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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[Key words: Archael diversity; Bacterial diversity; Oil-contaminated soil; 16S rRNA gene; Denaturing gradient gel electrophoresis]

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 were washed for three times using phosphate-buffered saline (PBS, pH 7.0) 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-μl PCR mixture containing: 2 μl of 10× buffer (containing 5 mM MgCl₂, 50 mM KCl, 10 mM Tris–HCl (pH 9), 0.01% gelatin, 0.01% Tween-20), 1 μl of each primer (10 μM final concentration), 1 μl of genomic DNA (10 ng/μl), and 1 μl of 0.5 U/μl TaKaRa Taq polymerase. The PCR products were visualized by electrophoresis in 1% agarose gel and purified using the Ultrafree MC system (Millipore, MA, USA). The purified PCR products were cloned into the pCR4–TOPO vector (Invitrogen, CA, USA) and transformed into competent E. coli. The resulting colonies were sequenced with the M13F and M13R primers using an ABI 3730 automated sequencer (Invitrogen, Shanghai, China).

### Phylogenetic analysis

The archaeal sequences were recovered using the same method described above and were checked against the Ribosomal Database Project II (RDP II) release 9.49 to determine the nearest matches. The Archaea sequences sharing 97% or greater similarity with the known Archaea were classified into 10 OTUs (Table 3). The bacterial sequences were classified into 26 OTUs (Fig. 1A). The archaeal and bacterial sequences were classified into 10 OTUs (Fig. 1B) and 26 OTUs (Table 3), respectively.

### Soil-sturdy experiments

The soil was mixed with water (1:1 ratio) and incubated at 25 °C with shaking at 150 rpm. The control samples were prepared in a similar manner but without the addition of petroleum. All of the slurry systems were prepared in triplicate. TPH was determined as described above.

### 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 amplified 16S rRNA gene fragments were cloned into the TOPO TA cloning vector (Invitrogen, CA, USA). The resulting clones were sequenced with the M13F and M13R primers using an ABI 3730 automated sequencer (Invitrogen, Shanghai, China).

#### 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.

### COMPARISON OF ARCHAEAL AND BACTERIAL COMMUNITY

![FIG. 1. Denaturing gradient gel electrophoresis profiles for partial 16S rRNA fragments showing archael (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.](image)

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### 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, 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 *Halobacteria* (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 *Halobacteria*); one OTU (SCAI10) 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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pristine soil</th>
<th>Contaminated soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>8.7</td>
<td>8.6</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Salt content (g/Kg)</td>
<td>5.5</td>
<td>4.2</td>
</tr>
<tr>
<td>Total P (%)</td>
<td>0.046</td>
<td>0.041</td>
</tr>
<tr>
<td>Total N (%)</td>
<td>0.087</td>
<td>0.076</td>
</tr>
<tr>
<td>Aromatics</td>
<td>13.6</td>
<td></td>
</tr>
<tr>
<td>Saturates</td>
<td>54.8</td>
<td></td>
</tr>
<tr>
<td>TPH (mg/Kg)</td>
<td>&lt;100</td>
<td>52709</td>
</tr>
<tr>
<td>Petroleum component (%)</td>
<td>0</td>
<td>54.8</td>
</tr>
</tbody>
</table>
| 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).
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, 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>
<thead>
<tr>
<th>Phylogenetic group</th>
<th>Number of OTUs from pristine soil</th>
<th>Number of OTUs from contaminated soil</th>
<th>Relative no. of clones from pristine soil</th>
<th>Relative no. of clones from contaminated soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euryarchaeota</td>
<td>Candidate division I</td>
<td>12(25)</td>
<td>42.4%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methanosarciinales</td>
<td>3(5)</td>
<td>8.5%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methanomicrobiales</td>
<td>2(8)</td>
<td>13.6%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Halobacteriales</td>
<td>10(28)</td>
<td>3.4%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Candidate division II</td>
<td>2(7)</td>
<td>11.9%</td>
<td></td>
</tr>
<tr>
<td>Crenarchaeota</td>
<td>Unknown</td>
<td>4(10)</td>
<td>16.9%</td>
<td></td>
</tr>
</tbody>
</table>

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 Methanolobus psychrophilus and SCA81 with Methanococcales alaskense AK-9). Two OTUs (SCA49 and SCA33) were affiliated with isolated Methanomicrobiales species known to utilize H₂-CO₂ and formate: OTU SCA49 was closely related to mesophilic Methanospirillum hungatii; 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 Methanoseta and Methanoculleus in crude-oil sludge (8). Kasai et al. found Methanoseta and Methanomethylovorans belonging to the order Methanosarciinales 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 Methanosarciinales were related to halophilic and methylphilic genera in the family Methanosarciinae. 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.
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).
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
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. Ganmaproteobacteria 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 hydrocarbonoclasti-
cus (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, Gammaproteobacteriun clone SC151 was closely related to uncultured soil bacterium clone PYR0d11 (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-miner-
alizing, 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 Salincola marensis and Pseudomonas halophila (97–99%) isolated from high saline environments, e.g. salt pan and salt lake. Clones clustered within the Alcani-
vorax–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. MARC45 (98%), and clone SN111 showing high similarity with halophilic oil-
dergading H. shengliensis (97%).

It has been reported that Pseudomonas spp. in Gammaproteobact-
eria and high G+C gram-positive Rhodococcus spp. in Actinobacteria are commonly detected bacteria in various oil-contaminating environ-
ments (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, Marinobac-
ter, 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 Kleinesteuber 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 hydro-
carbon-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.

ACKNOWLEDGMENT

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