, Indonesia (Kim et al., 2001). Bands F12 and F19 were identified as *Thermosyntropha lipolytica,* an anaerobic thermophilic lipolytic alkalitolerant bacterium, isolated from alkaline hot springs of Lake Bogoria (Kenva) (Sevtlitshnyi et al., 1996). Bands F13 and F23 were closely similar to *Coprothermobacter proteolyticus*, a thermophilic and proteolytic acetogen isolated from a methanogenic enrichment (Ollivier et al., 1985). Bands F2 was closely relate to *Moorella glycerini*, a homoacetogenic fermentative bacteria, isolated from a mixed sediment-water sample from a hot spring (Calcite Spring area) at Yellowstone National Park. Band F9 was closely related to *Desulfotomaculum geothermicum*, a thermophilic, fatty acid-degrading, sulfate-reducing bacterium isolated with H_2 from geothermal ground water (High-temperature oil field) (Daumas et al., 1988). Band F18 was closely affiliated with *Desulfotomaculum thermosubterraneum* a thermophilic sulfate-reducing bacterium which can reduce sulfate, sulfite, thiosulfate and elemental sulfur, isolated from an underground mine in a geothermally active area in Japan (Kaksonen et al., 2006). Band F15 was most relatively to *Caloramator viterbiensis*, a glycerol-fermenting bacterium isolated from a hot spring in Italy (Seyfried et al., 2002). Band F3 was closely affiliated with an unclassified Thermoanaerobacteriaceae, Thermoanaerobacteriaceae bacterium 46bZ, detected from Gangxi oil bed.

Thermotogae

Two sequences were placed into *Thermotogae*, band F1 was most related to *Fervidobacterium icelandicum*, an extremely thermophilic fermentive anaerobic bacterium isolated from an Icelandic hot spring (Huber et al., 1990). Band F24 was most relatively to *Thermotoga hypogeal*, a Xylanolytic, hyperthermophilic bacterium from an Oil-Producing Well (Fardeau et al., 1997).

Dictyoglomi

One bacterial sequence was placed into *Dictyoglomi*. Band F21 was most related to *Dictyoglomus. thermophilum*, an anaerobic, extreme thermophilic fermentive bacterium, isolated form a slightly alkaline hot spring (Saiki et al., 1985).

Actinobacteria

One band was placed into *Actinobacteria*. Band F11 was most similarity to *Symbiobacterium thermophilum*, a symbiotic and microaerophilic thermophile isolated in mixed culture with a Bacillus strain from compost in Hiroshima Prefecture, Japan (Ohno et al., 2000). The bacterium is capable of anaerobic mono-growth when supplied with CO_2 or bicarbonate (Watsuji et al., 2006).

Proteobacteria and unclutured bacterium

One band was placed into *Proteobacteria*. Band F10 was identified as *Roseomonas cervicalis*, a mesophilic bacterium isolated from cervix of a woman. Bands F4, F6, F7, F20 and F25 were uncultured bacterium, no bacterium related with them.

Phylogenetic affiliation of dominant archaeal Phylotypes

As seen in Figure 1b, 9 bands were excised and sequenced after DGGE analysis of the amplifications obtained by the application of Archaea-Specific primers. All of them yielded reliable sequences which were mostly affiliated with *Euryarchaeota* (Table 2). Two bands were belonged to hyperthermophilic archaea, Band Fb was most closely to *Geoglobus ahangari*, a fermentator could grow autotrophically on hydrogen and Fe(III), and oxidize long-chain acids (Kashefi et al., 2002). Band Fh was closely related to *Thermococcus aegaeus*, a *thermophila*,

Table 1. Bacterial phylotypes detected by PCR-DGGE.

Taxonomical group	DGGE band	Closet cultivated species or sequences	Sequence identity (%)	Temperature optimum of closest cultivated species (°C)
Thermotogae	F1(HM153484)	*Fervidobacterium icelandicum* (M59176)	97.2	65
	F24(HM153507)	*Thermotoga hypogea* (U89768)	99.4	70
Proteobacteria	F10(HM153493)	*Roseomonas cervicalis* (AY150047)	98.6	Mesophilic
Dictyoglomi	F21(HM153504)	*Dictyoglomus.thermophilum* (X69194)	97.2	78
Actinobacteria	F11(HM153494)	*Symbiobacterium thermophilum* (AP006840)	99.3	60
Firmicutes	F2(HM153485)	*Moorella glycerini* (U82327)	100	60
	F3(HM153486)	*Thermoanaerobacteriaceae* bacterium 46bZ (GU129098)	97.3	-
	F4(HM153487)	Uncultured bacterium clone 3BCL88(AM087647)	99.3	-
	F5(HM153488)	*Thermoanaerobacter brockii* (L09165)	99.3	65-70
	F6(HM153489)	Uncultured bacterium CL-090621 (EU809296)	99.4	-
	F7(HM153490)	Uncultured bacterium clone ZB_P14_C08 (GQ328684)	97.2	-
	F8(HM153491)	*Thermoanaerobacter yonsieensis* (AF212925)	99.3	75
	F9(HM153492)	*Desulfotomaculum geothermicum* (AJ294428)	96.5	54
	F12(HM153495)	*Thermosyntropha lipolytica* (X99980)	95.7	60-66
	F13(HM153496)	*Coprothermobacter proteolyticus* （GU363592）	98.6	65
	F14(HM153497)	*Thermoanaerobacter brockii* (L09165)	96.5	65-70
	F15(HM153498)	*Caloramator viterbiensis* (AF181848)	97.8	58
	F16(HM153499)	*Thermoanaerobacter brockii* (L09165)	100	65-70
	F17(HM153500)	*Thermoanaerobacter brockii* (L09165)	98.6	65-70
	F18(HM153501)	*Desulfotomaculum thermosubterraneum* (QD208688)	97.2	61-66
	F19(HM153502)	*Thermosyntropha lipolytica* (X99980)	96.5	60-66
	F20(HM153503)	Uncultured bacterium clone ZB_P14_C08(GQ328684)	100	-
	F22(HM153505)	*Thermoanaerobacter brockii* (L09165)	98.7	65-70
	F23(HM153506)	*Coprothermobacter proteolyticus* (GU363592)	100	65
	F25 (HM153508)	Uncultured bacterium clone Dan_Bac87 (FN356293)	100	-
	F26(HM153509)	*Thermoanaerobacter brockii* (L09165)	97.9	65-70
	F27(HM153510)	*Thermacetogenium phaeum* (AB020336)	98.6	58

Table 2. Archaeal phylotypes detected by PCR-DGGE.

Taxonomical group	DGGE band	Closet cultivated species or sequences	Sequence identity (%)	Temperature optimum of closest cultivated species (°C)
Euryarchaeota	Fa (HM153511)	*Methanomethylovorans thermophila* (AY672821)	97.8	50
	Fb (HM153512)	*Geoglobus ahangari* (AF220165)	96.3	88
	Fc (HM153514)	*Methanoculleus thermophilus* (AB065297)	98.5	55
	Fd (HM153513)	*Methanocalculus halotolerans* (AF033672)	98.5	38
	Fe (HM153515)	*Methanothermobacter thermautotrophicus* (AY196660)	98.6	65 - 70
	Ff (HM153516)	*Methanoculleus thermophilus* (AB065297)	99.3	55
	Fg (HM153517)	*Methanosarcina mazei* (AJ012095)	98.5	Mesophilic
	Fh (HM153518)	*Thermococcus aegaeus* (AY099171)	98.6	88-90
	Fi (HM153519)	*Methanosaeta thermophila* (AB071701)	97.8	55-60

hyperthermophilic heterotrophic archaea could utilize complex organic compounds like yeast extract, peptone and tryptone as carbon source (Arab et al., 2000). 7 bands were closely affiliated methanogens. One band was belonged to thermophilic methlotrophic methanogen, band Fa was most related with *Methanomethylovorans* which just uses methanol and mono-, di- and trimethylamine (Jiang et al., 2005). Four bands were belonged to hydrogenotrophic methanogen. Band Fd was most related to *Methanocalculus halotolerans*, a mesophilic halotolerant methanogen, which can use H_2+CO_2 and formate (Ollivier et al., 1998). Band Fe was most related with *Methanothermobacter thermautotrophicus* a thermophilic methanogen just use H_2+CO_2 (Wasserfallen et al., 2000). Bands Fc and Ff were most closely to *Methanoculleus thermophilus*, a thermophilic methanogen isolated from sediment of river, which uses either formate or H_2+CO_2 as a substrate for growth and methane formation (Rivard and Smith, 1982). Two bands belonged to acetogenotrophic methanogen. Band Fg was most related to *Methanosarcina mazei*, a mesophilic methanogen can convert acetate, methanol, methylamine, and trimethylamine to methane (Mah, 1980; Mah and Kuhn, 1984). Band Fi was most relative with *Methanosaeta thermophila,* a thermophilic methanoge, just uses acetate as the only substrate for growth and methanogenesis (Ohtsubo et al., 1991; Kamagata et al., 1992).

DISCUSSION

In this study, enrichment culture approaches and molecular analysis were used to investigate thermophilic microbial diversity in production water from a high-temperature water-flooded in the Dagang oil field. Many efforts have been made to explain the microbial diversity of petroleum reservoirs in recent years (Orphan et al., 2000; Bonch-Osmolovskaya et al., 2003; Grabowski et al., 2005; Li et al., 2007a). This study revealed more thermophilic microorganisms and less mesophilic microorganisms than that had been reported previously from such extreme environments. 27 bacterial and 9 archaeal bands were sequenced in all enrichment cultures, only 1 bacterial and 2 archaeal sequences were belonged to mesophilic microorganisms, the result indicates that enrichment cultures in high temperature for a long time is very useful way to decrease the effect of mesophilic microbes, and PCR-DGGE is available for characterizing the diversity of enrichment cultures. All samples were processed and compared in a fingerprint profile.

Many thermophilic microbes were detected in this study, but few of them were obtained from enrichment of hydrocarbon degradations enrichment culture. And they mainly belonged to *Thermoanaerobacter*, *Thermosyntropha*, *Symbiobacterium*, *Dictyoglomus*, *Methanomethylovorans* and *Methanoculleus.* Our result is very similar to previous reports (Widdel et al., 2006; Orphan et al., 2000; Li et al., 2007 a). It is generally believed that the microbial diversity in high temperature oil reservoir is low, consisted of some fermentative bacteria, syntrophic bacteria and methanogens. It may be attributed to the extreme conditions of the subsurface petroleum reservoir environment. Liquid hydrocarbons are the prevailing organic matter in geothermally heated oil reservoirs, and the anearobic bio-degradition of hydrocarbons is proved to be very slowly (Widdel et al., 2006). Most thermophilic microbe may not be the major components. Alternatively, they may have been missed due to PCR biases in the mixed assemblage DNA amplification.

More thermophilic bacteria and less archaea were found in enrichment cultures of SRB and NRB. This may be due to nitrate and sulfate can promote the anearobic bio-degradition of hydrocarbons and restrain the growth of archaea (Rueter et al., 1994; Aeckersberg et al., 1998). Still some archaea were found, but their bands were very dim in DGGE profiles, such as *Methanocalculus*, *Methanothermobacter.* More bacteria and archaea were detected in enrichment cultures of ferment, long-chain fatty-acid degrade and methanogens enrichment cultures, which indicated different low concentrations of exogenetic carbon sources can stimulate the growth of microorganisms of oil fields. The most microbial diverse was found in the enrichment sample of H_2/CO_2 and Na-acetate, which suggests that H_2/CO_2 and Na-acetate not only stimulate grow of methanogens but also hydrocarbon degradation.

The detected bacterial communities appeared to be dominated by fermentative bacteria and syntrophic bacteria in all enrichments cultures in this study. And they are also known to represent the majority of microorganisms in anoxic conditions (Magot et al., 2000; Schink, 2002), and play an important role in transforming organic compounds into chemical products favorable for terminal oxidizers such as methanogens, deniftrifiers and sulfate reducers.

No NRB and hydrocarbon degradation bacteria were found in all enrichments, which suggest they may be not dominant species in the oil fields. Moreover, sequencing results of some excised bands showed similarity to uncultured bacterial species in this study. The metabolisms of these uncultured species have not been clarified yet. Therefore, they may have nitrate reduction or hydrocarbon degradation capacity. SRB of *Desulfotomaculum* were found in all enrichment except NRB enrichment, which indicates that SRB may one of main bacteria in Dagang oilfield, and they can be inhibited by nitrate. Thermophilic archaeal phylogypes represented in the samples of enrichments were related to functionally diverse groups, including the hyperthermophilic fermentive *Geoglobus* and *Thermococcus*, the methylotrophic *Methanomethylovorans*, the hydrogenotrophic *Methanothermobacter* and *Methanoculleus*, and the acetogenotrophic *Methanosaeta*, suggesting that thermophilic methane be may the dominant archaea and most of them are hydrogenotrophic in high temperature oil field of Dagang.

21 thermophilic bacterial and 7 thermophilic archaeal sequences were found in this study. Results show that there were many thermophilic microbes in oil fields, including fermentative bacteria and archaea, syntrophic bacteria, SRB and methanogens, most of them might not the dominant microorganisms, and the co-microbial community would been changed because of different carbon sources and elector acceptors. The analysis of this information provides insight into the thermophilic microbial diversity, community in petroleum reservoirs, and inspiration for future studies.

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