J. Microbiol. Biotechnol. (2008), **18**(12), 1966–1974
doi: 10.4014/jmb.0800.279
First published online 1 August 2008

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Bioremediation Potential of a Tropical Soil Contaminated with a Mixture of Crude Oil and Production Water

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Received: April 18, 2008 / Accepted: June 2, 2008

A typical tropical soil from the northeast of Brazil, where an important terrestrial oil field is located, was accidentally contaminated with a mixture of oil and saline production water. To study the bioremediation potential in this area, molecular methods based on PCR-DGGE were used to determine the diversity of the bacterial communities in bulk and in contaminated soils. Bacterial fingerprints revealed that the bacterial communities were affected by the presence of the mixture of oil and production water, and different profiles were observed when the contaminated soils were compared with the control. Halotolerant strains capable of degrading crude oil were also isolated from enrichment cultures obtained from the contaminated soil samples. Twenty-two strains showing these features were characterized genetically by amplified ribosomal DNA restriction analysis (ARDRA) and phenotypically by their colonial morphology and tolerance to high NaCl concentrations. Fifteen ARDRA groups were formed. Selected strains were analyzed by 16S rDNA sequencing, and Actinobacteria was identified as the main group found. Strains were also tested for their growth capability in the presence of different oil derivatives (hexane, dodecane, hexadecane, diesel, gasoline, toluene, naphthalene, *o*-xylene, and *p*-xylene) and different degradation profiles were observed. PCR products were obtained from 12 of the 15 ARDRA representatives when they were screened for the presence of the alkane hydroxylase gene (*alkB*). Members of the genera *Rhodococcus* and *Gordonia* were identified as predominant in the soil studied. These genera are usually implicated in oil degradation processes and, as such, the potential for bioremediation in this area can be considered as feasible.

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Keywords: Bacterial communities, bioremediation, oil, PCR-DGGE, production water

Petroleum is one of the most important energy sources for the industrialized world. Environmental impacts caused by petroleum industries come from exploitation, transport, refinement, and product utilization [24, 45]. The technologies commonly used for soil remediation include mechanical removal, burying, evaporation, dispersion, and washing, amongst other physical and chemical treatments. However, many of these technologies are expensive and can lead to an incomplete decomposition of contaminants. The process of bioremediation, defined as the use of microorganisms (especially bacteria) to detoxify or remove pollutants owing to their diverse metabolic capabilities, is an evolving method for the removal and degradation of many environmental pollutants, including those produced by the petroleum industry [26, 30]. Several reports have already focused on the composition of natural occurring microbial populations contributing to biotransformation and biodegradation processes in different environments [6, 18]. Microorganisms able to degrade oil are considered ubiquitous [1, 7]; however, degrading community compositions may vary depending on the environmental conditions from where they have been isolated.

Culture-independent approaches have been successfully applied to study the composition of naturally occurring populations contributing to the biodegradation processes in different environments [14, 19, 34]. However, traditional cultivation methods are still being extensively used to isolate the bacteria that naturally inhabit contaminated sites that are involved in the degradation of petroleum hydrocarbons [22, 24]. Bacterial communities in contaminated soils tend to be dominated by the strains that can survive toxicity and are able to utilize the contaminant for growth. Moreover,

they have to be adapted to the organic matter content, nutrient supplies, temperature, pH, oxygen, and/or water availability of this soil. For these reasons, the naturally occurring *in situ* microflora is often used for the degradation of oil hydrocarbons in a specific site [44]. Identifying guilds of organisms that respond to the environmental conditions and how they can be used in bioaugmentation remain a challenge.

In 2004, an accidental spill occurred in a terrestrial oil field located in the northeast of Brazil. The soil was contaminated with a mixture of crude oil and production water. This salty water potentialized the oil contamination problem. Considering that biodegradation using microorganisms is an efficient process for the decontamination of oil-polluted environments as stated before, bioprospection for bacterial strains capable of degrading oil and tolerant of high salt concentrations in a contaminated site is of great interest. Therefore, the aims of our study were (i) to study the bacterial community structure in the oil-field-contaminated area compared with that of bulk soil (noncontaminated site) using molecular methods and (ii) to isolate bacterial strains able to degrade crude oil in the presence of salty production water. The determination of the oil (and different hydrocarbon compounds) degradation ability of these strains can help to predict the bioremediation potential of the impacted soil.

MATERIALS AND METHODS

Experimental Conditions, Soil, and Production Water

The experimental area was located at an important terrestrial petroleum extraction site in the northeast of Brazil. The soil was classified as clay-sandy (sand 66%, silt 12%, and clay 29%). Physical and chemical characteristics of the soil are presented in Table 1. In 2004, an accidental oil spill occurred in an area on sloping land and the oil moved away from the contamination origin. The crude oil was spilled together with production water (composition shown in Table 1). The vegetation present in this area was destroyed with the spill. Eight months after the accident, three soil samplings (0–10 cm deep) were collected from three different sites: (P1) the origin of contamination – highest site, (P2) in the middle of the sloping area, and (P3) the lowest site (where contaminants accumulated). Bulk soil (noncontaminated soil) sample was also collected. The 12 soil samples were kept at –20°C until DNA extraction.

Soil TPH Analysis

The biodegradation potential of the three different oil-contaminated sites was examined by gas chromatography, quantifying the remaining TPH (total petroleum hydrocarbons) using a modified EPA 8015 technique [13].

DNA Extraction from Oil-contaminated and Bulk Soils

DNA was extracted from oil-contaminated and bulk soil samples (0.5 g of each) using the Fast DNA Spin Kit for soil (Qbiogene, BIO 101 Systems, U.S.A.) and according to the manufacturer's instructions. The DNAs extracted were also visualized on 0.8% (w/v) agarose gels to assess their integrity and then stored at 4°C prior to their use in PCR reactions.

Table 1. Physical and chemical characteristics of soil and production water.

	Soil sample (0–20 cm)	Production water
pH	4.6	7.2
Salinity	–	54,230 mg/l
Ba	–	70.7 mg/l
Fe	312 mg/kg	4.6 mg/l
Ca^{2+}	2.7 $Cmol_c/dm^3$	1,416 mg/l
Mg^{2+}	1.2 $Cmol_c/dm^3$	656.1 mg/l
Na	0.17 $Cmol_c/dm^3$	23,768 mg/l
Cl	–	32,867 mg/l
K	0.22 $Cmol_c/dm^3$	422.4 mg/l
Al^{3+}	0.3 $Cmol_c/dm^3$	3.7 mg/l
Mn	3.8 mg/kg	0.6 mg/l
Ni	–	0.04 mg/l
Cd, Pb, Ag, Zn	–	<0.01 mg/l
Organic carbon	2.53%	–

Production water and soil samples were characterized as described in standard protocols and in the manual of methods of EMBRAPA [12]. (–), Not determined.

PCR Amplification of Bacterial 16S rRNA Genes and Denaturing Gradient Gel Electrophoresis (DGGE)

Fragments of 16S rDNA (correspondent to V6-V8 region of *E. coli* 16S rRNA gene) were amplified using the PCR primers U968F-GC1 and 1401R [28]. The 50-µl reaction mix contained 1 µl of template DNA (corresponding to approximately 15 ng), 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 2 mM of each dNTPs, 1.25 U of *Taq* DNA polymerase (Promega, WI, U.S.A.), and 0.2 µM of each primer. The amplification conditions were those described by Nübel *et al.* [28]. Negative controls (without DNA) were run in all amplifications. The PCR products were visualized by electrophoresis on 1.4% agarose gels stained with ethidium bromide (2 µg/ml). Amplicons were stored at –20°C until DGGE analysis.

DGGE of the 16S rRNA PCR products was carried out using a DCode Universal Mutation Detection System (Bio-Rad, CA, U.S.A.) apparatus. PCR amplicons were applied directly onto a 6% (w/v) polyacrylamide gel in 1× TAE buffer (40 mM Tris-acetate [pH 7.4], 20 mM sodium acetate, 1 mM disodium EDTA) containing a denaturing gradient of urea and formamide varying from 40% to 70%. The DGGE was run for 16 h at 60°C and 75 V and then stained with SYBER-Green I (Invitrogen-Molecular Probes, SP, Brazil) for 40 min. Stained gel was visualized under UV light and recorded using ImaGo Image Analysis System (B&L Systems, The Netherlands). DGGE fingerprintings were analyzed using the Image Quant software, and the clustering algorithm of Ward was used to calculate the dendrogram of the DGGE gel using the software package Statistica (ver. 5.1, StatSoft).

Isolation of Halotolerant Hydrocarbon-degrading Bacteria

Pre-enrichment cultures were performed by adding 5 g of contaminated soil from sites P1, P2, and P3 in flasks containing 45 ml of Bushnell-Haas medium (BH – Difco) prepared with production water and supplemented with 0.1% of crude oil from the local oil field. After

7 days of incubation at 28°C, 1 ml of each pre-enriched culture was transferred to a new flask and the same incubation conditions were maintained. Enrichment cultures were then plated onto two different culture media: TSB (Trypticase Soy Broth, Difco) and LB (1% tryptone, 0.5%, yeast extract pH 7.2), both added with 7% NaCl. The plates were incubated at 32°C for 72 h. Isolates (from the three different sites and from the different media) showing different colony morphologies were screened for their ability to degrade crude oil in the presence of production water.

Microplate Assay

Microplates containing 24 wells were filled with 1.8 ml of Bushnell-Haas medium prepared with the production water (instead of distilled water) and 200 µl of the different isolates grown in the same isolation medium (described above) and washed twice with sterile saline (0.8%). A drop (50–100 µl) of crude oil was added to each well, creating a film on the surface of the medium. During the 7-day incubation period at 32°C, oil biodegradation was determined visually by searching for any alteration of the oil film.

Phenotypic Characterization

Isolates that were positive in the oil degradation test in microplate assays were morphologically characterized by Gram staining and by their capability to grow in the presence of salt at different concentrations by using LB medium containing 0.5% to 17% (w/v) NaCl at 32°C.

Amplified Ribosomal DNA (rDNA) Restriction Analysis (ARDRA)

DNA of the selected isolates was extracted using the phenol/chloroform method described by Seldin and Dubnau [35]. The gene encoding 16S rRNA from each isolate was amplified by PCR using the pair of universal primers pA and pH and the conditions described in Massol-Deya *et al.* [25]. Negative controls (without DNA) were run in all amplifications and PCR products were visualized by 0.8% agarose gel electrophoresis in TBE at 80 V for 4 h at room temperature, and stained with ethidium bromide. Samples (10 ml) of the 16S rRNA amplified products were then digested with the endonucleases HinfI, HaeIII, and RsaI (Invitrogen), for 16 h, according to the manufacturer's protocols. Agarose (2%) gel electrophoresis of restricted DNA was performed at 80 V for 4 h at room temperature. The results of ARDRA were collected into a matrix indicating the presence or absence (scored as 1 or 0, respectively) of specific bands in the different restriction analyses. Simple matrixes were obtained by comparing pair of strains using the simple matching coefficient (SM), and the final dendrogram was constructed using the unweighted pair group method with arithmetic mean (UPGMA). For these analyses, the NTSYS software package (version 2.02, Exeter Software, Setauket, NY, U.S.A.) was used.

Cloning and Sequencing of 16S rDNA

Representative strains belonging to the different ARDRA patterns were selected for 16S rDNA sequencing. The PCR products (obtained with the primers pA and pH) were cloned using a pGEM T-easy vector according to the instructions of the manufacturer (Promega). After the transformation into *Escherichia coli* JM109 competent cells, clones were selected and sequenced using M13f and M13r primers by an ABI Prism 3100 automatic sequencer (Applied Biosystems, Foster City, CA, U.S.A.). Sequences were identified using the BLAST-N facility (http://www.ncbi.nlm.nih.gov/blast) of the National Center for Biotechnology Information with the GenBank nonredundant database.

Growth on Different Substrates

Growth of the 15 selected strains (200 µl of saline-washed cells) was determined using 50-ml flasks with 20 ml of Bushnell-Haas medium and containing 7% NaCl. Batch experiments were performed on a rotary shaker at 150 rpm at 30°C for 7 days. Different hydrocarbon compounds (at a concentration of 80 ppm) that included (a) *n*-alkanes such as hexane (C_6), dodecane (C_{12}), and hexadecane (C_{16}); (b) aromatic compounds such as toluene, naphthalene, *o*-xylene, and *p*-xylene; and (c) mixed compounds such as diesel and gasoline were used as the sole carbon source. Cell growth was determined visually as described in Cunha *et al.* [10]. Negative controls consisted of the same medium added with each carbon source but without the cell inoculum.

Detection of the Alkane Hydroxylase Gene (*alkB*)

The presence of *alkB* genes was determined by PCR using either the degenerate pair of primers TS2Sdeg1RE [36] or the primers RHOSE/RHOAS [17]. The amplification conditions were those described in both references. The PCR products were analyzed in 1.2% agarose gels at 80 V for 2 h at room temperature.

Results

Eight months after the accidental spill that occurred in a terrestrial oil field located in the northeast of Brazil, three soil samplings were collected from three different sites, named P1, P2, and P3. As previously stated, the studied area had a slight declivity. Therefore, site P1 corresponds to the origin of contamination, P2 to the middle of the sloping area, and P3 to the area where contaminants accumulated. Soil TPH analyses were used to determine the oil concentration in each of the three sites and in the control area. The results demonstrated that site 3 presented the highest TPH value (285 ppm), followed by sites 2 (259 ppm) and 1 (182 ppm). The control area presented a TPH value of 81 ppm.

Analysis of Bacterial DGGE Fingerprints

DNA was recovered and PCR products were obtained from the three sites and bulk soil (noncontaminated soil) samples. Hence, the mixture of oil and production water did not affect the quality of DNA extracted from soil. Community fingerprints were generated for each sampling, and reproducible DGGE profiles of bacterial 16S rDNA were obtained between triplicate bulk and oil-contaminated soil samples (Fig. 1A). Dominant bands in DGGE appeared both in the soils with and without oil contamination. However, different profiles were observed when the contaminated soils were compared with the noncontaminated control. Twenty-one markers (corresponding to the number of different bands) were considered to further cluster the DGGE patterns, according to the Ward algorithm and the Pearson coefficient. The dendrogram obtained is shown in Fig. 1B. Four main clusters were observed; three of which were made up of one oil-contaminated site and one consisted of the noncontaminated site. The DGGE profile from site P1 was closer to bulk soil

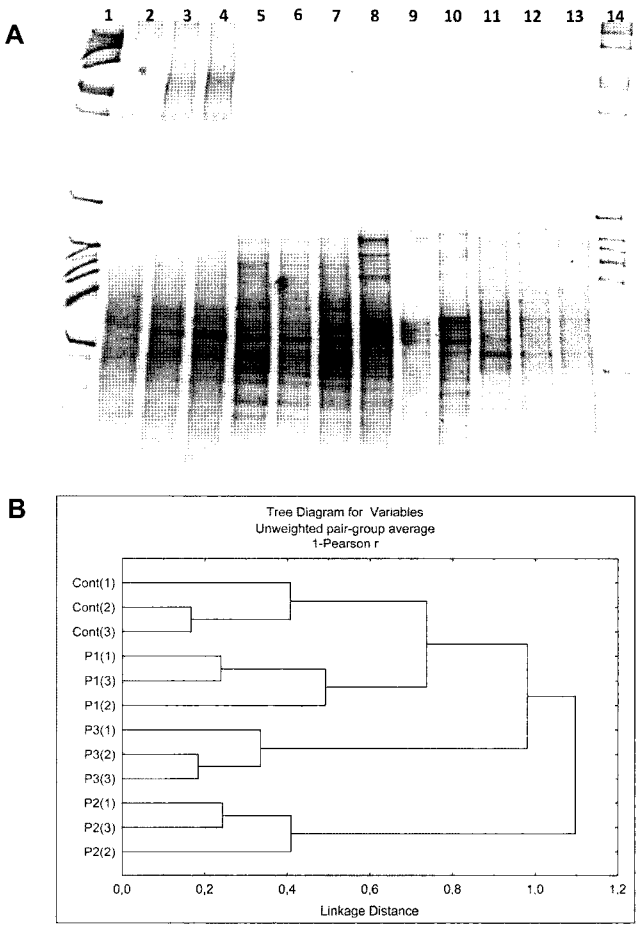

Fig. 1. A. Denaturing gradient gel electrophoresis (DGGE) fingerprints of bacterial 16S rRNA gene fragments amplified from bulk and oil-contaminated soil DNA templates.
Lanes 2, 3, and 4 correspond to three samplings of noncontaminated soil, lanes 5, 6, and 7 to site P1, lanes 8, 9, and 10 to site P2, and lanes 11, 12, and 13 to site P3. Lanes 1 and 14 correspond to 1 kb ladder (Promega). **B.** Dendrogram obtained following cluster analysis with Ward and Pearson correlation coefficient, comparing the bacterial communities in different soil samples.

than the other sites, whereas the cluster corresponding to the site P2 was the most distant. Furthermore, the number of bands observed in P3-originated profile was lower, probably due to the accumulation of crude oil and production water that occurred in this site.

Isolation of Hydrocarbon-degrading Bacterial Strains and Their Phenotypic and Molecular Characterization

From the LB and TSB plates, 131 colonies with different morphologies were selected for testing their ability to degrade hydrocarbons in 24-well plates. From the total number, 46, 43, and 42 were isolated from sites P1, P2, and P3, respectively (data not shown). The isolates were named according to the isolation medium and the site from where they were isolated (*e.g.*, for example: DLB 1.7: medium LB, site P1, strain 7). Among these isolates, 22 were able to degrade crude oil in the presence of production water. Fig. 2 shows an example of alteration of the oil film in a microplate assay by two bacterial strains (DTSB 1.6 and DLB 3.4).

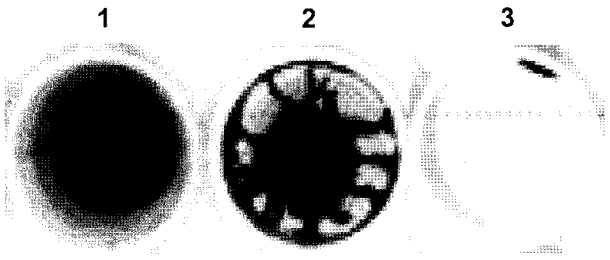

Fig. 2. Microplate assay for testing oil degradation in the presence of production water.
(1) Negative control. Two bacterial strains were used as an example of alteration of the oil film: (2) DTSB 1.6 and (3) DLB 3.4.

PCR products obtained from the 22 selected strains using universal primers for 16S rRNA were digested with HinfI, HaeIII, and RsaI, separately. A dendrogram was generated using the combined data from the different bands observed after digestion with these endonucleases (Fig. 3). At 100% similarity, 15 different 16S rRNA gene-based genotypes were observed. These groups were formed by 1 to 4 strains. Strain DLB1.7 diverged from the others to form a separate cluster at 59% similarity. Strains PLB 1.1, PLB 1.2, PLB 3.1, and PLB 3.4 were also separated from the others at about 59% similarity (Fig. 3).

One representative of each one from the 15 genotypes formed in ARDRA was then characterized by Gram staining and for their growth in different concentrations of NaCl (Table 2). From the 14 Gram-positive and one Gram-negative strains (PLB 3.1), all were able to grow in concentrations of NaCl that varied from 0.5% to 10%. Variable results were observed in LB plus 12% NaCl. Two strains were able to grow in up to 15% NaCl (DTSB 3.4 and DTSB 3.5) whereas none grew at 17% NaCl (Table 2). All these isolates were classified as halotolerant according to Kushner classification [20]. Moreover, the ARDRA representative strains were cultivated in different hydrocarbon sources (Table 2). None of them was able to grow in the presence of either *o*-xylene or *p*-xylene. Only strain DTSB 3.6 was able to grow using hexane, whereas all 15 strains grew in the presence of hexadecane. The only strain able to grow in toluene was DTSB 3.4. Variable results were obtained with dodecane, naphthalene, diesel, and gasoline (Table 2).

The presence of catabolic genes encoding alkane monooxygenase was determined by PCR using either the degenerate pair of primers TS2Sdeg1RE [36] or the primers RHOSE/RHOAS [17]. No PCR product was obtained when the DNA of strains DLB 1.7, DLB 1.9, or DLB 3.22 was used as template. For the remaining strains, both or at least one of the expected PCR products (343 bp for RHOSE/RHOAS and 550 bp for TS2Sdeg1RE) was observed (not shown).

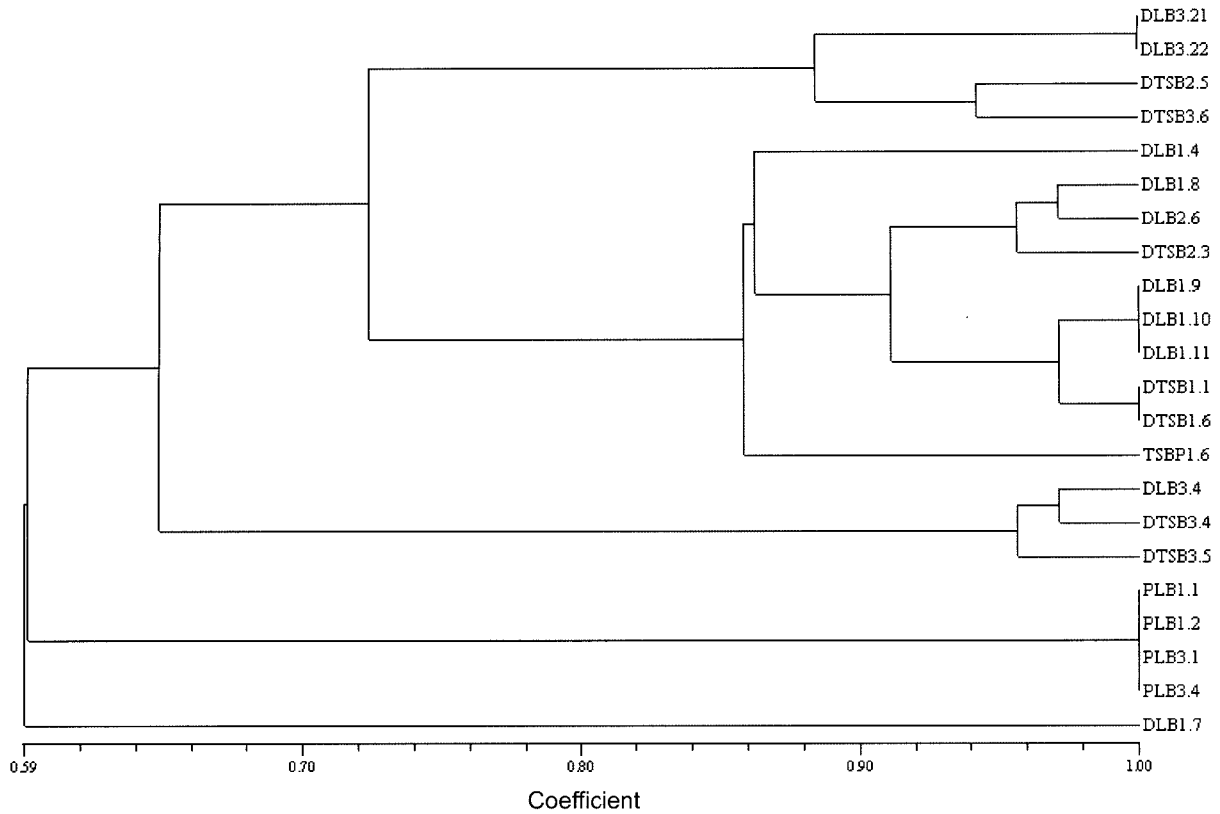

Fig. 3. Dendrogram based on data from ARDRA, followed by cluster analysis using Dice coefficient and UPGMA. Fifteen groups were formed at 100% similarity.

Table 2. Growth of oil-degrading strains using various petroleum derivatives as sole carbon sources, and their capability to grow in LB with different NaCl concentrations.

Bacterial strains (Gram staining)[a]	Petroleum derivatives[b]									Growth in LB with NaCl[c]		
	C6	C12	C16	DI	GA	TO	NA	*o*-XI	*p*-XI	10%	12%	15%
DLB 3.22 (pos)	–	+++	+++	+	–	–	+	–	–	+	W	–
TSBP 1.6 (pos)	–	–	+	+	–	–	–	–	–	+	–	–
DTSB 1.6 (pos)	–	+	++	–	–	–	–	–	–	+	W	–
DTSB 2.3 (pos)	–	+	+++	++	+	–	–	–	–	+	W	–
DTSB 2.5 (pos)	–	++	+++	+	–	–	+	–	–	+	W	–
DTSB 3.4 (pos)	–	+	++	++	–	+	+	–	–	+	+	W
DTSB 3.5 (pos)	–	+	++	++	–	–	–	–	–	+	+	W
DTSB 3.6 (pos)	+	++	++	++	+	–	+	–	–	+	W	–
DLB 1.4 (pos)	–	–	+	+	–	–	+	–	–	+	–	–
DLB 1.7 (pos)	–	–	+	++	–	–	+	–	–	+	W	–
DLB 1.8 (pos)	–	+	++	++	–	–	–	–	–	+	W	–
DLB 1.9 (pos)	–	+	++	++	–	–	+	–	–	+	W	–
DLB 2.6 (pos)	–	++	++	+++	+	–	+	–	–	+	–	–
DLB 3.4 (pos)	–	+	++	+	–	–	–	–	–	+	–	–
PLB 3.1 (neg)	–	–	+	+	–	–	–	–	–	+	–	–

[a]pos, Gram-positive; neg, Gram-negative

[b]C_6, hexane; C_{12}, dodecane; C_{16}, hexadecane; DI, diesel; GA, gasoline; TO, toluene; NA, naphtalene; *o*-XI, *ortho*-xylene; *p*-XI, *para*-xylene; (–) no growth; (+) weak growth; (++) good growth; (+++) very good growth;

[c](+) positive growth, (–) no growth observed, and (W) weak growth after 3 days of incubation at 32°C. All bacterial strains were able to grow in LB with up to 10% NaCl.

Table 3. Identification of the oil-degrading strains based in the 16S rRNA coding gene sequences by NCBI Blast-N.

Strains	NCBI BLAST-First hit	Accession No.	Max. identity	E-value
DTSB 1.6	*Rhodococcus* sp. 871-AN030	AF420420.1	98%	0.0
DTSB 2.3	*Rhodococcus* sp. 871-AN030	AF420420.1	97%	0.0
DTSB 2.5	*Gordonia rubripertincta* DSM43197[T]	X80632.1	99%	0.0
DTSB 3.5	*Rhodococcus* sp. Dasan	EF101927.1	99%	0.0
DTSB 3.6	*Gordonia alkanivorans*	AB065369.1	99%	0.0
DLB 1.4	*Rhodococcus equi*	AY741716.1	99%	0.0
DLB 1.7	*Microbacterium oxydans*	AB365061.1	98%	0.0
DLB 1.8	*Rhodococcus equi*	AY741716.1	99%	0.0
DLB 1.9	*Nocardia veterana* DSM 43557	AF490540.1	97%	0.0
DLB 2.6	*Rhodococcus equi*	AY741716.1	97%	0.0
DLB 3.22	*Gordonia alkanivorans*	AB065369.1	99%	0.0
PLB 3.1	*Stenotrophomonas* sp. EC-S105	AB200253.1	98%	0.0

The DNAs of 12 ARDRA representative strains were used for PCR amplification (primers pA and pH) and partial 16S rDNA sequencing. Phylogenetic analysis showed that the strains had 97–99% similarity to sequences retrieved from databases (Table 3). The majority of the isolates could be identified as Actinobacteria. Sequences from 16S rDNA of six strains were affiliated with the genus *Rhodococcus*, three strains with *Gordonia*, and the two remaining strains with the genera *Microbacterium* and *Nocardia*, respectively. The Gram-negative strain was identified as belonging to the genus *Stenotrophomonas*.

DISCUSSION

In the present study, DGGE fingerprints based on 16S rDNA fragments amplified by PCR from bacterial DNA were combined with traditional cultivation techniques to assess the bioremediation potential of a terrestrial oil field located in the northeast of Brazil. In this area, a spill occurred, contaminating the soil with oil and salty production water.

PCR-DGGE profiles obtained using primers based on the 16S rRNA gene usually yield complex patterns that reflect the composition of the dominant soil microbiota, including the nonculturable fractions [15]. The DGGE patterns obtained in this study with total community DNA from bulk and contaminated soils showed shifts in the composition of the dominant bacterial populations related to oil/production water pollution. The molecular profiling data (Fig. 1A) did not point to a strong selection of any specific bacterial population, although separate clusters made up of oil-contaminated samples (P1, P2, and P3) were formed in the dendrogram (Fig. 1B). The pattern observed in site P3 with fewer bands may reflect the accumulation of the contaminant. In this site, the highest amount of oil was detected in TPH analysis, although the differences between the values are low. Nevertheless, it is important to consider that the spill occurred eight months before the beginning of this study. Previous studies have also shown, through DGGE analysis, that the bacterial community of a soil with previous history of contamination showed significant changes when compared with less polluted soils [11, 14, 18].

Traditional cultivation methods have been previously used to isolate bacteria involved in the degradation of petroleum hydrocarbons, even though only a minority of the microorganisms in soil can generally be cultivated [40]. This includes organisms that are able to degrade oil *in situ* [22]. In this study, 22 strains able to degrade crude oil in the presence of production water were isolated. PCR products obtained from the 22 selected strains using universal primers for 16S rRNA were digested with different endonucleases, and a dendrogram was generated by using the combined data from the different bands observed. At 100% similarity, 15 different genotypes were observed. All selected strains (one representative of each ARDRA group) were able to grow in LB medium containing at least 10% NaCl and were considered as halotolerant strains as they also grew well in LB with 0.5% NaCl. Different reports have already demonstrated that a wide range of organic pollutants is mineralized or transformed by microorganisms able to grow in the presence of salts [29]. The use of these microorganisms could decrease the amount of money spent on remediation using dilution processes, ionic changes, and reverse osmosis often employed before biological treatment to diminish salinity from contaminant-affected areas [23]. Ashok *et al.* [3] isolated different bacterial strains able to degrade polycyclic aromatic hydrocarbons in the presence of 7.5% NaCl, and Nicholson and Fathepure [27] demonstrated the ability of a consortium composed of halotolerant and halophilic bacteria in degrading benzene, toluene, ethyl-benzene, and xylene (BTEX). Therefore, the isolation of halotolerant oil-degrading strains from a tropical soil (in the present study) may be useful in bioremediation processes where salty production water is present. Bento *et al.* [4] compared different bioremediation techniques (natural attenuation, biostimulation,

and bioaugmentation) and proved that bioaugmentation using indigenous bacteria was the most effective and appropriate process in contaminated soil bioremediation.

The fractions of the petroleum containing *n*-alkanes are usually mostly susceptible to biodegradation, whereas saturated fractions containing branched alkanes are less vulnerable to microbial attack. The aromatic fractions are even less easily biodegraded [33]. The results of growth in different hydrocarbon sources by the 15 selected strains have shown that they were able to grow not only using *n*-alkanes but also in the presence of naphthalene, toluene, gasoline, and/or diesel (Table 2). Different bacterial strains naturally inhabiting contaminated sites showing the ability to grow using PAHs have already been isolated and characterized, mainly belonging to the genera *Pseudomonas*, *Mycobacterium*, *Dietzia*, and *Bacillus* [9, 38, 39, 42].

PCR products of the anticipated sizes were generated in 12 of the strains tested using both or at least one of the pairs of primers for the gene encoding alkane monooxygenase [17, 36]. No PCR product was obtained when the DNA of strains DLB 1.7, DLB 1.9, or DLB 3.22 was used as template, although these strains were able to grow in hexane, dodecane, or hexadecane. This fact suggests that the primers and/or PCR conditions used are not ideal for the amplification of *alkB* in those strains or that degradation occurs *via* a metabolic/genetic pathway that is yet to be described.

The results of the phylogenetic analysis obtained here based on 16S rDNA sequences indicated that the contaminated samples showed a low variety distribution of types in the domain bacteria, with the predominance of Actinobacteria. Quatrini *et al.* [32] have already suggested that GC-rich mycolic acid containing Gram-positive actinomycetes of the genera *Rhodococcus*, *Gordonia*, and *Nocardia* might have a key role in bioremediation of *n*-alkane-contaminated sites under dry, resource-limited conditions, as observed in a hydrocarbon-contaminated Mediterranean shoreline. *Rhodoccocus* sp. was prevalent among our isolates, and this genus has been cited in many studies as degrading hydrocarbons, such as gasoline [21], a broad range of *n*-alkanes [5], phenol [31], and toluene [41]. Three other isolates were affiliated with different *Gordonia* species in the present study. The ability of different species of *Gordonia* to degrade or modify aliphatic and aromatic hydrocarbons, benzothiophene and xylene, has already been described [2]. The two other strains identified in this study belong to the genera *Microbacterium* and *Nocardia*. The genus *Microbacterium* was described as a degrader of phenanthrene and *n*-alkanes and considered useful for bioremediation applications [46]. The species *Microbacterium oxydans* has been detected by 16S rDNA DGGE band sequencing in a petroleum-contaminated tropical soil during biostimulation treatment [37], and demonstrated to utilize phenanthrene on leaf surfaces [43]. Therefore, Actinobacteria is a highly interesting group of microorganisms, not only for their degradation capability but mainly for their apparent resistance to the restrictive conditions of some harsh environments [32], including their tolerance to salt [8]. On the other hand, many other studies demonstrate that oil-contaminated sites are predominated by Gram-negative bacteria [22, 34]. In our case, only one halotolerant Gram-negative strain was isolated and its 16S rDNA sequence was related to *Stenotrophomonas* sp. This genus has previously been found in a mixed bacterial consortium responsible for the biodegradation of fuel oil hydrocarbons in sand and loamy soils [16].

In conclusion, our study demonstrated the potential for biodegradation of oil and saline production water by naturally occurring bacteria isolated from a contaminated site. Further studies are necessary to determine the biodegradation efficiency of these strains *in situ* before considering their application as an alternative treatment for soil bioremediation in the Brazilian oil field area studied here.

Acknowledgments

This study was carried out as a part of a research project supported by PETROBRAS. The authors want to thank Claudia D. Cunha for her assistance during the biodegradation tests and Dr. Andrew Macrae for his helpful comments on this manuscript.

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