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Bacterial Structure and Characterization of Plant Growth Promoting and Oil Degrading Bacteria from the Rhizospheres of Mangrove Plants

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Most oil from oceanic spills converges on coastal ecosystems, such as mangrove forests, which are threatened with worldwide disappearance. Particular bacteria that inhabit the rhizosphere of local plant species can stimulate plant development through various mechanisms; it would be advantageous if these would also be capable of degrading oil. Such bacteria may be important in the preservation or recuperation of mangrove forests impacted by oil spills. This study aimed to compare the bacterial structure, isolate and evaluate bacteria able to degrade oil and stimulate plant growth, from the rhizospheres of three mangrove plant species. These features are particularly important taking into account recent policies for mangrove bioremediation, implying that oil degradation as well as plant maintenance and health are key targets. Fifty-seven morphotypes were isolated from the mangrove rhizospheres on Bushnell-Haas (BH) medium supplemented with oil as the sole carbon source and tested for plant growth promotion. Of this strains, 60% potentially fixed nitrogen, 16% showed antimicrobial activity, 84% produced siderophores, 51% had the capacity to solubilize phosphate, and 33% produced the indole acetic acid hormone. Using gas chromatography, we evaluated the oil-degrading potential of ten selected strains that had different morphologies and showed Plant Growth Promoting Rhizobacteria (PGPR) features. The ten tested strains showed a promising degradation profile for at least one compound present in the oil. Among degrader strains, 46% had promising PGPR potential, having at least three of the above capacities. These strains might be used as a consortium, allowing the concomitant degradation of oil and stimulation of mangrove plant survival and maintenance.

***Keywords*:** bacterial structure, mangroves, oil-degrading rhizobacteria, PGPR

Mangroves are intertidal estuarine wetlands present along the coastlines of tropical and subtropical regions with great ecological and economic importance. Unfortunately, they are an endangered environment worldwide (Kathiresan and Binghan, 2001; Duke *et al*., 2007; Santos *et al*., 2010). Generally, most of these unique environments are situated in areas of high anthropogenic influence and, thus, they are usually exposed to pollutants, such as those released by oil spills (Burns *et al*., 1993; Li *et al*., 2007). The full extent of the consequences of this exposure is still unknown but will probably be disastrous. Obviously, methods for tracking the state of these environments and facilitating their recovery are essential to prevent the collapse of the mangrove ecosystems (Barbier *et al*., 2008; Peixoto *et al*., 2009; Santos *et al*., 2010).

Bioremediation techniques have been proposed to minimise the impact of oil contamination on mangrove ecosystems (Burns *et al*., 2000; Duke *et al*., 2000; Santos *et al*., 2010), and it known that the capacity to degrade oil hydrocarbons often resides in microorganisms. However, few studies have focused on the diversity or oil-degradation capacities of the microbial communities in mangroves (Burns *et al*., 2000; Brito *et al*., 2006; Gomes *et al*., 2008). Recently, Santos *et al*. (2010) highlighted oil degradation and the maintenance or restoration of mangrove vegetation as priorities in mangrove bioremediation strategies, indicating the need of a microbial consortia that have both the ability to degrade oil components and to assist in growth or recovery of impacted plants.

The rhizosphere is the portion of soil that surrounds plant roots; it forms a complex habitat for the soil biota, which might have its community altered by root activity. An important component of the rhizosphere is the actively growing microbial community that feeds on the organic nutrients in root exudates. In turn, microorganisms that colonise the rhizosphere may affect root and plant biology with regard to nutrition, development, and heath. The beneficial bacteria are known as plant growth-promoting rhizobacteria (PGPR). Among many characteristics, PGPR are often able to assist plant health and development by fixing nitrogen, solubilising phosphate, synthesizing siderophores, producing phytohormones and producing antimicrobial substances (Kloepper *et al*., 1980; Glick, 1995; Bashan and Holguin, 1997).

Few studies have evaluated the microbial diversity of mangrove plant rhizosphere and sediment by molecular tools such as PCR/DGGE (Gomes *et al*., 2008; Santos *et al*., 2010), moreover, even less studies focus on efficient bioremediation strategies for this environment. However, there are no studies that address the ability of rhizobacteria to degrade oil and concomitantly promote mangrove plant growth. This kind of strategy would be very useful, as the health of mangrove plant

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is fundamental for the recovery of this environment (Santos *et al*., 2010).

The aims of this study were to evaluate i) the profile of the bacterial community associated with the rhizospheres of the three most important Brazilian mangrove species, i.e. *Laguncularia racemosa*, *Rhizophora mangle*, *Avicennia schaueriana*, and with sediment samples by PCR-DGGE analysis; ii) to evaluate the diversity of potential oil degrading cultivated bacteria; iii) to find isolates that both degrade oil and show PGPR features.

Materials and Methods

Sampling

Rhizospheres of the plant species *A. schaueriana*, *L. racemosa*, and *R. mangle*, plus approximately 50 g of surface (0-5 cm) sediment were collected in triplicates and stored in sterile plastic bags and Falcon tubes, respectively. Each replicate sediment sample was composed of six subsamples collected near (within about 20 cm) each corresponding rhizosphere. The samples were obtained from the "Restinga da Marambaia" mangrove, located in Rio de Janeiro, Brazil (23°3'27"S 43°33'58"W) and maintained at 4°C during transport to the laboratory (about 3 h), where the enrichment experiment was performed immediately. After enrichment, samples were stored at -20°C.

Enrichment, isolation, and maintenance of bacterial strains isolated from *L. racemosa*, *R. mangle*, and *A. schaueriana*

Enrichment of biodegradative organisms was performed by adding 5 g of each rhizosphere or sediment sample to 45 ml of Bushnell-Haas (BH) broth (Difco, USA) supplemented with 5% oil as the sole carbon source. This mixture was incubated for 14 days at 30°C, with agitation (125 rpm).

After the enrichment process, 1 ml of each culture was mixed with 9 ml of a 0.85% NaCl solution (saline), and serial tenfold dilution of the enriched rhizosphere or sediment samples in saline were plated onto BH agar (Difco). The plates were then incubated for 24 h at 28°C. Colonies with different morphologies were selected for further purification (at room temperature) on Luria-Bertani (LB) agar. Purified cultures in LB broth were stored at -80°C with glycerol. DNA extraction from bacterial cultures, sediment, and rhizosphere soil samples.

Triplicate samples of 0.5 g sediment or rhizosphere pellet (Bakken and Lindahl, 1995) were subjected to DNA extraction using the BIO 101 DNA Extraction kit (QBiogene, USA), following the manufacturer's instructions.

Genomic DNA was extracted from all selected bacterial strains using the Wizard Genomic DNA Purification kit (Promega, USA). The integrity and quality of the extracted DNA were assessed by electrophoresis in a 0.8% agarose gel with 0.5 TBE buffer (45 mM Tris-borate, 1 mM EDTA; pH 8.0).

PCR amplification of the *nifH* gene

To assess the diversity of genes involved in nitrogen fixation, fragments (360 bp) of the *nifH* gene, which encodes the nitrogenase reductase enzyme, were amplified by PCR using genomic DNA of each bacterial strain using the primers PolF (5′-TGCGAYCCSAARGCBG ACTC-3′) and PolR (5′-ATSGCCATCATYTCRCCGGA-3′), following the conditions described by Poly *et al*. (2001).

Assay for production of antimicrobial substances

The method described by Rosado and Seldin (1993) was used to detect antimicrobial activity. Brain-heart infusion (BHI) agar plates were inoculated with 5-µl spots from overnight cultures of each isolate. After incubation at 32°C for 48 h, the cells were killed by exposure to chloroform vapour for 15 min. The plates were then flooded with suspensions containing the indicator strains *Micrococcus* sp., as described by von der Weid *et al*. (2005). The production of antimicrobial substance was indicated by growth inhibition zones around the bacterial colonies after incubation for an additional 24 to 48 h at 32°C.

Phosphate solubilisation

The phosphate solubilisation test described by Rosado *et al*. (1998) was used to determine the ability of the isolates to solubilise phosphate (calcium phytate). Strains were inoculated as a 5 µl spot on phosphate solubilisation medium plates and incubated at 32°C for 5 days (Rosado *et al*., 1998). Clear zones around the bacterial colonies after incubation were taken as evidence for phosphate solubilization.

Siderophore production

All isolates were screened for the production of siderophores in King's B agar supplemented with, chrome azurol S [CAS/iron(III)/ hexadecyltrimethyl ammonium bromide], as described by Schwyn and Neilands (1987). Strains were inoculated as a 5 µl spot on siderophore production medium plates and incubated at 32°C for 4 to 7 days (Schwyn and Neilands, 1987). Bacterial growth accompanied by the formation of a yellow halo around the colonies was recorded as positive for siderophore production.

Bacterial production of Indole Acetic Acid (IAA)

All isolates were grown in 3 ml LB medium for 48 h at 32°C. Then 100 µl aliquots of these cultures were added in triplicate, into 3 ml King's B medium and maintained at 27°C for 72 h in the dark on a rotatory shaker (100 rpm), as described by Bent *et al*. (2001). Uninoculated tubes were used as controls. The production of IAA in the culture was estimated by adding 1 ml of Salkowski's reagent (1.875 g $FeCl_3 \cdot 6H_2O$, 100 ml H_2O, and 150 ml H_2SO_4 at 96% purity) to 1 ml of each supernatant (Tang and Bonner, 1947). When a reddish colour appeared after the addition of the reagent, the test was considered positive.

Evaluation of total petroleum hydrocarbon (TPH) degradation

Based on the PGPR characteristics and morphological differences of their colonies, ten strains were selected for evaluation of their oil degradation ability. For this purpose, these bacterial cultures were introduced into 100 ml vials, containing 50 ml of BH broth (Difco) plus 1% of oil. The cultures were incubated with agitation (150 rpm) at 30°C for up to 14 days. After 14 days, the degradation of total petroleum hydrocarbons (TPH) was evaluated for each strain. Controls lacking inoculum were also assessed to determine losses of hydrocarbons through volatilisation (C-).

The analysis of the degradation of total petroleum hydrocarbons followed the recommendations of the *Reference Methods for Marine Pollution Studies* (UNEP, 1991). Total petroleum hydrocarbons were determined in a Varian Gas Chromatographe (GC) (CP 3800 MS Saturn 2200) coupled with mass spectrometry (GC-MS) and a CP8944A chromatographic column (5% phenyl polysiloxane). The initial temperature of the oven was 40°C, with an increase of 10°C/min up to 60°C, followed by an increase of 5°C/min up to 290°C, at which temperature the oven was kept for 5 min. Finally, the tem-

perature increased at the rate of 10°C/min to 300°C, and this temperature was maintained for 10 min. The injector temperature was 280°C, and the injected volume was 1 ml. In the MS detector, the source temperature was maintained at 200°C, while the temperature of the transfer line was maintained at 280°C.

For liquid/liquid extraction 40 ml of dichloromethane were added to a separating funnel with 50 ml of BH medium and 50 µl of oil. The contents were stirred vigorously for 3 min, followed by a rest period of 10 min, for the separation of the aqueous phase from the organic phase. A glass funnel packed with glass wool and containing about 10 mg of sodium sulphate (Na_2SO_4) was used to dry the extract. This organic phase was released directly through this funnel over a balloon collector. The extraction was repeated three times, after which the extracts were collected in the same flask and washed with dichloromethane sulphate at the end of the procedure.

PCR and denaturing gradient gel electrophoresis (DGGE)

PCR reactions for DGGE analysis were performed with a universal primer set for bacteria based on the 16S rRNA gene, U968*f*-GC1 and L1401, as previously described by Heuer and Smalla (1997). Before DGGE analysis, the presence of PCR products was confirmed by electrophoresis in a 1.2% agarose gel run at 80 V in Tris-Borate-EDTA buffer. The gel was stained for 15 min with 0.5 µg/ml ethidium bromide after which was examined under short-wavelength ultraviolet light. A 100 bp DNA ladder (Fermentas, Lithuania) served as the molecular size standard.

DGGE of the amplified gene sequences was performed using a DCode System (universal mutation detection system; Bio-Rad). The gel contained 6% acrylamide with a gradient of 45% to 65% denaturant (urea and formamide). All gels were loaded with DNA markers in the first and last lanes surrounding the lanes with samples to allow gel standardisation according to the manufacturer's instructions. Electrophoresis was performed in 1× Tris-acetate-EDTA buffer at 60°C at a constant voltage of 75 V for 16 h. Following electrophoresis the gels were stained with Sybr Gold (Invitrogen) and visualised using a Storm 860 Imaging System (GE Healthcare). The results were presented as dendrograms constructed after image capture and analysis by Pearson correlation coefficients (r). The cluster analysis was performed by the unweighted pair group method with average linkages (UPGMA) using BioNumerics software (Applied Maths, Belgium).

DNA sequencing and phylogenetic analysis

Bacterial primers 27f (5′-AGA GTT TGA TCA TGG CTC AG-3′) and 1492r (5′-GTT TAC CTT GTT ACG ACT T-3′) were used for amplification of the 16S rRNA gene from isolated colonies. 16S rRNA genes were amplified in a 50 µl reaction mixture consisting of 5 µl of 10× buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTP, 5 µM of each primer, 10 ng of template DNA, and 2.5 U of Taq DNA polymerase (Promega). Amplification conditions for the PCR included an initial denaturation step of 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2.5 min and a final extension step of 72°C for 10 min. The amplicons were purified with the PCR Purification System kit (QIAGEN). Sequencing was carried in ABI-3730 automatic capillary sequencer (Applied Biosystems), using the two primers from the ends, 27f and 1492, as well as two primers from the middle of the sequences, positions 532 (5′-CGT GCC AGC AGC CGC GGT AA-3′) and 907 (5′-CCG TCA ATT CMT TTG AGT TT-3′).

The sequences obtained were analysed using the Blast-N program through the NCBI web service (http://www.ncbi.nlm.nih.gov/) (Altschul *et al*., 1990). Sequences of closely related strains were recovered from GenBank and aligned to the sequences obtained in this study using CLUSTAL X (Thompson *et al*., 1997). A phylogenetic tree was constructed based on the almost-complete 16S rRNA gene sequences by Neighbor-Joining (NJ) with Jukes-Cuntor correction method, using MEGA 3.1 software (Kumar *et al*., 1993). The sequences obtained in this study were uploaded and are available at the GenBank under accessions numbers HQ020559 to HQ020615.

Results and Discussion

DGGE analysis of the bacterial community

The bacterial community profiles of the triplicate sediment and rhizosphere samples were investigated by DGGE. The analyses indicated the occurrence of two main clusters, with the exception of one sample (Avi 1). The first cluster encompassed all sediment samples, whereas the second one comprised all rhizosphere samples (Fig. 1). Within the second group, it was possible to detect two subclusters which separated the rhizospheres of *Avicennia schaueriana* and *Laguncularia racemosa* from the rhizosphere of *Rizophora mangle*. Many authors have described plant species or plant type as the key factor that determines the rhizosphere-associated microbial community composition and diversity (Costa *et al.*, 2006; Garbeva *et al*., 2007; Monteiro *et al*., 2009). The observed clustering could be partially explained by morphological and physiological differences between the mangrove plant species. *R. mangle* is a predominant species in mangrove areas that are prone to flooding. *R. mangle* forms stilt roots, which help in fixing poorly-structured soils and assist the exchange of gases in submerged portion of the root. It also furnishes an ultra-filter in its root system which can extract salts during the process of water absorption (Kathiresan and Binghan, 2001). In contrast, *L. racemosa* and *A. schaueriana* have no barriers to the entry of salts, but they can excrete salts through specialized

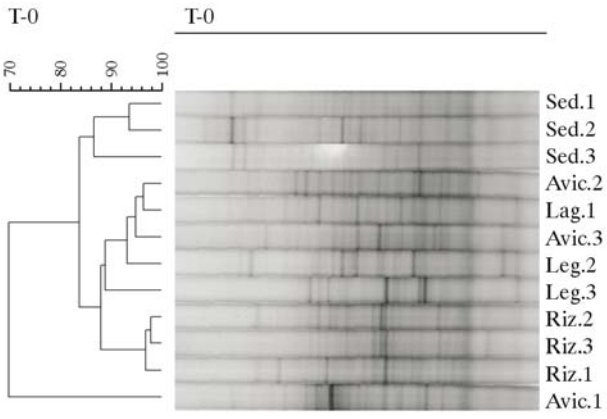

Fig. 1. Denaturing gradient gel electrophoresis (DGGE) fingerprints of 16S rRNA gene fragments amplified from sediment and rhizosphere soil DNA templates obtained from three mangrove plant types. Avi.1, Avi.2, and Avi.3 denote triplicate samples from *A. schaueriana*; Lag. 1, Lag.2, and Lag.3 denote triplicate samples from *L. racemosa*; Riz.1, Riz.2, and Riz.3 denote triplicate samples from *R. mangle*; and Sed.1, Sed.2, and Sed.3 denote triplicate samples from sediment.

glands in their leaves. Furthermore, they can produce solutes in their tissues that help to maintain osmotic balance when needed. Unlike *R. mangle*, these two species are more abundant on firmer grounds, in mangrove areas that more sheltered

Table 1. Plant growth promotion characteristics presented by the bacterial isolates from the mangrove plant rhizospheres and their identification

Isolates[a]	PCR *nif*H	Posphate	AMS	Siderophore	IAA	Species (Similarity) [Blast]
Avi 1, Avi3	-	-	-	-	-	*Paenibacillus* sp. (93%) [AM162350.1], *Rhodococcus* sp. (93%) [FJ752527]
Avi 2, Lag 2, Lag 8, Sed 13	-	-	-	+	-	*Diaphorobacter* sp. (93%) [FJ158841], Uncultured *Paenibacillus* sp. (94%) [EU135784], *Pseudomonas pseudoalcaligenes* (95%) [EU419938], *Delftia tsuruhatensis* (96%) [GQ 868495]
Avi 4, Lag10	+	+	-	-	-	*Azospirillum* sp (94%) [FJ594445], *Paenibacillus* sp. (99%) [EU135784]
Avi 10, Avi 12, Lag 6, Lag 9, Riz 1, Riz 13, Sed 2, Sed 7	+	+	-	+	-	*Pseudomonas pseudoalcaligenes* (94%) [AB276372.1], *Acinetobacter* sp. (94%) [GU566321], *Paenibacillus* sp. (96%) [AM162350], *Pseudomonas aeruginosa* (99%) [EU170480], *Pseudomonas stutzeri* (99%) [FJ411167], Delftia tsuruhatensis (99%) [EF421404], uncultured *Delftia* sp. (96%) [GU563748], *Acinetobacter* sp. (98%) [GU138160]
Avi 5	+	+	+	+	+	uncultured *Pseudomonas* sp. (99%) [HM152755]
Avi 6, Avi 7, Avi 8, Lag 11, Lag 12, Lag 13, Riz 5, Riz 12, Sed 14	+	-	-	+	-	*Paenibacillus* sp. (94%) [AM162350.1], *Pseudomonas aeruginosa* (100%) [GQ375800], *Pseudomonas pseudoalcaligenes* (99%) [EU440977.1], *Alcaligenes* sp. (91%) [FJ529025], *Arthrobacter* sp. (94%) [FJ851358], *Sinorhizobium adhaerens* (95%) [AJ420773], *Stenotrophomonas* sp. (99%) [FJ976542], *Pseudomonas alcaligenes* (99%) [EU240201], uncultured *Rhodococcus* sp. (96%) [FJ863098]
Avi 13, Riz 4, Riz 10	+	-	-	+	+	*Ralstonia* sp. (90%) [HM194608], *Pseudomonas aeruginosa* (99%) [GU384224], *Pseudomonas pseudoalcaligenes* (99%) [EU440977]
Lag 3, Lag 5, Riz 14, Sed 8, Sed 10	-	-	-	+	+	*Pseudomonas* sp. (99%) [EU107175], *Pseudomonas stutzeri* (99%) [GU339277], *Pseudomonas alcaligenes* (99%) [EU 596481], *Pseudomonas pseudoalcaligenes* (98%) [EU440977], *Delftia* sp. (81%) [GU566337]
Avi 9, Lag 4, Lag 7, Riz 15, Sed 15	-	+	-	+	-	*Pseudomonas* sp. (87%) [GU056312.1], *Pseudomonas* sp. (97%) [FJ897721], *Acinetobacter* sp. (93%) [EU000454], *Pseudomonas pseudoalcaligenes* (95%) [EU440977], *Pseudomonas aeruginosa* (98%) [GQ926936]
Avi 11, Riz 11, Sed 3	+	+	-	+	+	*Acinetobacter* sp. (92%) [GU566321], *Arthrobacter* sp. (99%) [EU571174], *Alcaligenes* sp. (95%) [FJ529025]
Lag 1	-	+	-	-	+	*Paenibacillus* sp. (91%) [AB461760]
Lag 14	+	-	+	-	+	*Pseudomonas pseudoalcaligenes* (99%) [EU419938]
Riz 2	+	-	-	-	+	*Pseudomonas* sp. (99%) [EU107175]
Riz 3, Riz 6, Riz 9, Sed 1	+	+	+	+	-	*Pseudomonas aeruginosa* (100%) [GU384267], *Pseudomonas putida* (99%) [GU329915], *Pseudomonas aeruginosa* (88%) [FJ976651], *Pseudomonas aeruginosa* (97%) [FJ972527]
Riz 7, Riz 8	+	-	+	+	-	*Pseudomonas aeruginosa* (100%) [GQ217529], *Stenotrophomonas* sp. (99%) [FJ976542]
Sed 4	-	+	+	+	-	*Pseudomonas pseudoalcaligenes* (95%) [EU419938]
Sed 5	-	+	-	-	-	*Pseudomonas pseudoalcaligenes* (95%) [EU419938]
Sed 6	-	-	-	-	+	*Acinetobacter* sp. (98%) [EU000454]
Sed 9, Sed 11, Sed 12	-	+	-	+	+	uncultured *Pseudomonas* sp. (94%) [FJ863108], *Pseudomonas aeruginosa* (95%) [EU170480], *Paenibacillus* sp. (99%) [FJ 719328]
Total (57)	34	29	9	48	19	

[a] different isolates were designated Lag, Riz, and Avi, followed by progressive numbers of isolation.

from the tide (Kathiresan and Binghan, 2001).

Isolation of bacterial strains from the rhizospheres of mangrove plants and from sediments

On the basis of morphological differences (morphotypes), we selected a total of 57 strains (Table 1) from samples of the rhizospheres of *L. racemosa*, *R. mangle*, and *A. schaueriana*, as well as from sediment samples. LB agar medium containing oil as the sole carbon source was used. Among the strains, 13 morphotypes were isolated from *A. schaueriana*, 14 from *L. racemosa*, 15 from *R. mangle*, and 15 from sediment. The different isolates were designated as Avi, Lag, Riz, and Sed, according to their origin, followed by successive numbers.

The partial sequencing of the 16S rRNA genes of the isolates revealed a predominance of Proteobacteria (81%). Among then, 78% belonged to the Gammaproteobacteria, followed by Betaproteobacteria (17%), and Alphaproteobacteria (4%). The dominant order of the Gammaproteobacteria was *Pseudomonadales* (94%), with only two isolates from the order *Xanthomonadales*. Within the *Pseudomonadales*, the majority of the isolates belonged to the genus *Pseudomonas* (85%) (Fig. 2).

Other phyla observed were Actinobacteria (4 strains, corresponding to 7% of all isolates recovered) and Firmicutes (12% of all strains), of which 100% belonged to the genus *Paenibacillus* (7 strains), which thus represented the second most-abundant genus (Fig. 2).

Almost all genera recovered in this work (*Pseudomonas*, *Azospirillum*, *Azotobacter*, *Paenibacillus*, *Arthrobacter*, *Acinetobacter*, and *Alcaligenes*) have previously been described as having PGPR features (Vazquez *et al*., 2000; Lucy *et al*., 2004; Karakurt and Aslantas, 2010). A total of 12 genera were recovered, where 7 were present in *Avicennia shaueriana* rhizosphere (*Paenibacillus*, *Rhodococcus*, *Diaphorobacter*, *Pseudomonas*, *Azospirillum*, *Acinetobacter*, *Ralstonia*), 6 in *Laguncularia racemosa* (*Paenibacillus*, *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Sinorhizobium*) and sediment (*Paenibacillus*, *Rhodococcus*, *Delftia*, *Pseudomonas*, *Acinetobacter*, *Alcaligenes*) samples and only 4 in *Rhizophora mangle* rhizosphere samples (*Delftia*, *Pseudomonas*, *Arthrobacter*, *Stenotrophomonas*) (Fig. 3). Despite the low diversity of genera, the majority of strains recovered (16) were isolated from *Rhizophora mangle* samples, being 12 *Pseudomonas* sp. representatives (Fig. 2). Only the genus *Pseudomonas* was recovered from all plant species rhizospheres as well as from sediment samples. This genus has been described as one of the best root colonizers, being used as a model root colonizer (Lugtenberg *et al.*, 2001). *Paenibacillus* and *Acinetobacter* genera were also widely distributed, being present in 3 of the 4 analyzed samples, being only absent in *Rhizophora mangle* samples (Fig. 3). Species of these two genera are also described associated with different plant rhizospheres (Mota *et al*., 2002; Timmusk *et al*., 2005; Ritcher and Smalla, 2007; Gulati *et al*., 2009) with a broad host range.

Plant growth-promotion properties of bacterial strains isolated from mangrove plant rhizospheres

A 360 bp *nifH* PCR product (expected size) was observed in 34 (60%) strains: 9 from *Avicennia schaueriana*, 7 from *Laguncularia racemosa*, 13 from *Rizophora mangle*, and 5 from sediment (Table 1).

Nitrogen fixation has previously been described in mangrove ecosystems, and high rates have been previously detected in association with dead leaves and in pneumatophores, in

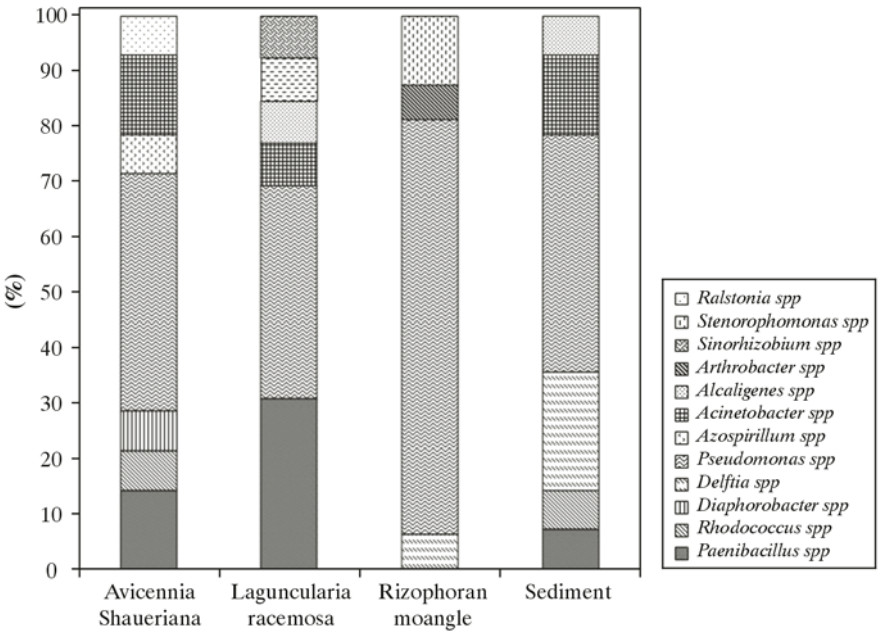

Fig. 2. Phylogenetic tree of partial 16S rRNA gene sequences (900 bp) showing the relationships between the 57 isolates and related species found by Blast-n searches. The tree was constructed based on the neighbour-joining method. Bootstrap analyses were performed with 1,000 replicates, and the results are represented at the branch points. The GenBank accession no. of each species is indicated in parentheses.

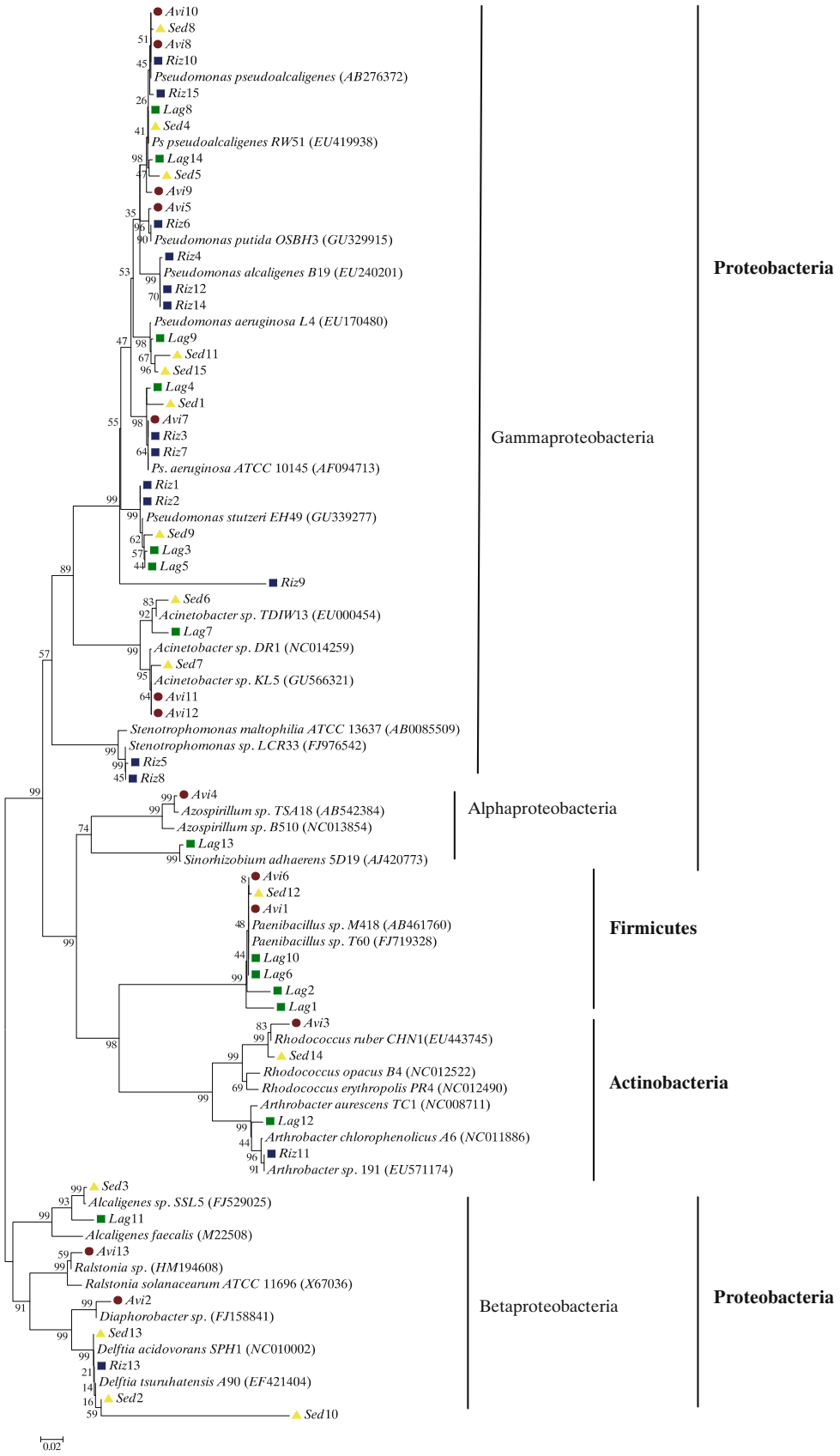

Fig. 3. Distribution and abundance of genera recovered from rhizosphere samples (*A. shaueriana*, *L. racemosa*, *R. mangle*) and from sediment.

the rhizosphere, and in the sediment itself (Toledo *et al*., 1995; Bashan and Holguin, 2002). Nitrogen-fixing strains belonging to several genera (*Azospirillum*, *Vibrio*, *Listonella*, *Azotobacter*, *Rhizobium*, *Clostridium*, and *Klebisiella*) have previously been isolated from the sediment, rhizosphere, and roots of different mangroves (Rojas *et al*., 2001; Bashan and Holguin 2002).

The ability to solubilise phosphate was detected in 29 (51%) isolates; 6 from *Avicennia schaueriana*, 6 from *Laguncularia racemosa*, 7 from *Rizophora mangle*, and 10 from sediment (Table 1). The presence of phosphate-solubilising bacteria may provide a great advantage for mangrove plants because phosphorus is often precipitated in marine sediment due to the abundance of cations in the interstitial water, making it unavailable to local plants (Bashan and Holguin, 2002). Very few studies have focused on the role of phosphate solubilizers in coastal environments. Vazquez and colleagues (2000) isolated 12 proficient strains of phosphate-solubilising bacteria from roots of mangrove plants in Mexico. They demonstrated that the average phosphate solubilisation capacity of one isolated strain, *Bacillus amyloliquefaciens*, was sufficient to supply a small terrestrial plant with its daily requirement of phosphate.

From the 57 strains, only 9 (16%), all *Pseudomonas* and *Stenotrophomonas* species, were able to inhibit the indicator strain *Micrococcus* sp. in plate assays (Table 1). It is possible that the use of only one indicator may have underestimated the antimicrobial potential of the recovered strains, although *Micrococcus* sp. has been extensively used as an indicator for the production of antimicrobial substances by different bacterial strains (von der Weid *et al*., 2005; Monteiro *et al*., 2009). The majority of the isolates (48, corresponding to 84% of the selected strains) produced siderophores. Overall, 10 siderophore-producing strains were recovered from *A. schaueriana* rhizosphere samples, 11 from *L. racemosa*, 14 from *R. mangle*, and 13 from sediment samples (Table 1). Siderophores secreted by bacteria in the mangrove habitat may stimulate plant growth directly, by increasing the availability of iron in the soil, or indirectly, by inhibiting plant pathogen growth by limiting their access to iron (Monteiro *et al*., 2009). The genus *Pseudomonas* has been extensively described as encompassing siderophore-producing species (Kloepper *et al*., 1980; Burd *et al*., 2000; Rodriguez *et al*., 2008; Cheng *et al*., 2009). As many of our strains belonged to the genus *Pseudomonas*, it

Table 2. Total petroleum hydrocarbons (TPH) profile 14 days inoculation with the selected strains

Strain/Oil compound	Control	Avi4	Avi5	Avi10	Avi13	Lag5	Lag10	Riz3	Sed7	Sed8	Sed9
C8	0.73	0	0	0	0.52	0.70	0	0	0	0.60	0.72
C9	1.06	0	1.06	0	0.39	0.87	0.53	0	1.10	0	0
C10	5.27	0.34	3.80	0	0	3.15	1.97	1.49	3.64	1.55	0
C11	20.08	3.56	14.31	1.43	4.17	11.71	7.73	9.82	12.11	6.34	0
C12	61.12	20.79	41.16	5.39	12.42	35.00	23.42	36.53	29.65	16.60	4.97
C13	148.20	56.19	95.96	17.91	28.47	84.88	54.26	76.92	53.22	33.82	0
C14	265.40	107.70	164.00	37.42	53.13	143.50	107.40	144.60	94.82	62.54	0
C15	369.50	151.70	233.20	53.69	67.67	194.60	144.90	183.70	123.90	81.99	8.46
C16	335.90	150.80	217.70	50.01	64.09	196.70	145.30	139.60	101.40	76.03	0
C17	0	0	0	0	0	0	0	0	0	0	0
Pristane	271.70	128.30	95.47	48.00	50.24	99.11	107.90	124.40	69.20	70.44	0
C18	139.90	93.47	75.61	47.95	70.48	62.18	63.38	82.73	0	52.41	0
Phytane	230.40	105.80	157.90	50.10	51.29	141.50	102.50	116.70	57.68	68.71	11.55
C19	200.10	99.82	138.30	44.05	47.18	123.50	89.59	114.20	49.84	62.63	20.01
C20	167.60	79.46	104.00	37.87	40.79	96.22	69.85	86.12	38.85	50.95	19.25
C21	138.80	66.92	91.53	32.52	35.61	85.14	60.27	70.57	33.37	44.31	14.67
C22	115.80	53.48	74.71	23.16	32.08	70.35	52.77	59.82	25.94	36.50	0
C23	94.82	43.74	60.17	20.29	26.92	59.28	41.66	48.64	24.37	31.53	0
C24	78.65	37.19	48.80	16.99	23.37	45.20	31.13	34.65	18.51	23.58	0
C25	58.53	25.80	35.12	12.27	18.98	35.91	25.09	27.90	15.76	19.98	0
C26	53.03	20.46	26.62	9.91	16.65	28.22	19.48	22.47	12.25	13.27	0
C27	33.24	16.21	18.97	6.92	11.65	19.90	13.61	15.77	7.39	10.31	3.64
C28	26.79	10.18	13.28	4.99	8.51	14.63	9.65	10.53	5.72	7.30	0
C29	0	0	0	0	0	0	0	0	0	0	0
C30	0	0	0	0	0	0	0	0	0	0	0
C31	0	0	0	0	0	0	0	0	0	0	0
C32	0	0	0	0	0	0	0	0	0	0	0
C33	0	0	0	0	0	0	0	0	0	0	0
C34	0	0	0	0	0	0	0	0	0	0	0
C35	0	0	0	0	0	0	0	0	0	0	0
C36	0	0	0	0	0	0	0	0	0	0	0
Total TPH	2816.63	1271.92	1711.68	520.88	664.63	1552.26	1172.41	1407.17	778.74	771.39	83.28
% of oil degradation	0	54.84	39.22	81.50	76.40	45.95	58.37	50.04	72.35	72.62	97.04

was expected that many of them would reveal the ability to chelate iron.

The capacity to produce IAA in the culture supernatants was observed in 19 (33%) of the strains: 3 from *A. schaueriana*, 4 from *L. racemosa*, 5 from *R. mangle*, and 7 from sediment (Table 1). Among the auxins, IAA is the most studied and the most produced by bacteria. IAA can affect root morphology by increasing the length and number of root hairs (Barbieri *et al*., 1986). Some studies have reported that members of the genera *Paenibacillus* and *Acinetobacter* are proficient producers of extracellular IAA (Rosado *et al*., 1998; Mota *et al*., 2008; Kang *et al*., 2009). Patten and Glick (1996) even suggested that the input of microbial IAA into rhizosphere ecosystems can modify the plant endogenous auxin level to an optimum level or even above it, resulting in optimized plant growth.

Only two strains, *Paenibacillus* sp. (Avi 1) and *Rhodococcus* sp. (Avi 3), had negative results in all five tests carried out in the present study. On the other hand, one strain, i.e. *Pseudomonas* sp. Avi 5, revealed positive results in all five tests. This strain thus showed positive amplification of *nifH*, the ability to produce IAA and siderophores, to solubilise phosphate, and to inhibit the growth of *Micrococcus* sp. (Table 1). Moreover, 26 strains revealed 3 or more positive results, thus demonstrating their great PGPR potential.

Oil degradation and bioremediation potential

We selected ten of the 26 most promising strains for evaluation of their oil degradation capacities. These ten strains were all able to degrade TPH. Specifically, between 39% to 97% of the total TPH were degraded in 14 days (Table 2). It is important to note that different strains degraded different fractions of the oil, as observed for C8, which was completely degraded by *P. aeruginosa* Riz 3, *Azospirillum* sp. Avi 4, *Pseudomonas* sp. Avi 5, *Acinetobacter* sp. Sed 7, *P. pseudoalcaligenes* Avi 10, and *Paenibacillus* sp. Lag 10. The C10 fraction was completely degraded by the strains *Pseudomonas* sp. Sed 9, *P. pseudoalcaligenes* Avi 10, and *Ralstonia* sp. Avi 13. The majority of the TPH fractions were completely degraded by at least one strain (except C12, C15, C16, C19, C20, C21, and C22). However, even fractions that were not completely eliminated significantly decreased, as observed for fraction C12 by *Pseudomonas* sp. Sed9; strain Sed9 was able to degrade almost 92% of the C12 fraction in 14 days.

Several authors have previously described the potential of the use of members of the above genera in oil degradation. Richard and Vogel (1999) evaluated the degradation of diesel oil by two *P. fluorescens* strains and observed that 10.4% and 12.3% of the oil hydrocarbons were degraded by these strains during the 50 day experiment. Moreover, in another study, *R. eutropha* H16 and *P. oleovorans* were shown to be able to use oil residue from industrial rhamnose production as a carbon source for growth (Füchtenbusch *et al*., 2000). Maciel-Souza and colleagues (2006) isolated oil-degrading bacterial strains from *L. racemosa* and *R. mangle* rhizospheres and indicated that, among the eleven most promising isolates obtained, ten belonged to the genus *Bacillus* and one to *Acinetobacter.*

Our results demonstrated the great potential of mangroves as sources of bacterial isolates to be used in bioremediation approaches which simultaneously depend on PGPR and oil degrading capacities. Because of the variable abilities of each strain in degrading oil or promoting plant growth, the selected strains could be potentially used as a consortium for mangrove bioremediation. The use of such a consortium could provide an effective tool for the bioremediation of oil-contaminated mangroves.

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