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Polyphasic Analysis of the Bacterial Community in the Rhizosphere and Roots of *Cyperus rotundus* L. Grown in a Petroleum-Contaminated Soil

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Cyperus rotundus* L. is a perennial herb that was found to be dominating an area in northeast Brazil previously contaminated with petroleum. In order to increase our knowledge of microorganism–plant interactions in phytoremediation, the bacterial community present in the rhizosphere and roots of *C. rotundus* was evaluated by culture-dependent and molecular approaches. PCR–DGGE analysis based on the 16S rRNA gene showed that the bacterial community in bulk soil, rhizosphere, and root samples had a high degree of similarity. A complex population of alkane-utilizing bacteria and a variable nitrogen-fixing population were observed *via* PCR–DGGE analysis of *alkB* and *nifH* genes, respectively. In addition, two clone libraries were generated from *alkB* fragments obtained by PCR of bulk and rhizosphere soil DNA samples. Statistical analyses of these libraries showed that the compositions of their respective populations were different in terms of *alkB* gene sequences. Using culture-dependent techniques, 209 bacterial strains were isolated from the rhizosphere and rhizoplane/roots of *C. rotundus*. Dot-blot analysis showed that 17 strains contained both *alkB* and *nifH* gene sequences. Partial 16S rRNA gene sequencing revealed that these strains are affiliated with the genera *Bosea*, *Cupriavidus*, *Enterobacter*, *Gordonia*, *Mycoplana*, *Pandoraea*, *Pseudomonas*, *Rhizobium*, and *Rhodococcus*. These isolates can be considered to have great potential for the phytoremediation of soil with *C. rotundus* in this tropical soil area.

Keywords: Petroleum, phytoremediation, *Cyperus rotundus*, nitrogen fixation, alkane degradation

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Petroleum is the major energy resource consumed throughout the industrial world [18]. As with any large scale-industrial process, petroleum production can lead to contamination of soil and groundwater [48], and therefore necessitating remediation of oil pollutants. Over the last few years, the use of plants for environmental cleanup has gained scientific acceptance and popularity among governmental agencies and the industry [28]. This technology, known as phytoremediation, makes use of the naturally occurring processes by which plants and rhizosphere microbial flora degrade and sequester organic and inorganic pollutants [47].

One benefit of phytoremediation is that a unique plant can remediate a contaminated environment in several ways. Low-molecular-weight compounds can be removed from the soil and released through leaves *via* evapotranspiration processes (phytovolatilization), whereas some of the nonvolatile compounds can be degraded or rendered nontoxic *via* enzymatic modification and sequestration within the plant (phytodegradation, phytoextraction). Other compounds are stable in the plant and can be removed along with the biomass by sequestration or incineration [47]. However, the main contribution of the plant to the remediation of petroleum hydrocarbons is through the rhizoremediation process, which is based on its interaction with the microbes associated with its rhizosphere [10, 22]. Complex interactions involving roots, root exudates, rhizosphere soil, and microbes result in accelerated degradation of toxic organic compounds to nontoxic (or at least less toxic) forms.

Sugars, organic acids, and larger organic compounds are released by plants to the environment, stimulating the growth of surrounding microorganisms. Bacteria found in the rhizosphere can positively influence plant growth and health by a variety of mechanisms, such as increasing the bioavailability of nitrogen (through nitrogen fixation); repressing soilborne pathogens (by the production of hydrogen

cyanide, siderophores, antibiotics, and/or competition for nutrients); improving plant tolerance to drought stress, high salinity, and metal toxicity; and producing phytohormones and solubilizing inorganic phosphate [10, 13]. The bacteria responsible for one or more of these characteristics are known as plant growth-promoting rhizobacteria (PGPR) [16]. Moreover, in polluted sites, plant exudates can stimulate specific bacterial populations such as contaminant-degrading microorganisms, thereby reducing the toxic effects of the pollutants on the plants. For a phytoremediation process to be successful, both plants and microbes must survive and grow in contaminated sites [25, 28, 48].

In Carmópolis, located in northeast Brazil, a plant identified as *Cyperus rotundus* L. is capable of growing in an area of soil previously contaminated with crude oil. This plant species, also known as coco-grass, purple nut sedge, or red nut sedge, is a cosmopolitan weedy sedge usually found growing in all soil types, from sea level to high mountains, in cultivated fields, grasslands, roadsides, neglected areas, at the edges of woods, and in sandy or gravelly shores [11]. Once established, this plant is very persistent and can withstand prolonged drought or inundation [24]. The antibacterial, antioxidant, cytotoxic, and apoptotic activities of tuber extracts of *C. rotundus* have been observed *in vitro* [14]. In addition, previous studies have demonstrated the involvement of *C. rotundus* in phytoremediation of herbicides and heavy metals [4, 29].

In the present study, we hypothesized that there is a bacterial community present in the rhizosphere and/or rhizoplane/root interior of *C. rotundus* grown in oil-contaminated soil that supports or promotes plant growth by reducing the toxic effects of the crude oil and/or acting as PGPR. Therefore, the aims of our work were (i) to study the bacterial community present in the rhizosphere soil and roots of *C. rotundus* using molecular methods based on polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis (DGGE) and clone libraries, and (ii) to isolate bacterial strains capable of both degrading crude oil and potentially fixing atmospheric nitrogen. Such strains would be considered potentially valuable for the phytoremediation process in this tropical area.

Materials and Methods

Site Description and Sampling

The experimental area was located at a large on-shore petroleum exploration and production site in Carmópolis, in northeast Brazil. The soil had a very clay texture (37% sand, 5% silt, and 58% clay) with a slightly acidic pH (6.0) and no history of previous oil contamination. Chemical characteristics of the soil can be summarized as follows: organic carbon, 0.58%; N and P content, 1.16 and 0.34 g/kg soil, respectively; Ca^{2+}, 0.52 g/kg; Mg^{2+}, 0.47 g/kg; and Fe, 21.8 g/kg of soil. The study site (4 soil plots, 3 m×3 m each) was constructed to analyze the bacterial community during the *in situ* bioremediation of oil and/or produced water-contaminated soil for a 2-year period by members of PETROBRAS, the Brazilian oil company. One of the plots was contaminated three times with a total of 40 l of crude oil over a 14-month period. At the end of the experiment (when the amount of the total petroleum hydrocarbon, or TPH, was decreased to 42% of the initial value; data not shown), this plot was found to be naturally colonized by one kind of plant, later identified as *Cyperus rotundus* L. To study the bacterial community associated with the rhizosphere and roots of this species, nine plants with no significant variation in height (about 15 cm) were harvested and the roots were shaken to remove loosely attached soil. To minimize the number of replicates, the plants were grouped into three samples (A, B, and C, each composed of three plants). The soil adhering to the roots of each group of plants was pooled and considered to be the rhizosphere soil. Roots (1 g from A, B, and C) were mixed with 9 ml of distilled water and shaken for 10 min. Thereafter, the water was discarded and another 9 ml of water was added to the roots. This procedure was repeated three times. The washed roots were then chopped and were considered to represent the rhizoplane plus root interior. In addition, samples of bulk soil (not associated with roots) were also collected from the same plot. The samples were kept at −20°C until DNA extraction was performed, or at room temperature for the isolation of bacterial strains.

Isolation and Maintenance of Bacterial Strains Associated with *C. rotundus*

Pre-enrichment cultures were obtained by adding 1 g of rhizosphere soil or washed root/rhizoplane from each combined sample in flasks containing 10 ml of Bushnell–Haas medium (BH; Difco) supplemented with 0.1% crude oil obtained 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 with 10 ml of BH and incubated under the same conditions. Enrichment cultures were then plated onto two different culture media: LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.2% agar, pH 7.2) and thiamine–biotin–nitrogen (TBN) agar [36]. LB plates were incubated at 32°C for 48 h and TBN plates were incubated for 5 days at 28°C in Gaspak jars with an atmosphere of 80% N_2, 10% CO_2, and 10% H_2 to facilitate isolation of nitrogen-fixing strains. Colonies presenting different morphologic characteristics were selected for further purification under aerobic conditions. Thereafter, bacterial cultures were stored at −80°C in LB medium containing 20% glycerol.

Microplate Assay

Isolates from rhizosphere soil and washed root/rhizoplane obtained from both solid media were screened for their ability to degrade crude oil. The isolates grown in LB medium (described above) were washed twice with sterile saline (0.85%). Twenty-four-well microplates were filled with 1.8 ml per well of BH and 200 µl of each isolate. A drop (approx. 50 µl) of crude oil was added to each well, forming an oil film on the surface of the medium, and plates were incubated for 7 days at 32°C. Oil biodegradation was determined visually during incubation by observing alterations in the appearance of the oil film.

DNA Extraction

DNA was extracted directly from bulk and rhizosphere soils and from root samples (0.5 g of each) using a FastPrep Spin Kit for soil DNA (BIO 101 Systems, CA, U.S.A.) as described by the manufacturer. Genomic DNA was extracted from all bacterial strains using the

protocol described in Pitcher *et al*. [30]. DNA preparations were separated by electrophoresis on an 0.8% agarose gel in 1× TBE buffer [35] and visualized to assess their integrity, and then stored at 4°C prior to PCR amplification.

PCR Amplification for DGGE Analysis

Part of the 16S rRNA gene was PCR-amplified from DNA from bulk and rhizosphere soils and root samples using the primers U968F-GC (5'- GC clamp+G AAC GCG AAG AAC CTT AC-3') and L1401R (5'-CGG TGT GTA CAA GAC CC-3') described by Nübel *et al*. [23]. The *alkB* sequences in bulk and rhizosphere soils and root samples were amplified using the primers alkH1FGC2 (5'-GC clamp+CIGIICACGAIITIGGICACAAGAAGG 3') and alkH3R (5'-IGCITGITGATCIIIGTGICGCTGIAG-3') [5]. The 50-ml reaction mix contained 1 µl of template DNA, 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 5 µl of 5× PCR buffer (100 mM Tris-HCl, pH 9.0, and 500 mM KCl), 0.5 µM each primer (U968F-GC/L1401R or alkH1FGC2/alkH3R), and 2.5 U of *Taq* DNA polymerase. The amplification conditions were an initial denaturation for 5 min at 96°C, 30 cycles of 1 min at 94°C, 1 min at 48°C for 16S rRNA or 55°C for *alkB,* and 1 min at 72°C, and then a final extension for 3 min at 72°C.

The *nifH* sequences were amplified using nested PCR as described by Demba Diallo *et al*. [7]. The first PCR was performed with the forward primer FGPH19 (5'-TACGGCAARGGTGGNATHG-3'; [38]) and the reverse primer PolR (5'-ATSGCCATCATYTCRCCGGA-3'; [31]). The second PCR was carried out using 1 µl of the first PCR product as the template, the forward primer PolF (5'-GC clamp+ TGCGAYCCSAARGCBGACTC-3') and the reverse primer AQER (5'-GACGATGTAGATYTCCTG-3') [31]. The 50-µl reaction mix contained the same general components in the same concentrations as for 16S rRNA and *alkB* PCR. The amplification conditions were 30 cycles of 1 min at 94°C, 1 min at 55°C for the first PCR or 50°C for the second PCR, and 2 min at 72°C, and then a final extension at 72°C for 5 min.

Negative controls (without DNA) were included in all amplifications. PCR products were separated by electrophoresis on a 1.4% agarose gel, and then stained with ethidium bromide to confirm their sizes. Amplicons were stored at −20°C until DGGE analysis.

Preparation of *alkB* and *nifH* Probes

Probes containing part of the *alkB* and *nifH* sequences of strains Arh10 (isolated in this study) and *Paenibacillus durus* P3L5^T (from our laboratory culture collection), respectively, were generated by PCR. The primers used for *alkB* amplification were those described by Chenier *et al*. [5]. The sequence of the *alkB* PCR product from Arh10 was determined and found to be closely affiliated with the gene encoding the enzyme alkane monooxygenase in *Rhodococcus* sp. (data not shown).

For *nifH* PCR, the primers PolF and PolR were used [31]. The amplification conditions were the same as described above. The PCR products thus obtained were digested with *Eco*RI and then labeled with digoxigenin-11-dUTP (DIG) using a DIG DNA Labeling and Detection Kit (Roche Diagnostics, São Paulo, Brazil) following the manufacturer's protocol.

Dot-Blotting and Hybridization Conditions

Genomic DNA from isolates showing an alteration in the oil film in the microplate assay was transferred onto positively charged nylon membranes as previously described [46]. Prehybridization and hybridization using DIG-labeled probes were performed as described in the manual from the DIG Nucleic Acid Detection Kit (Roche). Hybridization was performed at 65°C for 16 h, and then the nylon membrane was subjected to stringent washing steps. Signals were detected using a CSPD-based chemiluminescence detection kit (Roche) as recommended by the manufacturer.

PCR Amplification of the *nifH* Gene

The presence of the *nifH* gene in the genomes of isolates showing a positive result in the dot-blot analysis was confirmed by PCR amplification with the two sets of *nifH* primers described by Poly *et al*. [31] and Demba Diallo *et al*. [7] using the conditions described above.

DGGE Analysis

All DGGE assays were carried out using a Bio-Rad DCode Universal Mutation Detection System (Bio-Rad Laboratories, Munich, Germany). PCR products (approximately 150 ng) were applied directly onto 8% (w/v) polyacrylamide gels 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 60%. The gels were run for 16 h at 60°C and 65 V. After electrophoresis, the gels were stained for 30 min with SYBR Green I (Invitrogen, CA, U.S.A.) and photographed under UV light using a STORM apparatus (Amersham Pharmacia Biotech, Munich, Germany). DGGE profiles were analyzed with GelCompar II 4.06 (Applied Maths, Kortrijk, Belgium). The Pearson correlation coefficient for each pair of lanes within each gel was calculated as a measure of the similarity between profiles as described previously [6]. A binary matrix based on the presence (1) or absence (0) of individual bands of DGGE fingerprints was constructed independently for each gel. Hierarchical cluster analysis was performed using the Pearson coefficient of similarity and the unweighted pair group method with arithmetic mean (UPGMA) in GelCompar. In addition, Shannon H diversity [19] and evenness [27] indices were calculated for each DGGE profile and compared using a two-sided *t*-test.

Cloning and Sequencing of *nifH* DGGE Bands, 16S rRNA Gene Fragments from Bacterial Isolates and *alkB* Gene Fragments from Bulk and Rhizosphere Soils

Selected bands (marked in Fig. 1C) were retrieved from the DGGE gel, reamplified, and purified with a Wizard Rapid PCR Purification System (Promega, Madison, WI, U.S.A.). After purification, DNA fragments were cloned into the pGEM-T Easy Vector (Promega) according to the manufacturer's instructions. After transformation of competent *Escherichia coli* JM109 cells, clones were picked and the presence of inserts of the correct size was assessed by PCR using the primers M13f (5'-TAAAACGACGGCCAG-3') and M13r (5'-CAGGAAACAGCTATGAC-3') following the recommendations in the pGEM-T Easy Vector manual. Clones were sequenced using these primers on an ABI Prism 3100 automated sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

The 16S rRNA gene sequence in bacterial isolates was PCR-amplified using the primers U968F (without GC clamp) and L1401R. Fragments were purified using the Wizard system and sequenced using the primer U968F as described above. PCR-amplified *alkB* fragments (without clamp) were obtained from each bulk and rhizosphere soil sample. Amplicons originating from the same sample type were pooled, purified using the Wizard system, and

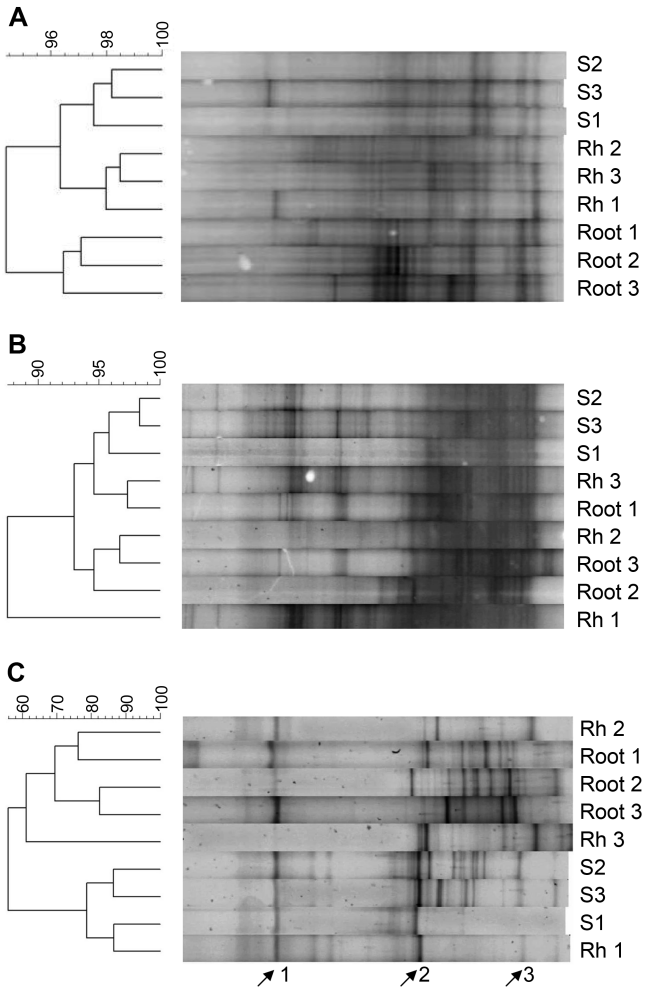

Fig. 1. Denaturing gradient gel electrophoresis (DGGE) fingerprints of (**A**) bacterial 16S rRNA, (**B**) *alkB*, and (**C**) *nifH* gene fragments amplified from bulk (S) and rhizosphere soils (Rh), and rhizoplane/root interior (Root) of *C. rotundus* DNA templates, and their respective dendrograms.
Arrows indicate bands that were excised from gels prior to cloning and sequence analysis.

cloned as described above to generate two libraries. Cloned fragments of the expected size were sequenced using the primers M13f and M13r as described above. The *alkB* sequences generated in this study have been deposited in the National Center for Biotechnology Information (NCBI) GenBank under the accession numbers GU585527–GU585548.

Phylogenetic Analysis

All sequences were identified using the NCBI BLASTN tool (http://www.ncbi.nlm.nih.gov/blast) against the GenBank nonredundant database. The *alkB* sequences of closely related strains were recovered from GenBank and then aligned with the sequences obtained in this study using the software package Clustal X [41]. BioEdit v. 7.0.0 (http://www.mbio.ncsu.edu/Bioedit/bioedit.html) was used for manual editing of sequences. A phylogenetic tree was constructed based on partial *alkB* sequences by the neighbor-joining (NJ) method using MEGA 4.1 software [17]. In addition, the sequences were used to test whether the two libraries were different using the Web-based LIBSHUFF program (version 0.96; http://libshuff.mib.uga.edu) [39].

Table 1. Statistical analyses of DGGE fingerprints.

	Shannon's diversity index (H)	Equitability (Evenness-E_H)
rRNA 16S		
Soil	2.59±0.01	0.91±0.03
Rhizosphere	2.74±0.22	0.89±0.03
Root	2.73±0.12	0.94±0.01
alkB		
Soil	2.78±0.26	0.71±0.07
Rhizosphere	2.69±0.42	0.66±0.17
Root	2.88±0.37	0.70±0.08
nifH		
Soil	2.78±0.30	0.78±0.07
Rhizosphere	2.67±0.14	0.77±0.04
Root	2.86±0.22	0.79±0.07

Results

PCR–DGGE Analysis of the Bacterial Community Associated with *Cyperus rotundus*

DNA was recovered from all samples of rhizosphere, rhizoplane/root interior (A, B, and C, each composed of three plants), and bulk soil evaluated in this study. When these DNA preparations were used as templates for PCR amplification with primers based on 16S rRNA, *alkB*, and *nifH* sequences, fragments of the expected sizes (470, 550, and 320 bp, respectively) were obtained in all reactions.

Total bacterial DGGE profiles were highly similar across replicates (Fig. 1A). These profiles were used to construct a dendrogram *via* hierarchical cluster analysis. The profiles from bulk soil (S1, S2, and S3) and rhizosphere (Rh1, Rh2, and Rh3) samples were more similar one another other than to those from root samples, although the three profiles showed a high degree of similarity, separating only at 94%. Moreover, comparison of the Shannon's diversity index and equitability (evenness) using the *t*-test among soil, rhizosphere, and root samples did not show a statistically significant difference (Table 1).

For alkane-utilizing bacterial populations (Fig. 1B), triplicates of bulk and rhizosphere soil and root samples of *C. rotundus* also showed reproducible patterns, although slight differences could be observed. At 96% similarity, five groups could be observed in the dendrogram: one formed by soil samples, two formed by a single rhizosphere and root sample each, and two formed by a mixture of rhizosphere soil and rhizoplane/interior of root samples. However, comparison of the Shannon's diversity index and evenness using the *t*-test among soil, rhizosphere, and root

samples showed that they were not statistically different (Table 1), suggesting that the diversity of *alkB*-containing bacteria was similar within intergroup profiles.

The profiles of nitrogen-fixing bacteria revealed considerable within-replicate variation (Fig. 1C). In the UPGMA-assisted cluster analysis, triplicates of bulk and Rh1 soils grouped together and separated from the remaining rhizosphere soil and root samples at 55% similarity. In contrast, the diversity and evenness values based on the analysis of *nifH* sequences were similar between bulk soil, rhizosphere, and root samples (Table 1). Three bands (indicated by arrows in Fig 1C) were sequenced; two of these bands occurred in almost all replicates, whereas the third band appeared simultaneously in bulk and rhizosphere soils. BLASTN analysis showed that the sequences of bands 1 and 2 were closely related to that of *nifH* of *Bradyrhizobium* sp. ORS278 (89% identity in both sequences; GenBank Accession No. GU585549 and GU585550, respectively). The third sequence (band 3; GenBank Accession No. GU585551) was affiliated with the *Burkholderia ferrariae* FeGI01 *nifH* gene (91% identity).

Analysis of *alkB*-based Clone Libraries Obtained from Bulk and Rhizosphere Soils

To extend our knowledge of the complex community observed *via* DGGE analysis, two libraries were constructed using *alkB* PCR products from bulk soil and rhizosphere samples of *C. rotundus*. A total of 19 and 18 clones were obtained from the two types of samples, respectively. BLASTX analysis revealed that all of the sequenced clones were closely affiliated with genes encoding the enzyme alkane monooxygenase (AlkB), a result that confirms the specificity of the primers used. The results of the phylogenetic analysis based on *alkB* sequences performed herein (Fig. 2) indicate that, in both the bulk soil and rhizosphere, a wide distribution of types in the domain Bacteria was present: a predominance of Actinobacteria (77% and 89% of the clones obtained from rhizosphere and bulk soil samples, respectively), Alphaproteobacteria (5% of the clones obtained from both types of samples), and *alkB* sequences of uncultured bacteria (16% and 5% of the clones obtained from rhizosphere and bulk soil samples, respectively). Moreover, BLASTN analysis showed a predominance of genes closely related to *alkB* from *Mycobacterium* species in both populations. When these two libraries were further compared using LIBSHUFF, the population compositions they represent were found to be significantly different in terms of *alkB* sequences, with a confidence of 95% (YX comparison had a *p*-value of 0.001).

Analysis of Bacterial Community Based on Culture-Dependent Methods

Pre-enrichment cultures of bacteria from rhizosphere soil and washed roots from each composite sample were prepared in BH supplemented with 0.1% crude oil, and then plated

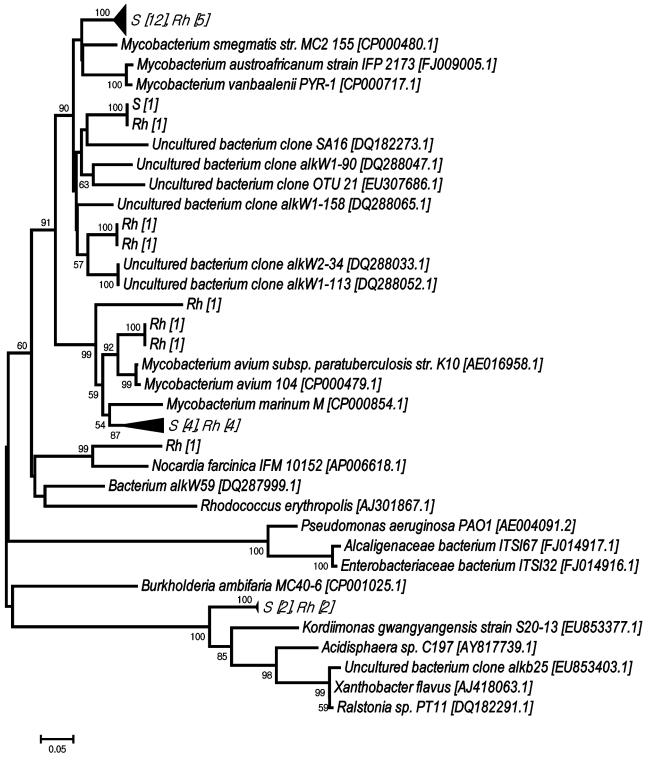

Fig. 2. Phylogenetic tree showing the affiliation of *alkB* clone sequences obtained from bulk and rhizosphere soil samples (two clone libraries) to selected reference sequences.
The tree was constructed based on the neighbor-joining method. Bootstrap analyses were performed with 1,000 repetitions and only values higher than 50% are shown. The scale bar represents the estimated sequence divergence. Numbers in brackets after the letters S (bulk soil) and Rh (rhizosphere soil) correspond to the number of clones analyzed.

onto LB or TBN agar. A total of 209 colonies presenting a variety of morphologies were selected to test their ability to degrade hydrocarbons in 24-well plates. Of the colonies tested, 102 and 107 were isolated from the rhizosphere and rhizoplane/root interior, respectively, and 151 and 58 were isolated from LB and TBN plates, respectively (data not shown). Among these isolates, 79 were able to disturb the oil film formed in each corresponding well.

Dot-blot hybridization with a DIG-labeled probe (*alkB* PCR product of strain Arh10) was performed to detect the presence of *alkB* homologous sequences in the genomes of the 79 isolates capable of degrading crude oil in the plate assay. Among these isolates, 35 strongly hybridized with the *alkB* probe, whereas 21 generated weak signals. No hybridization was detected with the DNA from *P. durus* P3L5^{T}, *E. coli* JM109, and *Desulfovibrio alaskensis* A11 used as negative controls (data not shown). Additionally, to select bacterial strains able to both degrade crude oil and fix atmospheric nitrogen, dot-blot hybridization of the DNA of the 79 isolates with a DIG-labeled probe (*nifH* PCR product of the nitrogen-fixing strain P3L5 ^{T}) was also

Table 2. Identification of bacterial isolates by NCBI BLASTN (first hit).

Isolate[a]	Closest match	Identity (%)	Phylogenetic position[b]	GenBank Accession No
Arh 1	*Enterobacter* sp. NR_024641.1	98	γ; Enterobacteriales	GU585553
Aro 50	*Pseudomonas* sp. NR_024734.1	98	γ; Pseudomonadales	GU585552
Arh 50	*Mycoplana* sp. NR_025831.1	98	α; Rhizobiales	GU585566
Aro 1	*Pandoraea* sp. EU022593.1	99	β; Burkholderiales	GU594320
Arh 10	*Rhodococcus* sp. NR_026524.1	98	Actinomycetales	GU585554
Bro 5	*Pseudomonas* sp. FJ493142.1	99	γ; Pseudomonadales	GU585562
Bro 2		99		GU585563
Bro 1		99		GU585564
Bro 10		99		GU585565
Bro 3	*Pseudomonas* sp. DQ306309.1	99	γ; Pseudomonadales	GU594319
Brh 51	*Cupriavidus* sp. EF189112.1	98	β; Burkholderiales	GU585555
Brh 50	*Gordonia* sp. FJ459995.1	98	Actinomycetales	GU585556
Cro 47				GU585557
Cro 21	*Rhizobium* sp. AM691584.1	99	α; Rhizobiales	GU585560
Cro 49				GU585561
Cro 46	*Bosea* sp. AF273081.1	99	α; Rhizobiales	GU585558
Cro 48				GU585559

[a]The isolates were named according to the samples (A, B, or C, followed by ro for root and rh for rhizosphere samples; for example: Arh 1 is sample A, rhizosphere soil, strain number 1).
[b]α, Alphaproteobacteria; β, Betaproteobacteria; γ, Gammaproteobacteria.

performed to detect the presence of *nifH* homologous sequences. Seventeen isolates tested positive for the presence of both *alkB* and *nifH* genes (data not shown). To confirm the presence of the *nifH* gene in the genomes of these isolates, their DNA was further subjected to PCR amplification using two sets of *nifH*-based primers (FGPH19/PolR-PolF/AQER and PolR/PolF). Fragments of the expected size were obtained in at least one of the amplification reactions using all strains tested (data not shown).

To aid in the phylogenetic placement of *alkB* (+) and *nifH* (+) strains, fragments corresponding to a part of the 16S rRNA gene from these 17 isolates were sequenced. BLASTN analysis revealed that these isolates are affiliated with the genera *Bosea*, *Cupriavidus*, *Enterobacter*, *Gordonia*, *Mycoplana*, *Pandoraea*, *Pseudomonas*, *Rhizobium*, and *Rhodococcus*, with identity values varying from 98% to 99% (Table 2).

Discussion

Some plants stimulate pollutant-degrading microorganisms in their rhizospheres by releasing carbon sources throughout the roots (rhizosphere effect), thus increasing the microbial density and enhancing microbial catabolism [22, 37]. In the present study, the total bacterial community associated with *Cyperus rotundus* grown in a petroleum-contaminated site in northeast Brazil was analyzed by 16S rRNA gene-based PCR–DGGE. Although the bacterial populations in the soil, rhizosphere, and rhizoplane/root interior of *C. rotundus* were grouped separately (Fig. 1A), they shared a high degree of similarity (94%) as determined by hierarchical cluster analysis. Therefore, the selection of a specific bacterial community associated with the rhizosphere/root of *C. rotundus* was not observed. This result was corroborated by the statistical analyses presented in Table 1. In the same way, Siciliano *et al*. [37] could not demonstrate any changes in bacterial diversity using a 16S rRNA gene-based approach, although an increase in the catabolic potential of rhizosphere soil associated with tall fescue and rose clover was detected. However, the authors were able to demonstrate that the change in bacterial composition was linked to specific functional genotypes with relevance to petroleum hydrocarbon degradation.

When analyzing the hydrocarbon-utilizing bacterial populations associated with *C. rotundus* by *alkB* PCR–DGGE, complex profiles were observed in all of the sample types studied (Fig. 1B). The results of the clustering analysis suggest that *alkB*-containing populations present in the rhizosphere and rhizoplane/root interior of *C. rotundus* were different from those present in the bulk soil. Some studies have demonstrated that alkane-utilizing bacteria or *alkB* genotypes are present in high levels in the rhizosphere when compared with bulk soil [15, 25, 37]. Although our study could not demonstrate differences in the diversity of *alkB* sequences between sample types based on the Shannon index (Table 1), hierarchical cluster analysis showed the dissimilarity of *alkB* sequences between the rhizosphere plus rhizoplane/root interior and bulk soil profiles.

Considering that changes in the composition (linked to specific functional genotypes) of bacterial communities associated with plants that promote petroleum hydrocarbon

degradation are known to occur [25, 37], two clone libraries were constructed (with *alkB* PCR products from bulk and rhizosphere soils) to learn more about the *alkB*-containing bacterial population associated with the rhizosphere of *C. rotundus*. When considering the phylogenetic position of the 37 clones, a high prevalence of clones related to *alkB* from Actinobacteria was observed in both the rhizosphere and bulk soils (Fig. 2). Other studies have highlighted the ability of Actinobacteria to degrade a wide range of short- to long-chain alkanes [43, 44]. In addition, Gram-positive GC-rich bacteria are considered to be K-strategists, being generally adapted to resource-limited conditions. In contrast, most Gram-negative alkane-utilizing bacteria, such as *Pseudomonas*, are r-strategists and are able to grow using only short- and medium-chain alkanes [45]. We hypothesize that after the soil in Carmópolis was contaminated with oil, the most easily degradable short- and medium-chain alkanes might have been used as a C source by r-strategist bacteria, whereas the heaviest portion of petroleum remaining might have selected K-strategists capable of using these larger molecules, as has also been suggested by Quatrini *et al.* [33]. In addition, when the two libraries were compared using LIBSHUFF, they were found to be statistically different despite the prevalence of Actinobacteria in both the bulk soil and rhizosphere. This result suggests firstly that, after colonization, *C. rotundus* stimulates a specific alkane-utilizing population, enabling it to coexist in the rhizosphere, and secondly that the clone library strategy was more sensitive to differences between bacterial populations present in the rhizosphere and bulk soils than the DGGE analysis using *alkB* primers.

The microbial community associated with the rhizosphere of plants found in contaminated areas can also be involved in protecting them against chemical injury [40] and promoting their growth, acting as PGPR [13]. Nitrogen is an element essential for life, and is often a limiting factor for both plant growth and microbial hydrocarbon degradation [32, 42]. Only some bacterial and archaeal species are capable of transforming N_2 to ammonia, and this process, called biological nitrogen fixation (BNF), is the major source of nitrogen in many terrestrial sites [34]. However, the effect of petroleum hydrocarbons on nitrogen-fixing bacteria is almost unknown [32]. For this reason, potential nitrogen-fixing bacterial populations associated with *C. rotundus* were characterized by *nifH* PCR–DGGE. The resulting DGGE profiles showed low values of similarity in the UPGMA-assisted cluster analysis between bulk soil and rhizosphere/root samples, suggesting that *nifH*-containing bacteria were selected by *C. rotundus*. However, the profiles were highly variable among triplicates, in contrast with the results obtained for the total bacterial community and the alkane-utilizing bacterial population. Since it is known that a high concentration of hydrocarbons is toxic to many microorganisms [26], this variability could be explained by a deleterious effect of the oil contamination on some of the nitrogen-fixing bacteria. BLASTN analysis of the sequences of the two bands that occurred in most replicates of *nifH* PCR–DGGE and a third band that appeared in both bulk and rhizosphere soil samples showed that they were related to the *nifH* gene of *Bradyrhizobium* sp. and *Burkholderia* sp. Although *Bradyrhizobium* species are mostly associated with leguminous plants, previous studies have demonstrated the importance of this genus to nitrogen input in non-leguminous plants [3]. In the same way, species of *Burkholderia* are found colonizing the rhizosphere and roots of diverse plants, showing a capacity to fix nitrogen, to produce siderophores, and to solubilize phosphate [2, 21]. Moreover, species belonging to this genus are frequently found in contaminated sites [49], and the capacity of *Burkholderia* to utilize hydrocarbons has been studied in detail [20].

We also attempted to isolate bacterial strains from the rhizosphere soil and roots of *C. rotundus* that were able to simultaneously degrade hydrocarbons and present plant growth-promoting characteristics. Despite the potential enrichment with hydrocarbon degraders, only 37.8% of the 209 isolates were capable of growing in the presence of petroleum hydrocarbons as the sole carbon source in the microplate assay.

Positive *alkB* dot-blot hybridization results were obtained with the DNA of 56 of the 79 hydrocarbon degraders using a DIG-labeled probe. Vomberg and Klinner [45], when studying the distribution of *alkB* genes among alkane-utilizing bacteria, showed the difficulty of analyzing hybridization results when working with the DNA of different bacteria. In our study, data were obtained by comparison with the negative controls, which did not hybridize with the *alkB* probe, thus demonstrating its high specificity. The same result was obtained using the *nifH* probe. Of the *alkB* (+) strains, 17 have sequences with homology to *nifH* in their genomes. Additionally, the presence of part of the *nifH* sequence was confirmed in all of these isolates by PCR amplification using different sets of *nifH* primers.

Considering the potential use of these 17 isolates in phytoremediation with *C. rotundus*, their 16S rRNA genes were partially sequenced. BLASTN analysis showed that these isolates are affiliated with Alpha-, Beta-, and Gammaprotebacteria and Actinobacteria (Table 2). Gram-negative bacteria belonging to Burkholderiales, Enterobacteriales, Pseudomonodales, and Rhizobiales are commonly found in association with plants, and many studies have demonstrated their importance to phytoremediation. For example, members of the genus *Enterobacter* have been described as plant growth promoters in a hydrocarbon-contaminated site [12]. Interestingly, three isolates presenting homology to *alkB* and *nifH* were identified as *Rhodococcus* sp. and *Gordonia* sp. Members of these two Gram-positive, high GC-containing genera are

not usually cited in the literature as nitrogen fixers; however, they are recognized for their ability to degrade environmental pollutants, and are considered potentially useful for applications in environmental and industrial biotechnology [1, 9]. Escalante-Espinosa *et al*. [8] have demonstrated the participation of *Gordonia* sp. in phytoremediation using *Cyperus laxus* Lam. However, the present study is the first to describe strains belonging to the genus *Gordonia* as harboring at least part of the gene encoding nitrogenase reductase.

In conclusion, the present study extends our current knowledge regarding the diversity of the microbiota associated with *C. rotundus*. Different bacterial strains from the rhizosphere soil and roots of *C. rotundus* with the ability to simultaneously degrade hydrocarbons and potentially fix nitrogen have now become available for further study. It is hoped that these strains will efficiently promote phytoremediation in association with *C. rotundus* grown in a tropical soil.

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