Comparison of archaeal and bacterial community structures in heavily oil-contaminated and pristine soils

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Archaeal and bacterial community structures in heavily oil-contaminated and pristine soils were compared using denaturing gradient gel electrophoresis and 16S rRNA gene libraries. The results showed that archaeal diversity was more complex in the contaminated soil than in the uncontaminated control soil. Archaeal populations in the contaminated soil consisted mainly of *Euryarchaeota*, with abundant methanogen-like operational taxonomic units (OTUs) and OTUs related to the phylogenetically diverse group, candidate division I, corresponding to rice cluster V. In contrast, only halophilic archaea were found in the pristine soil. Bacterial community structures also differed significantly between the contaminated and pristine soils. More clones from the contaminated soil were related to known hydrocarbon-degrading bacteria, implying that microorganisms with the potential to degrade petroleum were well-established. These results provide further insights into the composition of microbial communities in oil-contaminated soils.

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The toxic, mutagenic, and carcinogenic properties of petroleum mean that soil contamination by crude oil is an important environmental problem during oil drilling and production (1). Bacteria are important for the biodegradation of petroleum hydrocarbons, and many hydrocarbon-degrading bacteria have been isolated from different environments. These isolated bacteria are able to degrade alkanes and/or aromatic hydrocarbons under aerobic or anaerobic conditions. The bacteria have been assigned to a number of genera, including *Pseudomonas*, *Alcanivorax*, and *Marinobacter* etc. Some of them have been widely used for bioremediation of oil-contaminated environments (2, 3). Consequently, much research has focused on the characteristics of bacterial community structures in oil-contaminated sites, as well as changes in these community structures associated with oil contamination (4, 5).

Recent molecular-based studies have shown archaea, which were once thought to be synonymous with extreme environments, to be present in almost all the environments examined to date (6). Archaea have been detected in several oil-containing environments, such as petroleum reservoirs (7), underground crude oil storage cavities (8), and hydrocarbon-polluted aquifers (9). Furthermore, researchers have shown that archaea are involved in the mineralization of petroleum hydrocarbons, especially by methanogenesis under anaerobic conditions (10, 11). However, relatively little is known about archaeal community structures in heavily oil-contaminated soils.

Denaturing gradient gel electrophoresis (DGGE) fingerprinting and cloning and sequencing of polymerase chain reaction (PCR)-amplified 16S rRNA gene fragments have been successfully used to analyze bacterial and archaeal community structures in a wide range of environmental samples (12, 13). PCR-DGGE has the advantage of quickly and economically analyzing the diversity of microbial assemblages present in different environments, and sequence analysis of clones from 16S rRNA gene clone libraries can provide detailed, reliable information about the structure and diversity of microbial communities in specific ecosystems. Thus, in the present study, DGGE and 16S rRNA gene libraries were used for the comparative analysis of the archaeal and bacterial communities in oil-contaminated and pristine soils.

MATERIALS AND METHODS

Soil samples The sampling sites, including oil-contaminated and pristine soils, were located in the Jidong Oilfield near Bohai Bay, China. The oil-contaminated site had been used as a storage area for petroleum leaks during petroleum exploitation for about 10 years. Oil stains were visible on the surface of the soil. The pristine site was located about 50 m from the oil-contaminated site. In December 2006, soil samples were collected from the top of each site (0–2 cm) using an ethanol-swabbed hand shovel. Immediately after collection, samples were stored in sterilized glass jars at 4 °C. Samples were collected from multiple areas within each site and mixed thoroughly to produce homogenized samples. Total petroleum hydrocarbon (TPH) concentration was determined by the method previously described (14). The saturated and aromatic components were analyzed by gas chromatography-mass spectrometry (Agilent 6890/5973, Santa Clara, CA, USA) (15). The total nitrogen (T-N) and total phosphorus (T-P) concentrations were determined as described previously (16). The physicochemical characteristics of the soil samples are shown in Table 1.

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DNA extraction The soil samples was washed for three times using phosphate-buffered saline (PBS, pH7.0) and centrifuged at 4 °C, 10,000 rpm for 15 min. Then the genomic DNA was extracted by the method described by Tsai et al (17).

PCR-DGGE In order to minimize PCR bias in PCR-DGGE and subsequent cloning steps, three separate reactions were run for each sample and the results were pooled. For bacteria, 16S rRNA gene fragments were amplified by PCR with primers 341F, using a 40-bp GC-clamp attached to the 5' end, and 534R (18). For archaea, 16S rRNA gene fragments were amplified using a nested PCR approach. The first-round PCR was performed with primers Arch571F and UA1406R (19), followed by amplification using primers Arch915F (20) with a GC-clamp, and UA1406R. The resulting amplification products were separated using DGGE with the Dcode Universal Mutation System (Bio-Rad, Hercules, CA, USA). The PCR products (20–25 µl) were loaded onto 6% (w/v) polyacrylamide gels in Tris-acetate-EDTA buffer (pH 7.4). The gels had denaturing gradients of 35–57% (100% denaturant contains 7 M urea and 40% formamide) and were run at 150 V at 60 °C for 4.5 h. After electrophoresis, the gels were stained for 15 min with ethidium bromide and photographed on a UV transillumination table using a Gel Documentation System (Bio-Rad Laboratories-Segrate, Milan, Italy). DGGE gels were processed using Bio-Rad Quantity One 4.3.0 software.

Cloning and sequencing of 16S rRNA gene The 16S rRNA gene fragments were amplified by PCR using primers Arch571F and UA1406R for archaea, and 27F and 1492R (21) for bacteria. PCR products were purified using TaKaRa purification kits. The amplified 16S rRNA gene fragments were cloned into the TOPO TA cloning vector pCR2.1, and then TOP10 chemically competent *E. coli* was transformed with the plasmids according to the manufacture's instructions (Invitrogen). Transformants were selected by blue-white selection on Luria-Bertani (LB) agar plates containing ampicillin (100 µg/ml). Cloned inserts were amplified from lysed colonies by PCR with plasmid-vector specific primers M13F and M13R. Clones were sequenced with an ABI 3730 automated sequencer (Invitrogen, Shanghai, China).

Phylogenetic analysis The archaeal sequences sharing 97% or greater similarity were considered to represent the same operational taxonomic unit (OTU) using software DOTUR (<http://schloss.micro.umass.edu/software/>). Sequences were searched against the Ribosomal Database Project II (RDP II) release 9.49 and the GenBank database by using the BLASTn program to determine the nearest matches. Phylogenetic trees were constructed using MEGA, version 3.1 by the neighbor-joining algorithm and the Jukes-Cantor distance estimation method with bootstrap analyses for 1000 replicates was performed. The presence of chimera in the obtained sequences was determined by using the CHECK_CHIMERA (RDP; <http://rdp.cme.msu.edu>) prior to phylogenetic analysis.

Soil-slurry experiments The basal salt medium used in this study was composed of 5 mM KH₂PO₄, 5 mM K₂HPO₄, and 14 mM NH₄Cl. Approximately 2 g of soil and 100 ml of the sterile salt medium were dispensed into flasks (300 ml in capacity). Sterilized petroleum was added to each flask to a final amount of 120 mg TPH. Flasks were incubated at 25 °C with shaking at 150 rpm. Sterile controls were prepared under the same conditions by autoclaving the flasks for 20 min at 121 °C before adding petroleum. All the slurry systems were prepared in triplicate. TPH was determined as described above.

Nucleotide sequence accession numbers The partial 16S rRNA gene sequences that were determined have been deposited in the GenBank, nucleotide sequence databases under accession nos. EU735565–EU735702.

RESULTS

DGGE analysis PCR products from DNA extracted from the pristine and oil-contaminated soils showed the expected 491-bp archaeal PCR fragment and the 193-bp bacterial fragment. The archaeal populations in the two samples were clearly different (Fig. 1A). Archaeal diversity in the pristine soil was low, with only one intense band visible. More DGGE bands were produced from archaea found in the contaminated soil. There was only 35.5% similarity in the archaeal DGGE profiles between the contaminated and pristine soils (Dice's coefficient) (Fig. 1A). The bacteria from the two samples,

FIG. 1. Denaturing gradient gel electrophoresis profiles for partial 16S rDNA fragments showing archaeal (A) and bacterial (B) diversities in the pristine (lane 1) and oil-contaminated (lane 2) soil samples. The diagrams were generated using Quantity One 4.3.0 software.

however, showed more diversity (Fig. 1B), and the DGGE patterns for bacteria from the two soil samples showed a similarity of 57.7%.

Diversity of 16S rRNA gene sequences Due to the fact that the number of bands generated by DGGE may not accurately reflect the number of different sequences present in a given DNA mixture (22), clone libraries were constructed to obtain more detailed information. Partial 16S rRNA gene fragments were PCR-amplified from the extracted DNA using the bacterial and archaeal specific primer sets, and four clone libraries were produced (SCA, archaea in the contaminated soil; SNA, archaea in the pristine soil; SC, bacteria in the contaminated soil; SN, bacteria in the pristine soil). For the two archaeal libraries, a total of 59 and 28 archaeal clones of around 840 nucleotides were obtained from the contaminated and pristine soils, respectively, and these were classified into 26 OTUs (SCA) and 10 OTUs (SNA) with a sequence similarity of 0.97 or greater, using DOTUR software. For each of the two bacterial libraries, 50 (SC) and 56 bacterial clones (SN) were randomly selected. Four sequences from the contaminated soil were identified as chimeric and were excluded from further analyses.

All of the 10 archaeal OTUs derived from the pristine soil were affiliated with *Halobacteriales* (Fig. 2). Twenty-two OTUs from the contaminated soil (83% of clones) fell into the *Euryarchaeota* (Fig. 2). Among these, seven OTUs (25.4% of clones) were affiliated with known euryarchaeotic orders (three with *Methanosarcinales*, two with *Methanomicrobiales* and two with *Halobacteriales*); one OTU (SCA110) formed a monophyletic clade; the other 14 OTUs were not affiliated with any of the known orders and were divided into two order-level phylogenetic groups (candidate divisions I and II). The remaining four OTUs were grouped into *Crenarchaeota*, and were related to unidentified environmental clones (Table 2).

For the bacterial libraries, taxa that were common to both soils included species from *Alpha*-, *Delta*-, and *Gammaproteobacteria*, and *Bacteroidetes* (Table 3). *Gammaproteobacteria* predominated in both bacterial libraries (Fig. 3). The contaminated soil contained a smaller proportion of *Gammaproteobacteria* (32.6% of clones) and *Bacteroidetes* clones (13% of clones) than the pristine soil, but more *Alpha*- and *Deltaproteobacteria* clones (Table 3). Taxa that were unique to the contaminated soil included four phyla that were represented by three clones from the *Planctomycetes*, two clones of *Chloroflexi*, one clone of

TABLE 1. Characteristics of soil samples used in this study.

Parameter	Pristine soil	Contaminated soil
TPH (mg/kg)	<100	52709
Petroleum component (%)		
Saturates	0	54.8
Aromatics	0	13.6
Total N (%)	0.087	0.076
Total P (%)	0.046	0.041
Salt content (g/Kg)	5.5	4.2
Moisture (%)	12	16
pH	8.7	8.6

Values are means of triplicates.

FIG. 2. Phylogenetic analysis of archaeal operational taxonomic units retrieved from the oil-contaminated soil (A) and pristine soil (B). Evolutionary dendrogram constructed using the NJ method. Scale bar denotes 0.05 indicated changes per nucleotide. (SCA, archaeal clone from the contaminated soil; SNA, archaeal clone from the pristine soil).

FIG. 2 (continued).

Acidobacteria, and two clones of *Firmicutes*. In contrast, *Actinobacteria* and *Betaproteobacteria* (each with five clones) were only detected in the pristine soil. In addition, three (SN) and four (SC) clones could not be classified into any known phyla.

Hydrocarbon degradation potential in the soil samples The potential of the contaminated and pristine soil samples for petroleum hydrocarbon degradation were compared in soil-slurry systems, and the results are shown in Fig. 4. The TPH removal rate by each soil was estimated by subtracting the average abiotic loss of TPH in three sterile controls from the loss in each sample flask. The system inoculated with the contaminated soil exhibited a much higher TPH removal rate than the system inoculated with the pristine soil. The 8-day TPH removals were 87.6% and 45.8% for the contaminated and pristine soil-slurry systems, respectively, showing that the contaminated soil possessed a significantly higher TPH removal potential.

DISCUSSION

In this study, both DGGE profiles and analysis of archaeal libraries showed that archaeal community structure was more complex in heavily oil-contaminated soil than in pristine soil. All archaeal clones retrieved from the pristine soil were grouped into the order *Halobacteriales*, and were closely related to *Natronomonas* and *Natronobacterium*. A similar result was observed by Valenzuela-Encinas et al. in a study of analyzing the archaeal community in an alkaline-saline soil (23). In contrast, the archaeal community in the contaminated soil was more diverse. In addition to the *Halobacteriales* phylotypes (two OTUs associated with the genera *Halobacterium* and *Haloarcula*), phylotypes belonging to other order-level phylogenetic groups in the *Euryarchaeota* (*Methanosarcinales*, *Methanomicrobiales*, candidate divisions I and II), and *Crenarchaeota* were detected.

TABLE 2. Distribution of archaeal operational taxonomic units and related clone numbers in phylogenetic groups.

Phylogenetic group		Number of OTUs from		Relative no. of clones from contaminated soil
		Pristine soil	Contaminated soil	
<i>Euryarchaeota</i>	Candidate division I	–	12(25)	42.4%
	<i>Methanosarcinales</i>	–	3(5)	8.5%
	<i>Methanomicrobiales</i>	–	2(8)	13.6%
	<i>Halobacteriales</i>	10(28)	2(2)	3.4%
	Candidate division II	–	2(7)	11.9%
<i>Crenarchaeota</i>	Unknown	–	4(10)	16.9%

In the contaminated soil, five OTUs representing 22% of sequences belonged to methanogens. Three OTUs were closely affiliated with isolated *Methanosarcinales* species known to use methanol, methylamine, and related methyl-substrates for the production of methane (SCA10 with halophilic *Methanohalophilus portucalensis*, SCA66 with *Methanobacterium psychrophilum* and SCA81 with *Methanococcus alaskense* AK-9). Two OTUs (SCA49 and SCA33) were affiliated with isolated *Methanomicrobiales* species known to utilize H_2 - CO_2 and formate: OTU SCA49 was closely related to mesophilic *Methanospirillum hungatei*; and OTU SCA33 was related to *Methanoculleus receptaculi* isolated from the Shengli oil field, China (95% similarity). Previous studies have shown that methanogens were present in various oil-contaminated environments. Yoshida et al. detected clones related to *Methanosaeta* and *Methanoculleus* in crude-oil sludge (8). Kasai et al. found *Methanosaeta* and *Methanomethylovorans* belonging to the order *Methanosarcinales* in petroleum-contaminated unsaturated soil, but no methanogens in uncontaminated soil (24). Watanabe et al. demonstrated that archaea related to *Methanosaeta* and *Methanomethylovorans*, as well as *Methanomicrobiales*, were present in oil-contaminated groundwater (25). Most of these studies detected acetoclastic *Methanosaeta*. Acetoclastic methanogenesis has been suggested to be the major terminal electron-accepting process associated with anaerobic hydrocarbon degradation (9). However, acetoclastic *Methanosaeta* was not detected in the contaminated soil in the present study, and all of the OTUs belonging to *Methanosarcinales* were related to halophilic and methylphilic genera in the family *Methanosarcinaceae*. This may be related to the relatively high salt concentration in the contaminated soil (Table 1), and methyl compounds are thought to be the primary substrates for methanogens

TABLE 3. Distribution of bacterial clones in phylogenetic groups.

Phylogenetic group	Number of clones from		Relative number of clones from	
	Pristine soil	Contaminated soil	Pristine soil	Contaminated soil
α -Proteobacteria	3	8	5.4%	17.4%
β -Proteobacteria	5	–	8.9%	–
γ -Proteobacteria	28	15	50.0%	32.6%
δ -Proteobacteria	2	5	3.6%	11.0%
<i>Bacteroidetes</i>	10	6	17.9%	13.0%
<i>Planctomycetes</i>	–	3	–	6.5%
<i>Chloroflexi</i>	–	2	–	4.3%
<i>Acidobacteria</i>	–	1	–	2.2%
<i>Firmicutes</i>	–	2	–	4.3%
<i>Actinobacteria</i>	5	–	8.9%	–
Unknown	3	4	5.4%	8.7%

FIG. 3. Phylogenetic analysis of bacterial clones retrieved from the oil-contaminated (A) and pristine soil (B). Evolutionary dendrogram constructed using the NJ method. Scale bar denotes 0.05 indicated changes per nucleotide. (SC, bacterial clone obtained from the contaminated soil; SN, bacterial clone obtained from the pristine soil).

FIG. 3 (continued).

in marine sediments (26). It has been reported that methanogenic archaea are detected in oil production water and stratal water from oil fields (7), and they are thought to be indigenous microorganisms in oil reservoirs and crude oil (8). Thus, it is likely that methanogens detected here may originate from the contaminating crude oil. Methanogens are strict anaerobes. According to Coates et al., contaminating petroleum can prevent ventilation to soil and stimulate

the oxygen consumption, resulting in the formation of anaerobic zones (27). It is possible that anaerobic zones suitable for growth of methanogens are present in the contaminated soil due to the serious oil pollution (Table 1).

The phylogenetic analysis shown in Fig. 2A indicated that 14 OTUs could not be affiliated with known orders of *Euryarchaeota*, these being grouped into two candidate divisions. Twelve OTUs were

FIG. 4. Degradation of petroleum hydrocarbons in the pristine (open squares) and oil-contaminated (closed rhombuses) soil-slurry systems.

affiliated with candidate division I, which corresponded to rice cluster V. Candidate division I included rather diverse rDNA sequences, suggesting that further studies were needed on this group. The rice clusters have originally been proposed for archaea inhabiting a rice paddy field, while the results of recent studies indicate that members of these groups of archaea are distributed more widely in anoxic environments (25). Rice cluster V-like phylotypes have been detected in oil-contaminated groundwater at the bottom of an underground crude oil storage cavity (25). In this study, one OTU (SCA70) in candidate division I was related to clone KuA18 retrieved from oil-contaminated groundwater with 91% similarity. Candidate division II included two OTUs that showed high sequence homology with an uncultured archaeon clone retrieved from sediments, and these sequences were associated with clone ASC8 obtained from oil-contaminated unsaturated soil (24). Isolation of these archaea in pure culture or environmental genomic analyses will help to know the ecological roles of these uncultivated archaeal phylotypes in oil-contaminating environments.

All of the four OTUs grouped into *Crenarchaeota* were related to unidentified environmental clones. The 16S rRNA gene sequences of *Crenarchaeota* from the upper layer of soils can be recovered on all continents from almost any terrestrial ecosystem. According to Christa et al., non-thermophilic *Crenarchaeota* found in soils might be chemolithoautotrophic ammonia oxidizers (7).

DGGE profiles and phylogenetic analysis revealed that bacterial community structures were significantly different in the contaminated and pristine soils. *Gammaproteobacteria* were predominant in the SC library. The majority of *Gammaproteobacteria* clones (11 of 15 clones) were clustered within the *Alcanivorax*–*Marinobacter*–*Halomonas* complex, members of which are known to degrade petroleum hydrocarbons in various saline environments, such as marine sediments, saline soils, and oil-contaminated seawater. Among them, five clones (SC32, SC46, SC70, SC79, and SC176) were associated with hydrocarbon-degrading *Alcanivorax* sp. K2-1 and K3-3; four clones (SC10, SC34, SC43, and SC108) showed high similarity with *Marinobacter* sp. MARC4V (97–99%) known to degrade polycyclic aromatic hydrocarbons (PAH), and were moderately related to halophilic hydrocarbon-degrading *Marinobacter hydrocarbonoclasticus* (95–96%); clones SC13 and SC152 were related to *Halomonas* spp. (95–98%), of which *Halomonas shengliensis* capable of utilizing crude oil was isolated from an oil-contaminated saline soil from Shengli oilfield, China. In addition, *Gammaproteobacteria* clone SC151 was closely related to uncultured soil bacterium clone PYR10d11 (97%) associated with the degradation of pyrene in a bioreactor treating soil contaminated with PAH.

Of the other clones in the SC library, two clones were similar with those obtained from anaerobic environments. One clone (SC2) in the class *Bacteroidetes* was closely related to clone SB-1 (97%) derived from a sulfate-reducing consortium responsible for benzene-mineralizing, and the other (SC72) was related to clone Eub-3 from a toluene-degrading methanogenic consortium, suggesting the presence of anaerobic niches in the contaminated soil. Additionally, one

clone (SC86) in the class *Alphaproteobacteria* was closely related to *Roseobacter* sp. 812 (98%) associated with PAH-degradation.

In contrast to the *Gammaproteobacteria* group in the SC library, the main *Gammaproteobacteria* clones (12 of 28 clones) in the SN library were closely related to extremely halophilic *Salicola marensis* and *Pseudomonas halophila* (97–99%) isolated from high saline environments, e.g. saltern and salt lake. Clones clustered within the *Alcanivorax*–*Marinobacter*–*Halomonas* complex were also detected, including three clones (SN122, SN159, and SN161) related to hydrocarbon-degrading *Alcanivorax* sp. K3-3 (92–94%), one clone (SN 40) closely related to PAH-degrading *Marinobacter* sp. MARC4S (98%), and clone SN111 showing high similarity with halophilic oil-degrading *H. shengliensis* (97%).

It has been reported that *Pseudomonas* spp. in *Gammaproteobacteria* and high G+C gram-positive *Rhodococcus* spp. in *Actinobacteria* are commonly detected bacteria in various oil-contaminating environments (28). In the present study, *Pseudomonas*- or *Rhodococcus*-like clones were not detected in the oil-contaminated soil, whereas those affiliated with hydrocarbon-degrading *Halomonas*, *Marinobacter*, and *Alcanivorax* were in abundance. This should be related to relatively high salt content in the soil samples (Table 1). Similar results were observed by Kleinstaub et al. in a study about the population dynamics within a microbial consortium during growth on diesel fuel in saline environments (29), suggesting that *Halomonas*, *Marinobacter*, and *Alcanivorax* might be the primary hydrocarbon-degraders under saline conditions.

In order to compare the potential for hydrocarbon degradation in the contaminated and pristine soil samples, soil-slurry experiments were performed. Both the contaminated and pristine soil-slurry system could biodegrade petroleum hydrocarbons. However, the contaminated soil-slurry system showed the higher TPH degradation rate and 8-day removal, suggesting that the microbial community with substantial hydrocarbon degradation potential had been well-established in the contaminated soil. Comparatively abundant hydrocarbon-degrading bacteria-related clones detected in the bacterial SC library may support this conclusion to some extent.

In conclusion, our molecular approach demonstrated that archaeal and bacterial community structures differed significantly between pristine and oil-contaminated soils. The contaminated soil contained more diverse archaeal populations, and included more clones related to halophilic hydrocarbon-degrading microorganisms than those from the pristine soil. These results provide further insights into the composition of microbial communities in heavily oil-contaminated soils.

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