RESEARCH ARTICLE

Mangrove microniches determine the structural and functional diversity of enriched petroleum hydrocarbon-degrading consortia

Newton C.M. Gomes[1], Cecilia G. Flocco[2,3], Rodrigo Costa[4], Howard Junca[5,6], Ramiro Vilchez[5], Dietmar H. Pieper[5], Ellen Krögerrecklenfort[2], Rodolfo Paranhos[7], Leda C.S. Mendonça-Hagler[8] & Kornelia Smalla[2]

[1]CESAM and Department of Biology, University of Aveiro, Campus Universitário de Santiago, Aveiro, Portugal; [2]Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Braunschweig, Germany; [3]Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Buenos Aires, Argentina; [4]Microbial Ecology and Evolution Research Group, Centre of Marine Sciences (CCMar), University of Algarve, Faro, Portugal; [5]AG Biodegradation, Department of Microbial Pathogenesis, Helmholtz-Zentrum für Infektionsforschung, Braunschweig, Germany; [6]National Research Network of Genomics and Bioinformatics of Extreme Environments – GeBiX, Microbial Ecology Research Group, Molecular Genetics & Biotechnology, CorpoGen Corporation, Bogotá DC, Colombia; [7]Laboratory of Hydrobiology, Institute of Biology, CCS, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil; and [8]Laboratory of Taxonomy and Ecology of Microorganisms, Institute of Microbiology, CCS, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

Correspondence: Newton C.M. Gomes, CESAM and Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal. Tel.: +351 234 370 990; fax: +351 234 370 309; e-mail: gomesncm@ua.pt

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Abstract

In this study, the combination of culture enrichments and molecular tools was used to identify bacterial guilds, plasmids and functional genes potentially important in the process of petroleum hydrocarbon (PH) decontamination in mangrove microniches (rhizospheres and bulk sediment). In addition, we aimed to recover PH-degrading consortia (PHDC) for future use in remediation strategies. The PHDC were enriched with petroleum from rhizosphere and bulk sediment samples taken from a mangrove chronically polluted with oil hydrocarbons. Southern blot hybridization (SBH) assays of PCR amplicons from environmental DNA before enrichments resulted in weak positive signals for the functional gene types targeted, suggesting that PH-degrading genotypes and plasmids were in low abundance in the rhizosphere and bulk sediments. However, after enrichment, these genes were detected and strong microniche-dependent differences in the abundance and composition of hydrocarbonoclastic bacterial populations, plasmids (IncP-1α, IncP-1β, IncP-7 and IncP-9) and functional genes (naphthalene, extradiol and intradiol dioxygenases) were revealed by in-depth molecular analyses [PCR-denaturing gradient gel electrophoresis and hybridization (SBH and microarray)]. Our results suggest that, despite the low abundance of PH-degrading genes and plasmids in the environmental samples, the original bacterial composition of the mangrove microniches determined the structural and functional diversity of the PHDC enriched.

Introduction

Sediment contamination with petroleum hydrocarbons (PH) is especially dangerous for mangrove forests, because low-molecular-weight aromatic hydrocarbons (e.g. BTEX, naphthalene and phenanthrene) can be phytotoxic and affect plants at all stages of growth (Proffitt *et al.*, 1995; Hoff, 2002; Kummerová & Kmentová, 2004). Curiously, despite their clear symptoms of stress (death of mangrove saplings, smaller trees and the loss of forest cover), mangrove trees are still often found in urban areas under chronic exposure to PH contamination (Proffitt *et al.*, 1995; Gomes *et al.*, 2007; Benson & Essien, 2009). The survival of these plants may be due to their inherent physiological resistance, but could also be the result of beneficial interactions with the associated microbiota. Specifically, it had been documented that microbial PH degraders can be enriched in the plant rhizosphere due to the release of root exudates and can either contribute to soil detoxification surrounding the root or inside of the plant (Kuiper *et al.*, 2001; Germaine *et al.*, 2009). Surprisingly, despite the well-known ability of inland plants to promote bioremediation of contaminated soil, no studies have hitherto performed an in-depth analysis of the catabolic potential of microorganisms associated with

mangrove roots for PH degradation. Regarding neotropical coastal areas, mangroves are native trees that can be planted in those areas, potentially acting as reservoirs of PH-degrading microbial guilds for bioremediation purposes.

It is assumed that horizontal gene transfer by mobile genetic elements plays an important role in the acclimation of bacterial populations to environmental contamination (Bale *et al.*, 1988; Rasmussen & Sørensen, 1998; Wilson *et al.*, 2003). However, in spite of its potential ecological importance, a limited number of studies have addressed plasmid diversity in coastal environments (Sobecky, 1999; Beeson *et al.*, 2002), and no reports exist thus far that specifically focus on mangrove ecosystems. Moreover, although it is well known that the degradation of environmental pollutants is enhanced in plant rhizospheres, the diversity and heterogeneity of plasmids and the functional genes involved in this process remain almost completely unknown in natural mangrove settings.

The *in vitro* enrichment of functional guilds has been used commonly for the isolation of hydrocarbon degraders from terrestrial, marine and freshwater sources (MacCormack & Fraile, 1997; Head, 1998; Yakimov *et al.*, 2003). Often, the resulting enrichments allow the acquisition of specific information, such as identification of microbial degraders and metabolic pathways. For example, due to the difficulties associated with the culturing of environmental microorganisms, the utilization of DNA from enrichment cultures can allow the study of several new microbial traits and improve access to new potential sources of genes of biotechnological interest (Entcheva *et al.*, 2001). Furthermore, enrichments can be used as environmental inoculants to speed up natural biological processes (bioaugmentation) (Venosa *et al.*, 1996).

The utilization of plant inoculants has also proven to be a reliable approach to promote plant growth and phytoremediation of PH-polluted environments (Kuiper *et al.*, 2001; Germaine *et al.*, 2009). In fact, the plant–microorganism interactions have become an important topic in restoration ecology (Harris, 2009). The development of root inoculants with the ability to colonize and to degrade phytotoxic PH compounds in the root system (rhizoremediation) might contribute to the development of novel strategies for environmental recovery, which may couple both reforestation and remediation of PH-impacted environments. A primary step to achieving this promise relies on the isolation and identification of the structural and functional components involved in the degradation of PH in the rhizosphere.

In this study, bulk sediment and rhizosphere (microniches) of healthy plants of three different species (*Avicenia schaueriana*, *Laguncularia racemosa* and *Rhizophora mangle*) were sampled in a mangrove site exposed to chronic levels of PH contamination. The microbial communities retrieved from the samples were then used in serial batch enrichment subculturing in the presence of petroleum to select for mangrove microbial guilds involved in the process of PH degradation. A range of molecular tools were used to investigate the influence of the original composition of the microbial communities of different mangrove microniches on the structural and functional diversity of enriched PH-degrading consortia (PHDC). The PHDC obtained will be used for future mangrove reforestation projects in PH-polluted sites.

Materials and methods

Growth of mangrove plants and sampling

The sampling approach of this study was designed to ensure sampling access to rhizospheres from *A. schaueriana*, *L. racemosa* and *R. mangle* at similar developmental stages. Briefly, mature mangrove propagules of *R. mangle*, *A. schaueriana* and *L. racemosa* were collected in mangrove forests located in Guanabara Bay (Rio de Janeiro, Brazil) and grown in a mangrove nursery. Sixteen plant saplings (~75 days old) from each mangrove species were replanted in a mangrove forest exposed to chronic levels of oil hydrocarbon contamination close to the petrochemical complex of Duque de Caxias (22°44′08″S/43°13′55″W) (Rio de Janeiro, Brazil). The sampling site characteristics were described previously (Gomes *et al.*, 2007, 2008).

The saplings were planted randomly in high intertidal zones with a distance of at least 1 m between each other. Bulk sediment and rhizosphere samples were taken 28 days after planting. Three composite replicates of bulk sediment (~20 cm of top sediment with 4 cm diameter) and roots of individual plants (three replicates) of each species were sampled. A spatula was used to remove the sediment that could be easily detached from the roots. Only the remaining sediment adhering to the plant root system was considered as the rhizosphere fraction. Each rhizosphere sample consisted of the total root system, which was cut and thoroughly mixed. Microbial cells were detached from rhizosphere and bulk sediment samples (5 g) as described previously in Gomes *et al.* (2007). The resulting microbial suspensions were used in the batch enrichment of PHDC.

Enrichment of PHDC

The enrichment of PHDC was performed by means of a serial batch culture approach. The first enrichment round consisted of inoculation with 5 mL of the microbial community suspensions obtained from rhizosphere and bulk sediment samples (as described above) in Erlenmeyer flasks containing 45 mL mineral salt medium (MSM) (Margesin & Schinner, 1997) supplemented with light petroleum (0.1% v/v) (cordially provided by Petrobras S.A.) as the sole added carbon source. Before medium amendment, the petroleum was sterilized in an oven at 180 °C for 2 h. Flasks without

inoculation were used as a control. The flasks were closed with nonabsorbent cotton wool and incubated at 27 °C in the dark under constant shaking for 5 days. Enrichments were continued for two more rounds by serial subculturing in the MSM medium amended with petroleum as described above, using a 10% inoculum from the previous culture. Aliquots of each enriched PHDC were stored with glycerol at −80 °C for follow-up studies. The concentration of total PH was determined in extracts from the batch cultures after the last enrichment round for each replicate by gas chromatography (GC), as described previously in the Environmental Protection Agency (http://www.epa.gov) standard method 8015. ANOVA was applied to determine whether there was significant removal of total PH by PHDC.

DNA extraction

After the third enrichment round, 1 mL from each enriched consortium was transferred to a Lysing Matrix E tube (Q Biogene) and the microbial cells were pelletized by centrifugation. Total community DNA was extracted using the BIO101 DNA extraction kit (Q Biogene) according to the manufacturer's recommendations. Mechanical lysis was achieved using the FastPrep FP120 bead-beating system (Q Biogene) 2 × for 30 s at a maximum vertical velocity of 5.5 m s^{-1}.

PCR amplification of 16S rRNA gene fragments and denaturing gradient gel electrophoresis (DGGE)

Amplified 16S rRNA gene fragments suitable for DGGE fingerprint analyses were obtained with bacterial DGGE primers F984-GC and R1378 (~473 bp) according to Heuer *et al.* (1997). A nested-PCR approach (25 thermal cycles) was also applied for the amplification of 16S rRNA genes of *Alphaproteobacteria*, *Betaproteobacteria* and *Pseudomonas* groups as described previously (Heuer *et al.*, 1997; Gomes *et al.*, 2001; Milling *et al.*, 2004). The DGGE of the amplified 16S rRNA gene fragments was performed using the INGENY PhorU System (INGENY, Goes, the Netherlands). Gels were prepared with a double gradient of 30–65% denaturants (100% denaturant: 7 M urea and 40% formamide) and 6–9% acrylamide. The electrophoresis run was carried out in 1 × Tris-acetate–EDTA buffer at 58 °C at a constant voltage of 240 V for 20 h. The gels were silver stained according to Heuer *et al.* (2001).

PCR amplification of naphthalene dioxygenase (*ndo*) gene fragments and *ndo* DGGE analyses

The detection of genes encoding enzymes involved in the degradation of low-molecular-weight polycyclic aromatic hydrocarbon (PAH) in the enrichment cultures was achieved by a nested-PCR approach targeting *ndo* genes using the method developed by Gomes *et al.* (2007). This PCR system targets environmentally relevant *ndo* genes belonging to the main clade of group III according to the classification system proposed by Nam *et al.* (2001). Amplified GC-clamped *ndo* gene fragments obtained after the second PCR round were applied to a double-gradient DGGE as described above for bacterial fingerprints.

Detection of broad host range (BHR) plasmids and *ndo* genes by PCR from total community DNA and Southern blot hybridization (SBH)

Before SBH, a PCR-based approach was used for the amplification of BHR plasmid-specific sequences and *ndo* genes from DNA extracted from environmental samples and from the enrichment consortia. The PCR amplification of *ndo* genes (first step *ndo* PCR) was performed as described previously (Gomes *et al.*, 2007). The amplification of plasmid-specific sequences of IncP-1 α and β (*trfA2* gene), IncP-7 (*rep* gene) and IncP-9 (*ori-rep* genes) was carried out as described previously (Götz *et al.*, 1996; Krasowiak *et al.*, 2002; Izmalkova *et al.*, 2005). The amplicons from *ndo* and BHR plasmid PCR were Southern blotted onto HYBOND N nylon membranes (Amersham Pharmacia Biotech) according to Sambrook *et al.* (1989). The probes for *ndo* genes were generated from PCR products of *nahAc* from *Pseudomonas putida* KT2442 (pNF142) (Gomes *et al.*, 2005), *phnAc* from a cloned *phnAc* gene fragment (Gomes *et al.*, 2005) and from the cloned *nagAc* gene fragment 4NDO-S3 (Gomes *et al.*, 2007). The probes for BHR plasmids were PCR-generated as described previously for IncP-1 α and β, IncP-7 and IncP-9 (Götz *et al.*, 1996; Krasowiak *et al.*, 2002; Izmalkova *et al.*, 2005). Hybridization was performed under conditions of medium stringency following the protocol published by Fulthorpe *et al.* (1995). Hybridization of DIG-labelled probes was detected using a DIG luminescent detection kit (Roche) as specified by the manufacturer and exposed to an X-ray film (Roche).

Comparative analyses of enriched PHDC

The DGGE gels were scanned transmissively and the digitalized DGGE profiles were analyzed using the software package GELCOMPAR 4.0 (Applied Maths, Sint-Martens-Latem, Belgium) as described by Smalla *et al.* (2001). The sets used for band detection were 5% minimal profiling (area along the densitometric curve) and 0.5% minimal area. The positioning and quantification of bands were carried out by setting tolerance and optimization at eight points, i.e. 0.8%. The band positions and their corresponding intensities from each PHDC were exported to Excel files and the band surface was converted to relative intensity by dividing its surface by the sum of all band surfaces in a lane. Bray–Curtis similarities were calculated based on the band position and intensity. The matrices of similarities were then used for multivariate analyses of DGGE

profiles using analysis of similarities (ANOSIM) with the PRIMER 5 software package (Primer-E Ltd, Plymouth, UK). The ANOSIM was used to test whether communities differed significantly. The *R* statistic in ANOSIM ranges from 0 to 1, with higher values indicating greater variation in the composition among the samples (Clarke, 1993). Moreover, the extent to which the variation in the presence and relative abundance of DGGE bands is explained by the origin of PHDC (i.e. sediment or rhizosphere samples) was addressed. To this end, ordination of PHDC fingerprinted by DGGE and their corresponding sources of isolation was performed following the procedures of Costa *et al.* (2006). Briefly, detrending correspondence analysis and detrending canonical correspondence analysis were carried out to estimate the gradient lengths of band relative intensity datasets. Based on these assessments, linear species response models – i.e., principal components analysis (PCA) and redundancy analysis (RDA) – were deemed to better fit the dataset and therefore used in unconstrained (PCA) and constrained (RDA) ordination analyses (Ramette, 2007). Focus on sample distances was used with further default parameters, as implemented in the software package CANOCO for windows 4.5 (Microcomputer Power, Ithaca, NY).

Sequence analyses

The most dominant bands selected from bacterial DGGE profiles were excised, cloned, screened and sequenced as described previously (Gomes *et al.*, 2008). One clone per band carrying the right insert with the DGGE mobility of each selected band was sequenced. The partial 16S rRNA gene sequences obtained were classified according to the Naive Bayesian ribosomal RNA Classifier (Version 1.0) of the Ribosomal Database Project II (http://rdp.cme.msu.edu/) and compared with sequences available in the GenBank database using BLAST-N (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were subjected to the Chimera Check program from RDP and deposited in the GenBank database under the accession numbers (HM003918–HM003925). The *ndo* gene sequence fragments for cloning were screened as described previously (Gomes *et al.*, 2007). Clones containing inserts that shared the electrophoretic mobility with dominant DGGE bands were selected for sequencing. BLAST-N was used for similarity searches for *ndo* gene sequences (~740 bp). Sequences of cloned *ndo* gene fragments were aligned with their closest relatives. A phylogenetic tree was then inferred using the neighbor-joining algorithm applied to a matrix of evolutionary distances calculated with the Maximum Composite Likelihood method and bootstrapping analysis using the Molecular Evolutionary Genetics Analysis (MEGA4) integrated software. The nonredundant *ndo* gene sequences obtained were deposited in the GenBank database under the accession numbers (HM003909–HM003917).

Microarray analyses

The microarray designed in this study was composed of probes targeting 16S rRNA gene sequences (137 probes) representing distinct bacterial groups across a variety of phyla (including known degraders of aromatic hydrocarbons) and gene members of the main evolutionary clusters (subfamilies) of extradiol dioxygenases type I (EXDO, vicinal chelate superfamily) (197 probes) and intradiol dioxygenases (INDO) (257 probes) involved in aromatic degradation. The probes targeting 16S rRNA genes were designed, according to the bacterial taxonomy of the Ribosomal Database Project (http://rdp.cme.msu.edu/), on a selection of sequences from type strains representing 19 phyla: *Chloroflexi*, *Thermomicrobia*, *Nitrospira*, *Deferribacteres*, *Cyanobacteria*, *Chlorobi*, *Actinobacteria*, *Planctomycetes*, *Chlamydiae*, *Spirochaetes*, *Fibrobacteres*, *Acidobacteria*, *Bacteroidetes*, *Fusobacteria*, *Verrucomicrobia*, *Dictyoglomus*, *Gemmatimonadetes*, *Firmicutes* and *Proteobacteria*. In the cases of *Firmicutes*, sequences from type strains of the different classes inside the families of *Clostridia*, *Mollicutes* and *Bacilli* were included. For *Proteobacteria*, we included expanded representation of type strain sequences of the following orders (in parentheses) inside Classes of *Alphaproteobacteria* (*Rhodospirillales*, *Rickettsiales*, *Rhodobacterales*, *Sphingomonadales*, *Caulobacterales*, *Rhizobiales*, *Parvularculales*); *Betaproteobacteria* (*Burkholderiales*, *Hydrogenophilales*, *Methylophilales*, *Neisseriales*, *Nitrosomonadales*, *Rhodocyclales*); *Gammaproteobacteria* (*Acidithiobacillales*, *Thiotrichales*, *Chromatiales*, *Xanthomonadales*, *Cardiobacteriales*, *Legionellales*, *Methylococcales*, *Oceanospirillales*, *Pseudomonadales*, *Aeromonadales*, *Alteromonadales*, *Vibrionales*, *Enterobacteriales*, *Pasteurellales*); *Deltaproteobacteria* (*Desulfurellales*, *Desulfovibrionales*, *Desulfobacterales*, *Desulfuromonales*, *Syntrophobacterales*, *Myxococcales*); and *Epsilonproteobacteria* (*Campylobacterales*).

The probes against catabolic genes were designed on two custom CDS databases for EXDO type I and INDO gene families, which were built based on protein sequences from reference enzymes, representing different evolutionary branches described for INDO (Eulberg *et al.*, 1998) and for EXDO type I (Eltis & Bolin, 1996). These sequences were used as seeds for BLASTP searches in order to upgrade the sequence diversity currently found in the databases. By collecting the CDS corresponding to the related proteins of BLASTP results and excluding the redundant representation of the same or near-identical sequences (> 99% similarity), the resulting databases had been used to design 50-oligomer probes (Chou, 2010) able to specifically detect, under the hybridization conditions assayed, each of their sequence targets with a similarity higher than 84%. According to MEGABLAST searches (GenBank nonredundant nucleotide database), all the probes designed have matches with highly related genes (95% similarity over the complete alignment of

the target and the related gene CDS or 16S rRNA gene). The full list of the genes targeted (including the GenBank number, abbreviation of the bacterial genus or species where the gene was found, strain name, gene name abbreviation) conforming to the final dataset used for probe design can be found in the Supporting Information, Fig. S1. These probes arrayed are able to collectively detect all the various types of evolutionary branches across the gene families or in the case of 16S rRNA gene, taxonomically highly related type strain sequences.

About 20 ng of DNA of the third PHDC replicate (Avi-c, Lag-c, Rhi-c and Sed-c) of each treatment was amplified by phi29 DNA polymerase using the Ultrafast Repli-G kit (Qiagen) according to the specifications of the manufacturer and the appropriate test of controls. After digestion with DNase turbo (Ambion), fragments were biotinylated using dUTP-biotin (Roche) and concentrated to a final volume of 20 μL in a vacuum centrifuge. Afterwards, the volume was adjusted to 100 μL with formamide buffer and hybridization was performed at 55 °C for 18 h. Slides were stained with streptavidine-Cy3 in TNB buffer at room temperature and washed with SSC buffer 0.1 × SDS 0.2% at 48 °C for 10 min. Before scanning, slides were rinsed with SSC buffer 0.1 × at room temperature. Scanning was performed in an Agilent system according to the manufacturer's instructions. Raw data intensities were normalized against the background using the formula (spot intensity − intensity background)/intensity background. Values for signals considered above the background as threshold were considered as positives. Controls of type genomes for which hybridization profiles were predicted as well as specific target gene copies vs. probes intensities were used to define the intensity threshold at which the experimental results fully matched the expected profiles (R. Vilchez-Vargas, unpublished data). All the microarray results presented are the average intensities of triplicate measurements per probe.

Results and discussion

Enrichment of PHDC

The quantitative and qualitative aspects of root exudates released by different plant species and at different plant growth stages will affect root colonization by responding bacteria and the synergistic interactions among these (Hedge & Fletcher, 1996; Jaeger *et al.*, 1999). Therefore, the sampling approach of this study was designed to ensure rhizosphere samples from different mangrove plant species at similar growth stages.

The GC analysis of *A. schaueriana* ($129.6 \pm 62\ mg\ L^{-1}$) and *L. racemosa* ($134.6 \pm 35.2\ mg\ L^{-1}$) rhizosphere PHDC (third transfer) showed a significant reduction in the total PH concentration in comparison with the control flasks ($301 \pm 109.3\ mg\ L^{-1}$) ($P < 0.05$). The final concentration of the total PH in the flasks containing *R. mangle* ($166.1 \pm 13.1\ mg\ L^{-1}$) and bulk sediment ($235.8 \pm 38.1\ mg\ L^{-1}$) PHDC were not significantly different from the control flasks ($P > 0.05$). Although not significant, the values for *R. mangle* and bulk sediment PHDC still show a trend toward an improved PH degradation. The petroleum volatile compounds (several *n*-alkanes and low-molecular-weight aromatic molecules) belong to the petroleum light fraction and are known to be more readily degraded than high-molecular-weight PH (Venosa *et al.*, 1996). The lack of significant differences between the control flasks and *R. mangle* and bulk sediment PHDC may be explained by the dominance of microbial guilds more specialized in the degradation of volatile PH compounds in these two cultures. Obviously, petroleum volatile compounds were also lost due to volatilization in the control flasks during the 5 days of the incubation period. However, this loss was not measured in this work. In contrast, flasks inoculated with *A. schaueriana* and *L. racemosa* rhizosphere PHDC showed a stronger decrease in the final concentration of the total PH. The higher efficiency of PH removal by *A. schaueriana* and *L. racemosa* rhizosphere PHDC suggests that the relative abundance and activity of specific PH-degrading guilds in these two enrichments were different from *R. mangle* and bulk sediment enrichments. Studies on plant–microorganisms interactions have shown that the influence of root exudation on the composition of microorganisms colonizing the rhizosphere is plant species specific and can selectively enhance specific microbial guilds (Grayston *et al.*, 1998; Neumann & Römheld, 2001). In order to demonstrate that the enrichments display a microniche-dependent structural and functional diversity of PHDC, a thorough DNA-based analysis was performed.

DGGE analyses of PHDC

Bacteria and taxon-specific DGGE analyses were used to compare the relative abundance of PHDC enriched from rhizosphere and bulk sediment microbial communities (see Fig. S2). While *Bacteria* and *Alphaproteobacteria* DGGE fingerprint analyses revealed complex profiles, a rather low complexity of DGGE ribotypes was detected for *Betaproteobacteria* and *Pseudomonas*. Differences in the complexity (number of bands) of *Bacteria* fingerprints were also observed between PHDC enrichments. With the exception of one replicate of *R. mangle* PHDC, the *Bacteria* fingerprints of rhizosphere PHDC showed a higher number of bands compared with PHDC from bulk sediment, suggesting that a larger number of populations adapted to growth on PH.

In this study, ANOSIM of bacterial DGGE profiles were used to test whether the differences observed between PHDC communities were significant (R) (Table 1). Values of $R > 0.75$ are considered well separated, values > 0.5 as moderately separated and values < 0.25 as poorly separated (Clarke, 1993; Ramette, 2007). With the exception of *R. mangle* PHDC, the statistical analysis of *Bacteria*

Table 1. ANOSIM statistics of Bray–Curtis similarity measures (*R*) of rhizosphere and bulk sediment PHDC

PHDC	*Bacteria* (*R*)	*Alphaproteobacteria* (*R*)	*Betaproteobacteria* (*R*)	*Pseudomonas* spp. (*R*)
Rhiz* vs. Sed†				
Avi‡	0.96	0.96	0	0.18
Lag§	0.96	0.59	0	0.18
Rhi¶	0.14	0.55	0	0
Rhiz vs. Rhiz				
Avi vs. Lag	1	0.14	0	0.40
Lag vs. Rhi	0.63	0.22	0	0
Rhi vs. Avi	0.11	0.70	0	0.14

*Rhizosphere.
†Bulk sediment.
‡Avi, *Avicenia schaueriana*.
§Lag, *Laguncularia racemosa*.
¶Rhi, *Rhizophora mangle*.

community fingerprints revealed significant separation between rhizosphere PHDC of different plant species and between rhizosphere and bulk sediment PHDC. The ANOSIM also indicated significant separation of *Alphaproteobacteria* communities from rhizosphere and bulk sediment PHDC. However, the comparison of rhizosphere PHDC only showed significant separation between *A. schaueriana* vs. *R. mangle* ($R = 0.70$). The analyses of *Betaproteobacteria* and *Pseudomonas* communities did not show significant differences between rhizosphere PHDC from different plant species nor between rhizosphere and bulk sediment PHDC. In general, the statistical analyses indicated that differences in the bacterial community structure of each PHDC were best depicted by *Bacteria* fingerprint analyses.

Further multivariate statistics performed using ordination techniques such as PCA and RDA supported the overall trends observed via ANOSIM. The PCA ordination biplot of PHDC bacterial DGGE fingerprints and their underlying environmental samples (Fig. 1) illustrate the clear differentiation between bulk sediment, *A. schaueriana* and *L. racemosa* PHDC across the ordination space. Taken together, these results indicate for the first time that the inoculum from different mangrove microniches determined the structural composition of the PHDC enrichments. Similar trends have been observed in previous studies based on terrestrial plants, which have shown that root exudates can induce plant species-specific changes on the structural diversity of rhizosphere bacterial communities (Gomes *et al.*, 2001; Smalla *et al.*, 2001; Costa *et al.*, 2006).

Sequence analysis of dominant DGGE bands

Selected dominant bands from *Bacteria* and taxon-specific DGGE fingerprints of PHDC were chosen for cloning and subsequent sequencing. The sequence analysis of all selected DGGE ribotypes revealed bacterial populations, which were assigned to three orders: *Pseudomonadales*, *Rhizobiales* and

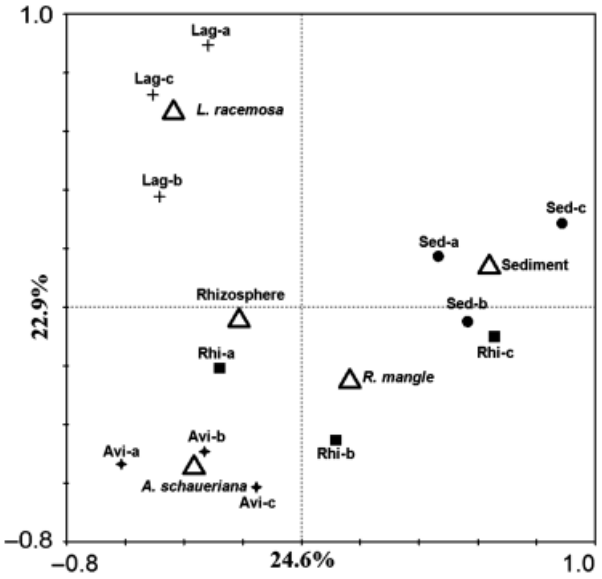

Fig. 1. PCA ordination biplot of PHDC of bacterial PCR-DGGE fingerprints and their corresponding sources of isolation. Sed, bulk sediment; Avi, *Avicenia schaueriana*; Lag, *Laguncularia racemosa*; Rhi, *Rhizophora mangle*.

Burkholderiales (Table 2). The sequences affiliated to the orders *Pseudomonadales* and *Burkholderiales* were associated with the most abundant populations detected in the PHDC enrichments. These sequences were often closely related to known PH degraders (Table 2). Sequence analyses of bands excised from *Bacteria* profiles revealed the dominance of *Acinetobacter venetianus* in nearly all PHDC, with the only exception of *L. racemosa* PHDC. In contrast, the results indicated that *Acinetobacter baumannii* was only enriched in *L. racemosa* PHDC. Members of the genus *Acinetobacter* are known for their ability to catabolize a wide range of carbon sources and for their importance in the process of emulsification and degradation of PH (Sar & Rosenberg, 1983; Pleshakova *et al.*, 2001; Koren *et al.*, 2003). The two

sequences derived from the *Betaproteobacteria* PHDC fingerprints were assigned with high confidence to *Comamonas testosteroni* and *Burkholderia* sp., bands 1bet and 2bet (see Fig. S2c, Table 2), respectively.

The 16S rRNA gene sequence analyses of bands excised from *Pseudomonas* profiles revealed the dominance of populations affiliated with *Pseudomonas aeruginosa* and *P. putida* (Table 2). These species are known versatile degraders of PH (Van Hamme *et al.*, 2003; Wongsa *et al.*, 2004). We have shown previously that *P. aeruginosa* populations were enhanced in mangrove sediments contaminated with PH (Gomes *et al.*, 2008). Brito *et al.* (2006) also reported the isolation of a *P. aeruginosa* strain from petroleum-contaminated mangrove sediments able to degrade fluoranthene and octadecane. Several environmental and clinical isolates of *P. aeruginosa* were also found to contain *alk* genes (Smits *et al.*, 2003), which can be assumed to belong to the core genome of this species (Palleroni *et al.*, 2010). The *alk* genes code for alkane hydroxylase, which is an important enzyme involved in the process of environmental degradation of petrogenic *n*-alkanes. These data suggest that *P. aeruginosa* might have a potential ecological role in the process of PH detoxification in mangrove sediments.

In contrast to the sequence analyses of bands retrieved from *Bacteria*, *Betaproteobacteria* and *Pseudomonas* fingerprints, which revealed typical PAH degraders in the enrichment consortia, the dominant bands retrieved from *Alphaproteobacteria* profiles (see Fig. S2b, 1alp and 2alp) were assigned to phylogenetic groups belonging to the *Rhizobiales* order. This order comprises several plant symbionts and nitrogen-fixing bacteria. Only recently have some members of this group been reported as degraders of aromatic hydrocarbon compounds (Baek *et al.*, 2003). Even though their potential role in the process of PH degradation in the plant root system remains largely unknown, a recent survey of aromatic degrading properties encoded in the genomes of > 900 sequenced bacteria indicated strains of the *Rhizobiales* order to be metabolically highly versatile (Pérez-Pantoja *et al.*, 2010). The sequence from the DGGE ribotype 1alp was similar to *Bosea* sp., a non-nitrogen-fixing *Rhizobiales* able to generate energy from the oxidation of reduced sulfur compounds (chemolithoheterotroph) (Das *et al.*, 1996), which was previously shown to be able to degrade PAH (Seo *et al.*, 2007). The closest relative of the cloned sequence retrieved from band 2alp could not be assigned with good confidence to any family and its closest relative in the GenBank was an uncultured bacterium clone (Table 2).

PCR-SBH analyses

The PCR-SBH analysis of total community DNA extracted from environmental samples revealed positive signals for only a few replicates (Table 3), suggesting that *ndo* genotypes (*nag*, *phn* and *nah*) and BHR plasmids (IncP-1α, IncP-1β, IncP-7 and IncP-9) were in low abundance in the rhizosphere and bulk sediments analyzed. Nevertheless, the different plasmid groups and *ndo* genes were enriched from these same samples after PHDC enrichments (Table 3). These results indicate that several biological components involved in the process of PH removal are initially present below the detection limit of the technique applied in both mangrove rhizosphere and bulk sediments. Despite the differences in the hybridization signals, SBH analysis showed that the *ndo* genotypes and BHR plasmids were strongly enriched in the PHDC. Populations carrying plasmid groups belonging to IncP-9, IncP-1α and IncP-1β were enriched in most of the PHDC (Table 3). The IncP-9 plasmid is a known group of self-transmissible plasmids responsible for horizontal transfer of catabolic genes

Table 2. Results of partial 16S rRNA gene sequence analysis and tentative affiliations of selected DGGE ribotypes

		Closest phylogenetic relative		
DGGE band type*	Accession no.†	BLAST-N identity	%	Accession no.‡
Bacteria				
1bac	HM003919	*Acinetobacter venetianus*	100	DQ912805
2bac	HM003923	*Acinetobacter baumannii*	100	FN563424
Alphaproteobacteria				
1alp	HM003918	*Bosea* sp.	100	DQ104981
2alp	HM003922	Uncultured bacterium	99	EU234233
Betaproteobacteria				
1bet	HM003920	*Comamonas testosteroni*	99	GQ259481
2bet	HM003924	*Burkholderia* sp.	99	AB299596
Pseudomonas				
1pse	HM003921	*Pseudomonas aeruginosa*	100	GU447238
2pse	HM003925	*Pseudomonas putida*	99	GU396284

*Codes refer to the bands shown in Fig. S1.

†GenBank sequence accession numbers of the respective clone.

‡GenBank sequence accession number of the most closely related bacterial sequence.

Table 3. PCR SBH analyses of plasmid groups and naphthalene dioxygenase genes of mangrove environmental samples (MES) (rhizospheres and sediment) and their respective enriched PHDC

	A. schaueriana			*L. racemosa*			*R. mangle*			Sediment		
MES	a	b	c	a	b	c	a	b	c	a	b	c
IncP-1α												
IncP1β			+					+		+		
IncP-7												
IncP-9												
nahAc												
phnAc												
nagAc					+					+		
PHDC												
IncP-1α	++	(+)	++	+	++	++	(+)		(+)	++	(+)	++
IncP1β	+	(+)	+	+	++	++	+		(+)	++	++	++
IncP-7	+	(+)						(+)			++	
IncP-9	++	++	++	+	+	++	++	++	++	++	+	
nahAc	(+)	(+)	(+)	(+)	+		(+)	+			(+)	(+)
phnAc	++	++	++				+	++				
nagAc	++	++	++	++	++	++	++	++	++	++	++	++

(+), weak hybridization (when faint bands were detected); +, hybridization (dark and sharp bands); ++, strong hybridization (dark oversized band area).

encoding PH (e.g. xylene, toluene and PAH) catabolism (Dennis, 2005; Sota *et al.*, 2006). Probably, the enrichment of this group of plasmids in nearly all PHDC occurred due to the selection of plasmid-encoded PH catabolic traits. Curiously, SBH signals for IncP-1α and β were stronger in *A. schaueriana*, *L. racemosa* and bulk sediment PHDC than in *R. mangle* PHDC. The IncP-1 group encompasses the most promiscuous self-transmissible plasmids known to date, which often carry genes coding for the degradation of man-made chloroaromatic compounds and resistance against antibiotics and metals (Dennis, 2005; Smalla *et al.*, 2006; Schlüter *et al.*, 2007). The IncP-1 group may have been spread through the PHDC bacterial population due to the growth of a plasmid-containing population or horizontal spread as different studies have shown that IncP-1 plasmids can also be efficiently transferred to bacterial populations even in the absence of selection pressure (Pukall *et al.*, 1996; Fox *et al.*, 2008). Surprisingly, plasmids belonging to IncP-7 were poorly enhanced in the PHDC. Plasmids belonging to this group are known to possess complex genetic rearrangements that can help the host cell to adapt to environmental changes (Dennis, 2005). This group is also deemed to include important mobile elements for spreading genes encoding PH degradation.

The SBH analyses of *ndo* genes detected the enrichment of *nahAc*, *nagAc* and *phnAc* genes in the PHDC (Table 3). However, while *nahAc* and *phnAc*-like genes were enhanced in few PHDC, the results indicated the enhancement of genotypes closely related to *nag* genes in all cultures. In agreement with these results, the *ndo* DGGE analyses (Fig. 2) revealed the dominance of one band (*ndo* DGGE band type 1) in nearly all PHDC. The phylogenetic analysis of cloned sequences matching these bands showed a close phylogenetic relationship with the protein encoded by the *pahAc* gene of *C. testosteroni* H (Fig. 3). The *pah* gene of *C. testosteroni* H has a high sequence similarity to the *nag* operon (Moser & Stahl, 2001). The abundance of populations carrying the *nagAc*-like genes was previously shown to be enhanced in a mangrove sample from sites under chronic exposure to PH (Gomes *et al.*, 2007). Bulk sediment samples from the same sampling site were used in the PH enrichments performed in this work. The *nagAc* genotype has been detected in PH-contaminated sites in different geographic locations all over the world (Widada *et al.*, 2002; Jeon *et al.*, 2003; Gomes *et al.*, 2007). A study by Dionisi *et al.* (2004) has shown that indigenous bacteria carrying *nagAc*-like genes play an important ecological role in the process of *in situ* degradation of PAH. Previous studies have also indicated that *phnAc* genotypes similar to those found in *Burkholderia* sp. RP007 are ubiquitous genes often detected in PH-contaminated soils (Laurie & Lloyd-Jones, 1999; Lloyd-Jones *et al.*, 1999; Wilson *et al.*, 2003; Gomes *et al.*, 2005). Both molecular approaches used in this study for *ndo* gene analyses (SBH and DGGE) revealed that *phnAc*-like genes related to *Burkholderia* sp. RP007 were abundant only in replicates of *A. schaueriana* PHDC and two replicates of *R. mangle* PHDC. *Burkholderia* sp. RP007 was isolated previously from contaminated soil samples in New Zealand (Laurie & Lloyd-Jones, 1999). Our results suggest that while *nag* genotypes might be ubiquitously distributed in bulk and rhizosphere sediments, bacterial guilds carrying *phn*-like genes may have a more specific association with the rhizospheres of *A. schaueriana* and *R. mangle*.

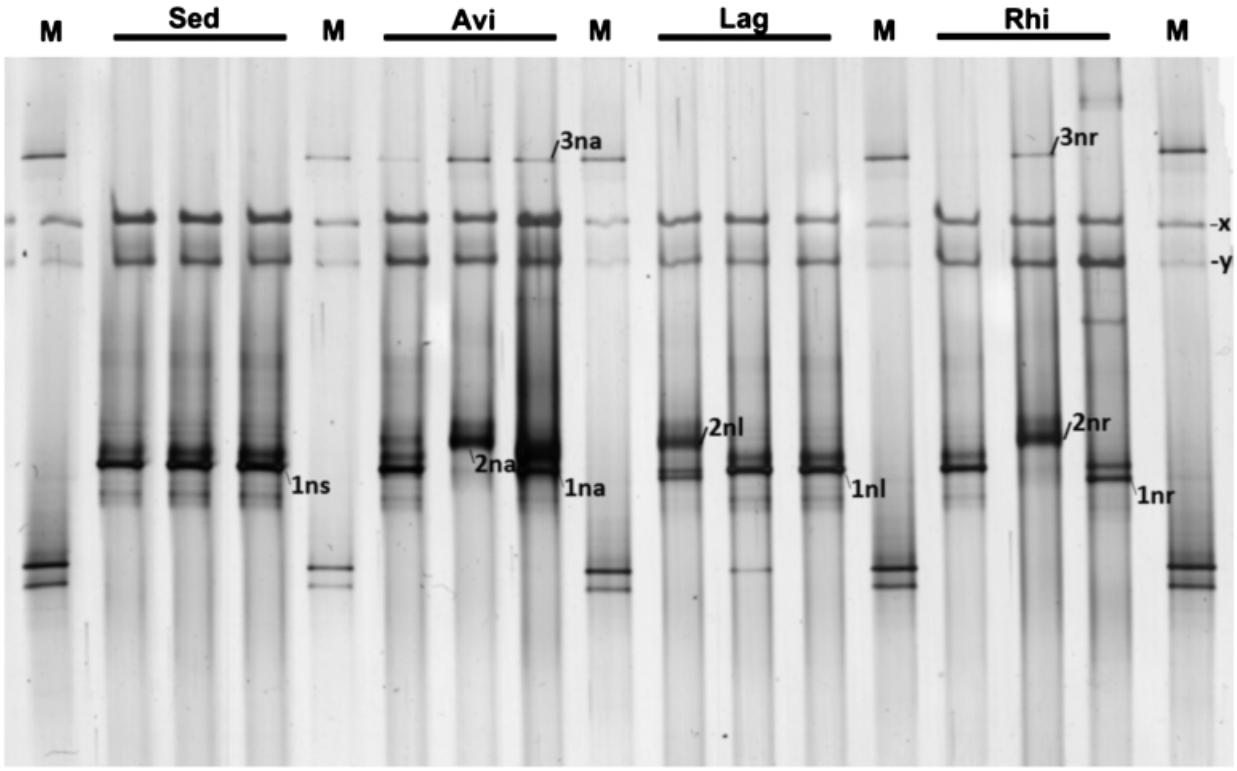

Fig. 2. DGGE fingerprints of *ndo* gene fragments amplified from DNA templates extracted from PHDC. Sed, Bulk sediment; Avi, *Avicenia schaueriana*; Lag, *Laguncularia racemosa*; Rhi, *Rhizophora mangle*. The bands x and y are silver-stained single-strand DNA. The band positions indicated in the gel correspond to the melting behavior of selected representative *ndo* gene clones that matched dominant genotypes. From top to bottom, *ndo* gene fragments used as markers (M): phnAc (environmental clone), nahAc (*Pseudomonas putida* KT2442 – pNF142) (Gomes *et al.*, 2005) and nahAc (*Pseudomonas* sp. ARS 10) (I. Kosheleva, unpublished data).

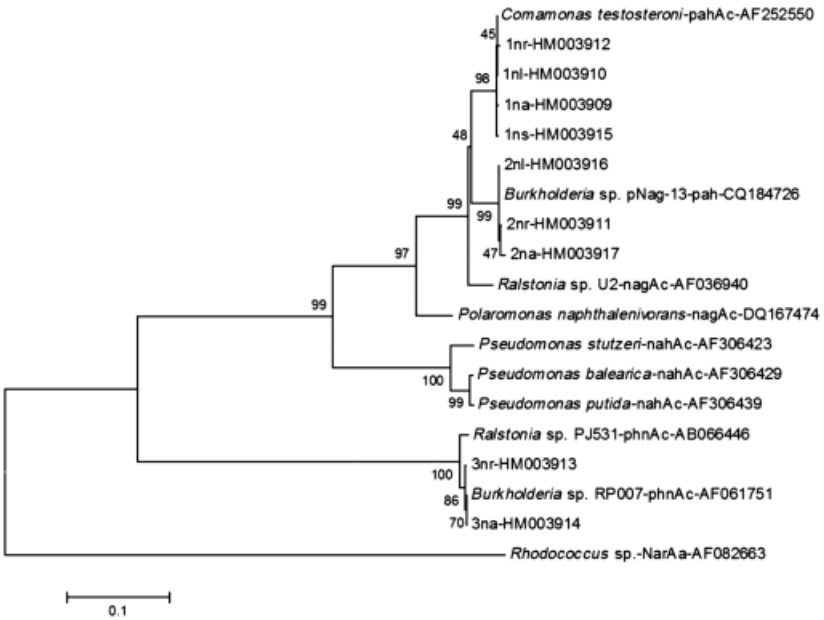

Fig. 3. Phylogenetic relationships of DNA sequences of the large α-subunits of naphthalene dioxygenase genes. The sequences were aligned with related sequences retrieved from GenBank. The *narAa* gene from a PAH-degrading gram-positive bacterium (*Rhodococcus* sp. NCIMB12038) was used as an outgroup. The numbers on the branches indicate the percent of bootstrap values.

Diversity and affiliation of 16S rRNA and aromatic ring-cleavage genes based on microarray analyses

A microarray was specifically designed (unpublished) to determine profiles of 16S rRNA genes and aromatic ring-cleavage (*meta*- and *ortho*-cleavage pathways) encoding genes. The arrays can detect all the main branches described for the families targeted, and as such, can detect a range of highly divergent branches in the family, but, nevertheless, rely on previous sequence knowledge and each probe is also able to detect highly similar genes (>90%) to the sequence targeted. With the exception of *L. racemosa* PHDC, patterns of positive signals above the background threshold were obtained for DNA samples extracted from PHDC. Microarray analysis of *L. racemosa* PHDC showed hybridization problems that could not be solved and only two taxon-specific probes (*Bacillus cereus* ATCC27877 Z84581 S000005826 and *Pseudomonas psychrophila* E-3 AB041885 S000002852) had signals above the background threshold (data not shown). It was found that the majority of taxa microarray signals (see Table S1) were detected by probes targeting *Pseudomonadales* 16S rRNA genes, in accordance with DGGE results (see Fig. S2 and Table 2). However, some dominant populations detected by PCR-DGGE were not detected by microarray analysis. Such a difference may be due to differences in the sensitivity of both techniques and most probably due to the fact that the microarray designed in this study has a limited number of taxon-specific (16S rRNA gene) probes designed based on species type strains.

Probes targeting EXDO and INDO detected genes encoding various enzymes responsible for the *ortho*- and *meta*-cleavage of catechols and chlorocatechols (catechol 1,2 dioxygenases, C12O) (Tables 4 and 5). The INDO family comprises different evolutionary branches, such as catechol 1,2-dioxygenases of *Proteobacteria*, those of *Actinobacteria*, chlorocatechol 1,2-dioxygenases, hydroxyquinol dioxygenases and the α and β subunits of protocatechuate 1,2-dioxygenases (Pérez-Pantoja *et al.*, 2010). Prominent signals were observed, indicating the abundance of catechol 1,2-dioxygenase and protocatechuate dioxygenases encoding genes of *Burkholderia* and *Pseudomonas* spp., genes typically belonging to the core genome of members of these genera (Pérez-Pantoja *et al.*, 2010). These results are in accordance with the observation of a high abundance of such species in the samples analyzed. Also, genes encoding catechol 1,2-dioxygenases of *Actinobacteria* could be observed in all samples. This group of bacteria has not been targeted in PCR assays, but may be important and must be tracked in detail in future studies of the ecosystem under analysis. Probes specifically designed for chlorocatechol 1,2-dioxygenase encoding genes, such as the ones targeting *Pseudomonas* sp. P51 (P27098), *Delftia acidovorans* P4a (AAC35836) or *P. aeruginosa* JB2 (AAC69474) only showed hybridization signals for *A. schaueriana* enrichment. The reason for the specific abundance of such genes remains to be elucidated. Hydroxyquinol dioxygenase encoding genes were observed in high abundance in rhizosphere PHDC, possibly due to the enrichment of organisms harboring such genes by root exudates.

Whereas multiple signals for INDO-encoding genes were observed, which, with the exception of chlorocatechol 1,2-dioxygenases, show a typical chromosomal location and therefore can be regarded as a kind of phylogenetic marker (Pérez-Pantoja *et al.*, 2010), only a few signals due to the presence of genes encoding EXDO of the vicinal chelate superfamily were evident. In fact, microarray results detected genes encoding EXDO of the subfamily I2A (according to the nomenclature of Eltis & Bolin, 1996), which have been described previously as being responsible for extradiol cleavage of catechol generated from toluene after monooxygenation of the side chain (Nakai *et al.*, 1983), from naphthalene after 1-hydroxylation of intermediate salicylate (Bosch *et al.*, 2000), from phenol by soluble diiron multicomponent oxygenases (Bartilson & Shingler, 1989) or from benzene and toluene after dioxygenolytic attack (Junca & Pieper, 2004), and that are typically harbored by *Pseudomonas* spp. strains. Also, the presence of genes encoding EXDO of the subfamily I2C, often observed as chromosomally encoded in *Burkholderiales* involved in phenol degradation, was observed. In contrast, signals indicating the presence of genes similar to *Ralstonia* sp. U2 *nagC* (GenBank accession number AF036940) encoding 1,2-dihydroxynaphthalene dioxygenases involved in naphthalene degradation and typically encoded in the same gene cluster as *nagAc*, observed in PHDCs by PCR-based profiling, were not detected. This obviously does not mean that the associated genes are not present or enriched in PHDCs, but may rather occur in quantities too low to yield signals above the threshold of normalization. Taking into account that a complex mixture of petroleum hydrocarbons was used as an enrichment substrate, this is not surprising and the enrichment may have overall selected for a complex community of alkane and monoaromatic degrading *Proteobacteria* and *Actinobacteria*, only some of which have the capability to degrade higher molecular weight aromatics. Light petroleum generally possesses about 15% of its weight in aromatic compounds, where the lightest aromatic fractions (benzene, toluene, ethylbenzene and xylenes) are by far the most abundant aromatic hydrocarbons (Wang *et al.*, 2003). Therefore, it is possible that in this study, the initial mechanisms to break high-molecular-weight polyaromatics could be a minority compared with the number of genes encoding mechanisms to cleave, for example, *n*-alkanes and monoaromatics. It is certainly an aspect to be studied in further works applying tools to achieve the detection of a broader set of catabolic gene families (i.e. including the families responsible for ring activation mechanisms such as initial dioxygenases

Table 4. Positive hybridization signals of DNA extracted from PHDC with the microarray probes against 257 CDS of protein members representing all the main phylogenetic branches of the INDO family (see Fig. S2)

INDO gene target*	*A. schaueriana*	*R. mangle*	Sediment
Acinetobacter lwoffii K24 catA U77658		0.68 ± 0.18†	
Arthrobacter sp. BA-5-17 catA BAD11154		0.99 ± 0.80	
Aspergillus fumigatus Af293 pcatA AAHF01000008	1.17 ± 1.09		
Brevibacterium linens BL2 pPC34DO NZAAGP01000002	0.85 ± 0.30	0.92 ± 0.82	1.75 ± 1.22
Brucella suis 1330 pcaC AE014292	0.61 ± 0.03	1.16 ± 1.27	0.54 ± 0.48
Burkholderia cenocepacia AU pPC34DO2 YP_624054	0.86 ± 0.32	1.23 ± 0.63	
Burkholderia cenocepacia AU10 pPC34DO NZAAHI01000020	0.71 ± 0.24	1.41 ± 1.59	0.55 ± 0.26
Burkholderia mallei 10399 pPC34Do NZAAHN01000025	0.56 ± 0.14		
Burkholderia mallei ATCC23344 pcaH NC006349	0.84 ± 0.43	0.95 ± 0.54	0.61 ± 0.38
Burkholderia mallei NCTC10247 pPC34DO NZAAHP01000043	1.67 ± 0.28		
Burkholderia pseudomallei 171 pPC34DO NZAAHS01000030	0.60 ± 0.39	0.61 ± 0.33	
Burkholderia pseudomallei 668 pPC34DO NZAAHU01000001	2.72 ± 1.68	2.90 ± 1.52	2.38 ± 1.12
Burkholderia pseudomallei 668 pPC34DO NZAAHU01000032	0.88 ± 0.52	0.76 ± 1.32	
Burkholderia pseudomallei K96243 pca NC006351	0.70 ± 0.47	0.72 ± 0.40	0.50 ± 0.36
Burkholderia pseudomallei NCTC 10229 pPC34DO YP_001024070	0.66 ± 0.38	1.05 ± 1.19	
Burkholderia pseudomallei S13 pPC34DO NZAAHW01000091	1.04 ± 0.25	0.66 ± 0.40	
Burkholderia sp. c12o TH2 BAC16769	0.54 ± 0.35	0.56 ± 0.44	
Burkholderia vietnamiensis G4 NZAAEH02000012			0.59 ± 0.24
Cupriavidus necator CH34 pC12O YP_587012			0.50 ± 0.32
Cupriavidus necator JMP134 catA YP_298335	0.57 ± 0.27		
Delftia acidovorans P4a chloro-C12O AAC35836	0.56 ± 0.29		
Jannaschia sp. pPC34DO YP_512105	1.37 ± 0.74	0.81 ± 0.68	0.83 ± 0.82
Nocardia sp. H171 catA AY613438		0.80 ± 0.85	0.79 ± 0.68
Polaromonas sp. JS666 pPC34DONZAAFQ02000022	0.97 ± 0.79	0.81 ± 0.57	0.69 ± 0.50
Polaromonas sp. pC12O YP_548892	0.54 ± 0.30	1.05 ± 0.59	
Pseudomonas aeruginosa FLH04754001 catA AAT51180	0.62 ± 0.46	0.59 ± 0.65	
Pseudomonas aeruginosa JB2 chloro-C12O AAC69474	0.69 ± 0.36		
Pseudomonas aeruginosa PAO1 catA NC002516	0.72 ± 0.18	0.52 ± 0.18	
Pseudomonas balearica catA CAG15365		1.23 ± 0.40	0.97 ± 0.45
Pseudomonas putida KT2440 pC12O NP_745846	0.70 ± 0.13	1.42 ± 1.06	1.97 ± 0.68
Pseudomonas putida mt-2 pCN12 D37782	0.87 ± 0.51	1.19 ± 0.96	0.68 ± 0.41
Pseudomonas putida PRS1 catA U12557	0.64 ± 0.29		
Pseudomonas sp. CA10 catA AB047272	0.82 ± 0.57	0.95 ± 0.57	0.59 ± 0.47
Pseudomonas sp. P51 chloro-C12o P27098	0.63 ± 0.36		
Pseudomonas stutzeri ATCC 27951 catA AJ617514	1.03 ± 0.50	1.17 ± 0.23	0.77 ± 0.57
Pseudomonas stutzeri CCUG11256 catA AJ617513		0.68 ± 0.25	
Pseudomonas synthetic construct catA AAT51180	0.61 ± 0.29	0.85 ± 0.16	0.98 ± 0.92
Pseudomonas syringae pv. *syringae* B72 pPC34DO CP000075	1.23 ± 0.60	1.14 ± 0.61	0.55 ± 0.37
Rhodobacterales bacterium Y4I pPC34DO1 YP_002693941	0.56 ± 0.14	0.70 ± 0.19	0.52 ± 0.35
Rhodococcus opacus 1CP pcaH AF003947		0.57 ± 0.29	
Rhodococcus rhodochrous NCIMB 13259 C12O AAC33003	0.51 ± 0.42		
Roseovarius nubinhibens ISM pPC34DO ZP_00961395	0.64 ± 0.31		
Rubrobacter xylanophilus DSM 9941 PC34DO YP_644355	0.61 ± 0.51		
Ruegeria pomeroyi DSS-3 pPC34DO YP_164875	1.45 ± 1.12	0.75 ± 0.38	1.26 ± 0.65
Solibacter usitatus Ellin6076 pPC34DO YP_821544	0.52 ± 0.18		1.15 ± 0.46
Streptomyces coelicolor A3(2) SCO66 AL939128	0.61 ± 0.29		
Streptomyces setonii catA AAK14065	0.56 ± 0.14	0.70 ± 0.19	0.52 ± 0.35
Streptomyces setonii catB AF277051	0.71 ± 0.28	1.65 ± 1.22	0.83 ± 0.39
Streptomyces setonii catA AAK14065	0.66 ± 0.30	0.60 ± 0.38	0.82 ± 0.52
Streptomyces sp. 2065 PC34DO AAD05270		0.56 ± 0.34	
Uncultured organism C12O AAW82364		2.56 ± 1.82	1.59 ± 0.67
Xanthomonas axonopodis pv. *citri* str. 306 pPC34DO NP_640723	1.76 ± 0.34	5.39 ± 2.76	5.10 ± 1.12
Xanthomonas campestris pv *campestris* pPC34DO NC003902a	0.52 ± 0.15		
Xanthomonas campestris pv. *campestris* str. ATCC pPC34DO NP_635762	0.77 ± 0.61		0.85 ± 0.40
Xanthomonas oryzae pv. *oryzae* KACC10331 pPC34Do YP_199121	0.88 ± 0.51	1.01 ± 0.39	0.78 ± 0.45

DNA extracted from PHDC; *Avicenia schaueriana*; *Rhizophora mangle* and bulk sediment.

*INDO gene targets are described with the genus or species name and bacterial strain identification where the aromatic ring-metacleavage protein member of the INDO family has been found, followed by the gene abbreviation and its GenBank accession number.

†Signal intensities detected in DNA extracted from the PHDC of bulk sediment (Sediment), *A. schaueriana* and *R. mangle* above the significant relative intensities are shown as the triplicate mean values with the corresponding standard deviations.

C12O, catA or catB: catechol 1,2-dioxygenase.

pPC34DO, pca, or pcaH: putative (predicted from annotations of genome projects) protocatechuate 3,4-dioxygenase.

Table 5. Positive hybridization signals of DNA extracted from PHDC with the microarray probes against 197 CDS of protein members representing all the main phylogenetic branches of the EXDO family (see Fig. S2)

EXDO gene target*	*A. schaueriana*	*R. mangle*	Sediment
Burkholderia vietnamiensis G4 c23o AAEH02000069	0.92 ± 0.59	0.77 ± 0.31	0.53 ± 0.39†
Comamonas testosteroni TA441 tesB AB040808		0.84 ± 0.23	
Nocardia farcinica IFM10152 nfa30490 AP006618	0.52 ± 0.02	1.01 ± 0.43	1.28 ± 0.63
Pseudomonas putida MT53 xylE pWW53 AF102891	1.14 ± 0.71	1.05 ± 0.62	0.86 ± 0.66
Pseudomonas stutzeri OX1 cdo AJ496739	0.94 ± 0.41	0.66 ± 0.28	0.65 ± 0.30
Sphingomonas aromaticivorans F199 xylE pNL1 AF079317		1.04 ± 0.26	1.13 ± 0.14
Sphingomonas sp. CHY-1 phnC AJ633552			0.57 ± 0.19

*EXDO gene targets are described with the genus or species name and bacterial strain identification of the microorganism where the aromatic ring-metacleavage protein member of the EXDO family has been found, followed by the gene abbreviation and its GenBank accession number.
†Signal intensities detected in DNA extracted from the PHDC bulk sediment (Sediment), *Avicenia schaueriana*, *Rhizophora mangle* above the significant relative intensities (above threshold of 0.5 normalized against the overall background) are shown as the triplicate mean values with the corresponding standard deviations.

and monooxygenases), analogous to the approach for INDO and EXDO performed in this work.

Conclusions and future research

The identification of functionally important microbial components involved in the degradation of environmental pollutants is a primary step toward understanding the ecological mechanisms of environmental recovery. In this study, the detection of the functional components involved in the process of PH decontamination in mangrove microniches was enhanced due to the combination of batch enrichment cultures and molecular analyses. This approach allowed us to identify the bacterial phylotypes, plasmids and degrading genes potentially involved in the removal of PH in rhizosphere and bulk sediments of a mangrove chronically polluted with oil hydrocarbons. In general, the functional genes and plasmids involved in the process of PH removal detected in the PHDC were below the detection levels in the environmental samples. The molecular analyses of PHDC support the notion that differences in the composition of the original communities used for the enrichments triggered the structural and functional differences between PHDC. The differences in the composition of enriched hydrocarbonoclastic bacterial communities and specific degrading genes between PHDC may reflect the adaptation of the microbial communities to preferential substrates available in their original microniches.

The isolation of plant beneficial microorganisms for plant inoculation is a common agricultural practice already established for centuries. Basically, microorganisms are isolated from environmental samples (e.g. rhizospheres, plant tissues) and screened for the desired phenotypic characteristic for further use in agriculture as plant growth promoters. This same principle has been hypothesized to promote phytoremediation and plant growth in the presence of phytotoxic PH (Barac *et al.*, 2004; Germaine *et al.*, 2009). This approach can be especially interesting for the recovery of PH-polluted mangrove forests. Rhizoengineering approaches based on the combination of replanting mangrove methodologies and plant root inoculation with PH-degrading bacterial guilds and plasmids can be a promising approach that may couple both reforestation and remediation of PH-impacted mangrove forests. The rhizosphere PHDC recovered in this study can be evaluated in future investigations as a starting point to engineer efficient plant–microorganism consortia aimed at improving replanted sapling survival and growth in PH-impacted mangrove forests. In addition, they most likely constitute suitable reservoirs for future metagenomics exploration of gene clusters or for the domestication of novel bacteria involved in PH degradation.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Phylogenetic distribution of the genes types belonging to INDO (A) and EXDO type I (B) associated to the probes producing microarray signals detected in DNA extracted from the PHDC cultures.
Fig. S2. Comparison between rhizosphere and bulk PHDC by DGGE of 16S rRNA gene fragments amplified from sediment DNA templates.
Table S1. Major relative signal intensities of the probes targeting 16S rRNA gene of 137 different phylotypes in the DNA extracted from PHDC, *Avicenia schaueriana*, *Rhizophora mangle* and Bulk sediment.

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