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Characterization of hydrocarbonoclastic bacterial communities from mangrove sediments in Guanabara Bay, Brazil

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Abstract

Hydrocarbonoclastic bacterial communities inhabiting mangrove sediments were characterized by combining molecular and culture-dependent approaches. Surface sediments were collected at two sampling sites in Guanabara Bay (Rio de Janeiro, Brazil) and used to inoculate in vitro enrichment cultures containing crude oil to obtain hydrocarbonoclastic bacterial consortia. In parallel, in situ mesocosms (located in the Guapimirim mangrove) were contaminated with petroleum. Comparison of bacterial community structures of the different incubations by T-RFLP analyses showed lower diversity for the enrichment cultures than for mesocosms. To further characterize the bacterial communities, bacterial strains were isolated in media containing hydrocarbon compounds. Analysis of 16S rRNA encoding sequences showed that the isolates were distributed within 12 distinct genera. Some of them were related to bacterial groups already known for their capacity to degrade hydrocarbons (such as *Pseudomonas, Marinobacter, Alcanivorax, Microbulbifer, Sphingomonas, Micrococcus, Cellulomonas*, *Dietzia*, and *Gordonia* groups). Other strains, with high capacity for degrading hydrocarbons (aliphatic or aromatic), were related to isolates from hydrothermal vents that have not been thus far detected in hydrocarbon-contaminated sites, nor described for their ability to grow or degrade petroleum hydrocarbons. Degradation studies showed the ability of *Marinobacter, Alcanivorax* and *Sphingomonas* isolates to degrade both PAH and alkane compounds. Our results point out the rich microbial diversity of the mangroves, whose potential for hydrocarbon degradation is promising for future studies on pollutant bioremediation.

Keywords: Biodegradation; Biodiversity; Bacteria

1. Introduction

Mangroves are intertidal ecosystems along the coastlines of tropical and subtropical regions. They constitute important nurseries for fish, crustaceans and birds. The mangroves of the Guanabara Bay (Rio de Janeiro, Brazil) are examples of such ecosystems. One of the major problems in Guanabara Bay is the discharge of untreated domestic effluents, originating from the metropolitan area of Rio de Janeiro, that daily enter this ecosystem at high fluxes. In addition, the entire system is affected by wastewater input from about 14 000 companies located around the Guanabara Bay area, and is subjected to high sedimentation rates [17]. Another problem, in the northwestern part of this bay, is the input of petroleum from tanker traffic, atmospheric input, and urban and industrial discharges, which together amount to 17 tons per day [17].

The Guanabara Bay was also subjected to catastrophic accidents like the oil spill which occurred in January 2000 when a collapsed pipeline in the NE part of the bay spread approximately 1.3 million tons of oil, contaminating the surrounding

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beaches and especially the protected area of Guapimirim mangrove. Despite the general environmental degradation of Guanabara Bay, the Guapimirim mangrove is still preserved.

Biodegradation by microorganisms is the primary mechanism in elimination of hydrocarbons and xenobiotic substances [5]. The capacity of a number of microorganisms from different aquatic environments to degrade hydrocarbons has already been described [5,41]. However, although the mangrove ecosystems have been extensively studied, few studies have focused on the capacity for oil biodegradation. Most of these studies report growth stimulation of hydrocarbon-degrading microorganisms [10,11,41], measure the effect of oil pollution on microbial communities and activities [14] and estimate the biodegradation potential of strains isolated from hydrocarbon-contaminated environments [48,49].

Further characterization of bacterial diversity, and an evaluation of the degradation capacities of the microorganisms inhabiting mangrove sediments, would provide new insights for improving the management of such environments. Recent advances in microbial ecology render it possible to combine molecular and culture-dependent approaches in order to describe bacterial diversity at environmental sites. Molecular methods based on 16S rRNA gene analyses are useful for describing community structures, while bacterial isolation is needed to characterize the degradation pathways. T-RFLP (terminal restriction fragment length polymorphism) has been shown to be effective in distinguishing between microbial communities in a range of environments [13,29].

The main objective of the present work was to characterize hydrocarbon-degrading bacterial communities inhabiting mangrove sediments. From environmental samples, a bacterial community was selected and its degradation potential was estimated in in situ mesocosms in the Guapimirim mangrove. Bacterial strains able to degrade hydrocarbon compounds were isolated from enrichment cultures inoculated with both mesocosms and environmental sediments. The bacterial composition of the different incubations was analyzed by T-RFLP, and the isolated bacterial strains able to degrade hydrocarbon compounds were characterized.

2. Materials and methods

2.1. Description of sampling sites

Guanabara Bay (Brazil, Rio de Janeiro) can be subdivided into four areas: (i) the northeastern part is the most well preserved area, presenting a lower human population density, drained by less polluted rivers and where the remaining mangroves present favorable conditions; (ii) in the central part lies the navigation channel, with good water circulation; (iii) the northwestern section is the most severely impacted area, in which are located petroleum refinery plants and activities of oil storage and transfer; (iv) the southern part is influenced by urban residues from the large metropolitan area of Rio de Janeiro city. The two sampling sites (Fig. 1) were located at Boa Viagem beach and the Guapimirim mangrove. Boa Viagem beach, at the entrance to Guanabara Bay, is exposed to chronic oil pollution due to boat traffic. Guapimirim is a preserved mangrove area, part of the larger mangrove that originally covered 285 km^2 of the Guanabara Bay littoral, but that is now restricted to a 135 km^2 area.

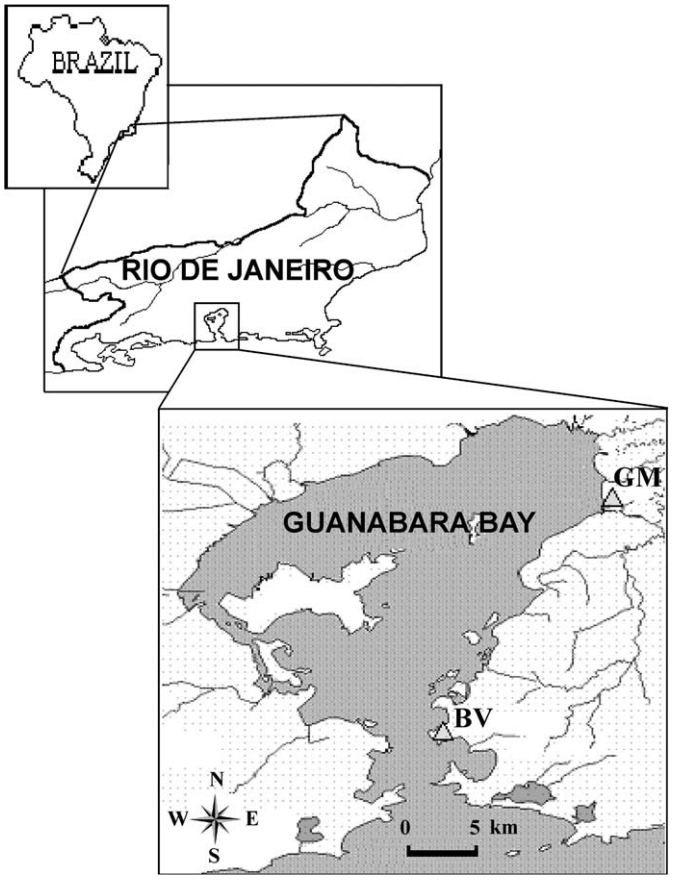


Fig. 1. Map of Guanabara Bay. The sampling sites are indicated by GM (Guapimirim mangrove: 22°44′ S, 43°02′ W) and BV (Boa Viagem beach: 22°54′ S, 43°07′ W).

Surface sediment samples (0–2 cm) were collected from both sampling sites at low tide in order to isolate bacterial consortia able to grow on petroleum enriched media. The samples were placed in sterile zip lock bags, transported to the laboratory, where enrichment cultures were immediately prepared.

2.2. Enrichment and isolation of hydrocarbon-degrading bacteria

Bacterial strains were isolated from (a) sediments from the Guapimirim mangrove (GME) and Boa Viagem (BVE); and (b) sediments from three in situ mesocosms (SP1, SP2 and SP3) installed in the Guapimirim mangrove.

Bacterial consortia able to survive in the presence of petroleum were obtained from GME and BVE sediments. Sucrose, urea and petroleum were added to the enrichment medium in order to obtain petroleum-resistant bacterial consortia with large metabolic diversity and high growth rate. Five hundred mL of seawater media were inoculated with 50 g of sediments. Seawater was filtered (0.45 μm membrane) to eliminate particulate material in suspension, and then sterilized by autoclaving at

1 bar for 30 min. 15 g L^{-1} sucrose and 10 g L^{-1} urea were added to provide the carbon and nitrogen sources for high biomass production. After the addition of 3 mL Arabian Light petroleum, the cultures were incubated under constant magnetic stirring at room temperature. After 30 days of incubation the obtained consortia were tested for their bioremediation capacity in situ mesocosm. Bacterial strains were isolated from this mesocosm after 130 days incubation period.

For bacterial strain isolation, synthetic seawater media supplemented with model hydrocarbon compounds as unique carbon source were inoculated with GME, BVE and mesocosm sediments. The obtained enrichment cultures were named according to the hydrocarbon used as carbon source (Nh: naphthalene, Fl: fluoranthene, Oc: octadecane, Pr: pristane and Py: pyrene) follow by the sediment origin (GME, BVE, or SP1, SP2 and SP3 mesocosms).

2.2.1. *In situ mesocosm experiments*

Three in situ mesocosms, each with 700 cm^2 surface area, were installed in the Guapimirim mangrove. 350 mL of Arabian Light petroleum were added to them. The mesocosms SP1, SP2 and SP3 are replicates settled 10 m away from each other. After 130 days, surface sediments of these contaminated mesocosms were collected and 1 g of each was used to inoculate synthetic seawater media supplemented with a model hydrocarbon compound as unique carbon source.

2.2.2. *Culture medium and strain isolation*

The synthetic seawater medium used in this study contained (per liter) 26.5 g NaCl, 750 mg KCl, 200 mg NH_4Cl, 1.47 g $CaCl_2$, 120 mg KBr, 24 mg $SrCl_2{\cdot}6H_2O$, 3 mg NaF, 5.28 g $MgCl_2$, 6.64 g $MgSO_4$, 265 mg Na_2CO_3, 1 ml of trace elements solution, 4 μM KH_2PO_4 and 1 ml vitamin solution. The pH was adjusted to 7.3. The trace elements solution contained (per liter): 50 mg $MnCl_2{\cdot}H_2O$, 300 mg H_3BO_3, 1.1 mg $FeSO_4{\cdot}7H_2O$, 190 mg $CoCl_2{\cdot}6H_2O$, 2 mg $CuCl_2{\cdot}2H_2O$, 24 mg $NiCl_2{\cdot}6H_2O$, 18 mg $NaMoO_4{\cdot}2H_2O$ and 42 mg $ZnCl_2{\cdot}7H_2O$. The solid synthetic seawater medium was obtained by adding of 15 g L^{-1} of agar. The enriched medium used to obtain biomass was prepared as follows: nutrient agar 15 g L^{-1}, peptone 5 g L^{-1}, beef extract 3 g L^{-1}, pH 6,8. Culture medium was further supplemented with model hydrocarbon compounds as sole carbon and energy source (pristane or octadecane at 250 mg L^{-1}; naphthalene or fluoranthene at 100 mg L^{-1} or pyrene at 50 mg L^{-1} were used for enrichment and isolation of cultures). Liquid cultures were incubated at 30 °C with shaking (170 rpm). Agar plates were incubated in the dark at 37 °C. Isolated strains were stored at −80 °C in culture medium containing 20% (v/v) glycerol.

2.3. *DNA extraction*

DNA was extracted from sediment, mesocosm and bacterial cell samples with the Ultra Clean Soil DNA isolation kit (Mo Bio Laboratories) as previously described [40]. The DNA was verified by a 1% agarose gel electrophoresis in Tris-acetate–EDTA buffer (TAE). DNA solutions were preserved at −20 °C.

2.4. *T-RFLP analyses*

16S rRNA encoding genes were amplified by PCR using primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 926R (5′-CCGTCAATTCCTTTRAGTTT-3′) fluorescence-labeled with TET and HEX, respectively. The reactions were cycled in a PTC 200 thermocycler (MJ Research) with an initial denaturation step at 94 °C for 10 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1:30 min and extension at 72 °C for 1 min, and then a final extension step at 72 °C for 15 min. PCR products were purified with the GFX PCR DNA purification kit (Amersham) and digested with 10 U of restriction enzymes *Hae*III or *Him*pI1 at 37 °C for 3 h. Approximately 50 ng of digested DNA were mixed with 15 μL of deionized formamide and denatured (94 °C for 5 min and chilled on ice). The lengths of the terminal restriction fragments were determined by capillary electrophoresis on an ABI prism 310 (Applied Biosystem, USA). After an injection step of 10 s, the electrophoresis was carried out for 30 min applying a voltage of 15 kV. The T-RFLP profiles were analyzed using Gene Scan Software (Applied Biosystem, USA).

Data sets were constructed considering the peak at which fluorescence was higher than 100 units for at least one sample, as previously described [9]. Statistical analyses were carried out with a hierarchical cluster analysis, based on a Jaccard coefficient by the unweighted-pair group method using arithmetic averages (UPGMA). Statistical analyses were carried out with MVSP software (Multi-Variate Statistical Package 3.1, Kovach Computing Services, UK).

2.5. *Phylogenetic analyses of isolated strains*

16S rRNA encoding genes were amplified by PCR using primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1489R (5′-TACCTTGTTACGACTTCA-3′). The amplified 16S rRNA encoding genes were partially sequenced (from 8 to 700 nt considering *E. coli* numbering) using the BigDye sequencing kit (Applied Biosystem, USA). Sequences were compared to those present in the databank at the infobiogen website (http://www.infobiogen.fr) using BLAST and aligned with the ClustalW program. Phylogenetic trees were obtained using Mega software using the neighbor-joining method. The confidence of the phylogenetic trees was analyzed by the bootstrap method. The EMBL accession numbers of the sequences obtained in this work are indicated in phylogenetic trees (Fig. 3).

2.6. *Hydrocarbon degradation assay*

Degradation capacity for all isolated strains was evaluated in culture medium containing model hydrocarbon compounds. After 21 days of incubation, hydrocarbons were extracted with 5 mL dichloromethane. Phenanthrene, fluoranthene, octadecane and pristane were quantified by gas chromatography (GC 8000 Serie, Fisons Instruments, Italy) equipped with a flame ionization detector, a splitless injector and capillary column DB-5625 30 m long × 0.25 mm in diameter. Dodecane was used as internal standard. Helium was used as the carrier gas, the oven

temperature program was: 100 °C for 1 min, 100–300 °C at 40 °C min^{-1}, 300 °C for 5 min and then decreased to 100 °C. Pyrene was quantified by HPLC (thermoseparation products, Spectra System TSP U/6000LP, USA), equipped with a Supercosil column (LC-PAH 25 cm × 46 mm) using isocratic conditions of 80% acetonitrile (v/v) at flow rate 4.5 ml min^{-1}. Assays were made in triplicate and percentage of degradation was calculated using as control inactivated strains.

3. Results

The distinct approaches to which the sediment was subjected resulted in differing diversities measured using T-RFLP profiles (Fig. 2). Higher similarity was found in profiles of bacterial communities from the Guapimirim mangrove in situ mesocosm sediments (with 27-68 T-RFs), whereas profiles of the bacterial communities able to grow on model hydrocarbon compounds obtained from SP mesocosms showed intermediate (3-30 T-RFs) diversity, and profiles of bacterial consortia obtained from petroleum-enriched in vitro cultures from the Guapimirim mangrove (GME) and Boa Viagem (BVE) sediments displayed lower values (2-5 T-RFs).

Table 1 presents characterization of the isolates, and Fig. 3 their phylogeny. While a relatively large number of strains were isolated (15 and 24, respectively, 11 and 7 different genera) from enrichments SP3 and SP2, only 4 (3 different genera) were obtained from SP1. Among 64 isolated strains, 32 were able to degrade the hydrocarbon molecules: 5 degraded naphthalene, 9 degraded octadecane, 8 degraded fluoranthene, 7 degraded pyrene and 3 degraded pristane. Most of the strains (79%) are Gram-negative bacilli. Analysis of 16S rRNA encoding sequences showed that 42% of the strains were linked to members of the gamma-proteobacteria (Fig. 3a), 37% to the alpha-proteobacteria (Fig. 3b), and 21% to the Gram-positive actinobacteria group (Fig. 3c). Twelve different genera were found, with the dominance of representatives related to the genera *Marinobacter* (7 strains) and *Alcanivorax* (5 strains) as well as to isolates of hydrothermal vents (7 strains).

Since pyrene, a polycyclic aromatic hydrocarbon containing four aromatic rings, is considered to be a recalcitrant compound, pyrene-degrading strains present a potential for bioremediation processes. We determined the ability of the isolated pyrene-degrading strains to degrade other hydrocarbon molecules. Table 2 shows that these strains were able to degrade both aliphatic and aromatic hydrocarbons at different rates. Furthermore, the dioxygenase α-subunit encoding gene was detected in these strains by hybridization with a DNA probe of the conserved region of the metal binding site of dioxygenases (data not shown).

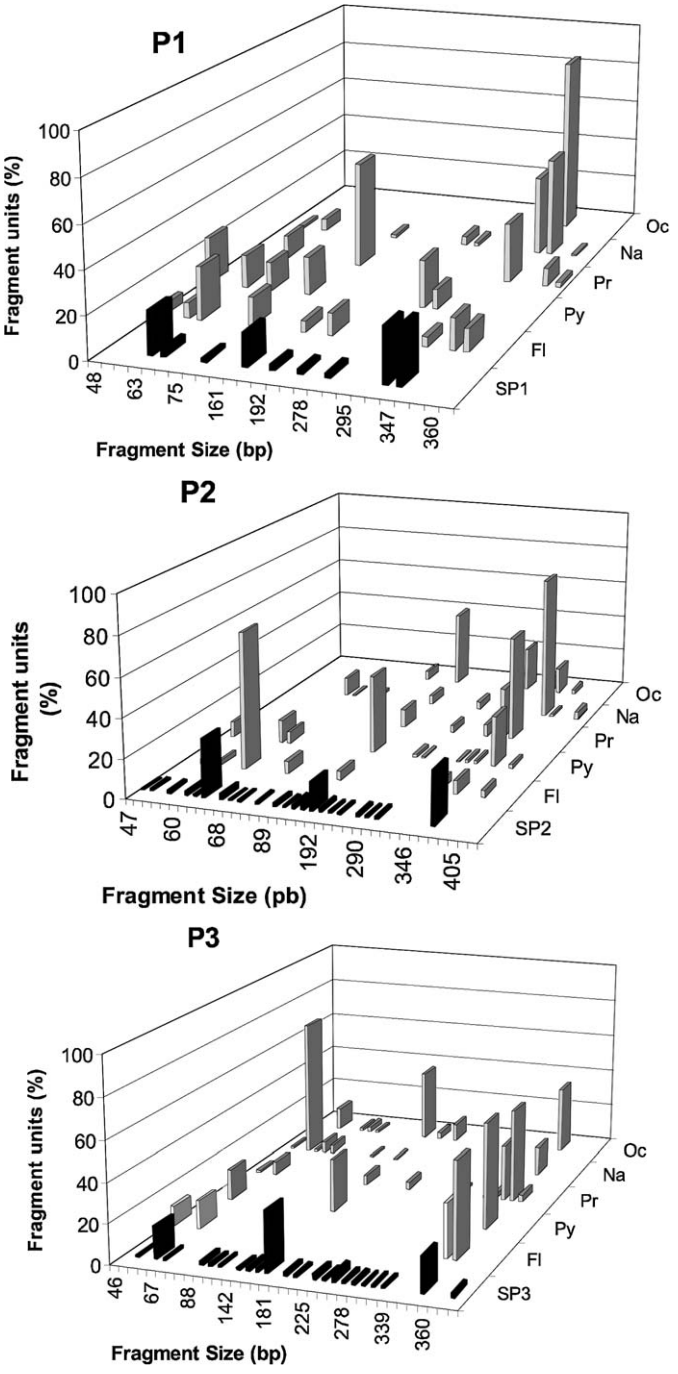


Fig. 2. Comparison of bacterial community structures. T-RFLP profiles of bacterial communities from enrichment cultures on model hydrocarbons (Oc: octadecane; Pr: pristane; Nh: naphthalene; Py: pyrene; and Fl: fluoranthene) are compared for each mesocosm sediment contaminated by petroleum—SP1, SP2 and SP3. T-RFLP profiles were obtained by digesting the 16S RNA encoding gene by *Him*pI1.

4. Discussion

4.1. T-RFLP analysis of selected communities

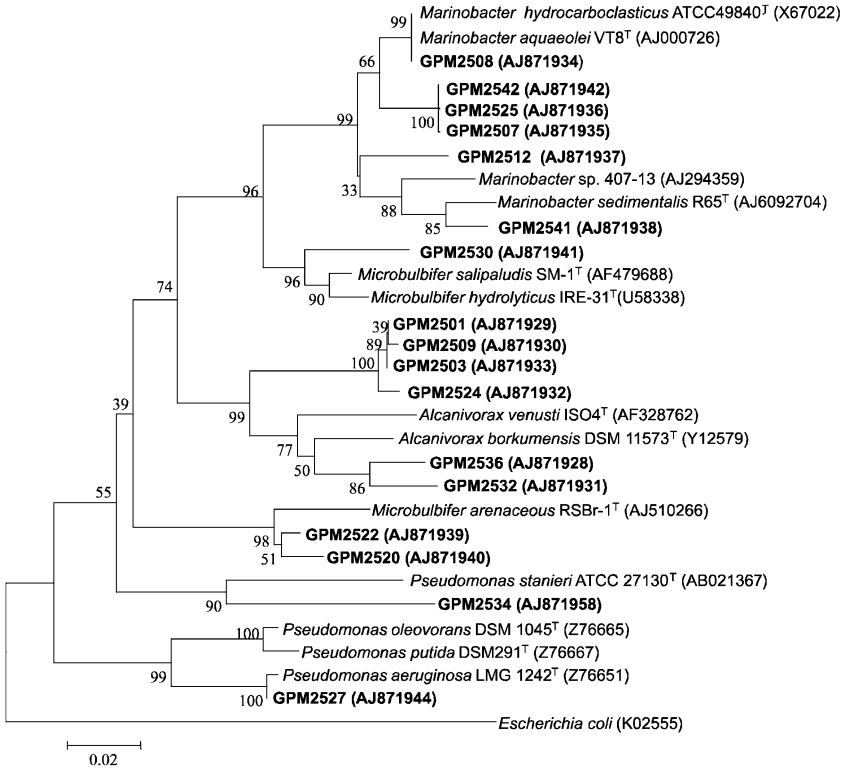
The low diversity presented by BVE and GME bacterial consortia as compared to mesocosm communities (SP1, SP2 and SP3) was expected, since the selective pressure imposed by laboratory experimental conditions is much stronger than what occurs in nature. This confinement also explains the similar behavior of bacterial communities able to grow on media containing single molecules of model hydrocarbons as carbon source, and the SP1, SP2 and SP3 mesocosm communities from which they originated. When confronted with environmental changes such as hydrocarbon contamination, microorgan-

Table 1
Origin and identification of hydrocarbon-degrading bacterial isolates from mangrove sediments

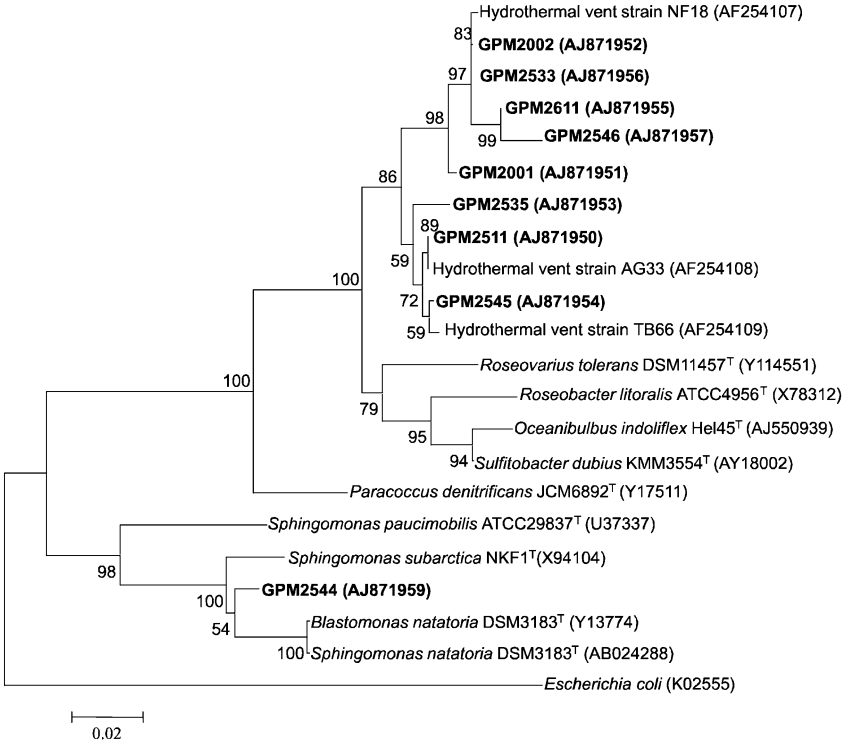
Strain	Identification[a]	HBCc[b]	HC source[c]	HC deg.[d] (%)
GPM2501	*Alcanivorax venusti* ISO4[e]	GME	Oc	23
GPM2502	*Micrococcus luteus* Ballarat[q]	GME	Oc	7
GPM2503	*Alcanivorax venusti* ISO4[e]	GME	Oc	45
GPM2509	*Alcanivorax venusti* ISO4[e]	GME	Py	16
GPM2511	Hydrothermal vent strain AG33[k]	GME	Py	11
GPM2524	*Alcanivorax venusti* ISO1[f]	GME	Oc	20
GPM2603	*Micrococcus luteus* Ballarat[q]	BVE	Pr	99
GPM2527	*Pseudomonas aeruginosa* BHP7 6[r]	SP1	Oc	67
GPM2542	Gamma-proteobacterium HTB148[j]	SP1	Fl	21
GPM2546	Hydrothermal vent strain NF18[k]	SP1	Nh	14
GPM2522	*Microbulbifer arenaceous* RSBr 1T[o]	SP2	Fl	5
GPM2525	*M. hydrocarbonoclasticus* ATCC 27132T[l]	SP2	Oc	32
GPM2536	*Alcanivorax venusti* ISO4[e]	SP2	Oc	12
GPM2541	*Marinobacter sp.* 407.13[n]	SP2	Py	27
GPM2604	*Dietzia maris* SAFR 020[h]	SP2	Nh	8
GPM2609	*Cellulomonas sp.* IFO16066[g]	SP2	Py	55
GPM2611	Hydrothermal vent strain NF18[k]	SP2	Nh	41
GPM2001	Hydrothermal vent strain AG33[k]	SP3	Py	21
GPM2002	Hydrothermal vent strain AG33[k]	SP3	Py	21
GPM2507	*M. hydrocarbonoclasticus* ATCC 27132T[l]	SP3	Fl	20
GPM2508	*M. hydrocarbonoclasticus* ATCC 27132T[l]	SP3	Pr	37
GPM2512	*M. hydrocarbonoclasticus* ATCC 27132T[l]	SP3	Oc	8
GPM2519	*M. hydrocarbonoclasticus* DPUZ[m]	SP3	Oc	25
GPM2520	*Microbulbifer arenaceous* RSBr 1T[o]	SP3	Fl	12
GPM2530	*Microbubifer salipaludis* SM 1[p]	SP3	Nh	16
GPM2533	Hydrothermal vent strain NF18[k]	SP3	Py	24
GPM2534	*Pseudomonas stanieri* ATCC 27130T[s]	SP3	Fl	51
GPM2535	Hydrothermal vent strain TB66[k]	SP3	Fl	21
GPM2544	*Sphingomonas subarctica* KF3[t]	SP3	Pr	100
GPM2545	Hydrothermal vent strain TB66[k]	SP3	Nh	100
GPM2602	*Micrococcus luteus* Ballarat[q]	SP3	Fl	20
GPM2606	*Gordonia polyisoprenivorans* VH2[i]	SP3	Fl	12

[a] Closest relative after 16S rRNA encoding gene analysis.
[b] Hydrocarbon-degrading bacterial communities (HBC), origins of isolates (see text).
[c] Hydrocarbon source (HC): naphthalene—Nh, fluoranthene—Fl, octadecane—Oc, pristane—Pr and pyrene—Py.
[d] Hydrocarbon degradation was measured after 21 days of incubation.
Strains isolated from:
[e] Mediterranean seawater, coast of Santa Pola, Spain [15];
[f] Mediterranean seawater, coast of Alicante, Spain [15];
[g] mangrove rhizosphere, Japan [47];
[h] spacecraft (Venkateswaran, K., unpublished);
[i] rubber tree plantation, Vietnam [26];
[j] deep-sea near the southern part of Japan [46];
[k] Galapagos hydrothermal vent samples [51];
[l] Mediterranean seawater near a petroleum refinery [16];
[m] ephemeral stream bed contaminated with industrial wastewater in the Negev Desert, Israel [43];
[n] cultures of *Alexandrium* sp. (Dinophyceae) associated with the production of paralytic shellfish toxins (Gallacher, S., unpublished);
[o] red sandstone from coastal areas of Scotland [50];
[p] salt marsh around the junction of the Youngsan River and the Yellow Sea, Korea [57];
[q] activated-sludge plant in Ballarat, in Victoria, Australia [55];
[r] arid soils in southern Arizona, USA [8];
[s] seawater [7];
[t] biofilm of a continuous-flow fluidized bed reactor inoculated with activated sludge and polychlorinated phenols [37].

isms have developed adaptive responses including metabolism switches, cell-cell interactions and acquisition of new functions by mutations or transfer of mobile genetic elements. In structured and dispersed systems, like natural ecosystems and in situ mesocosms, bacteria are spatially distributed as a function of environmental variables that can result in specialized ecological niches. Consequently, the selection pressure is more dispersed, undefined and occurs at several levels (individual, colony or population levels). Thus, when environmental variability is imposed by the presence of a pollutant, the development of new metabolic pathways can increase diversity within one population or result in the formation of a new niche, which is a way of avoiding direct competition. In contrast, when strong selection pressure is applied, as occurs with in vitro isolation procedures,



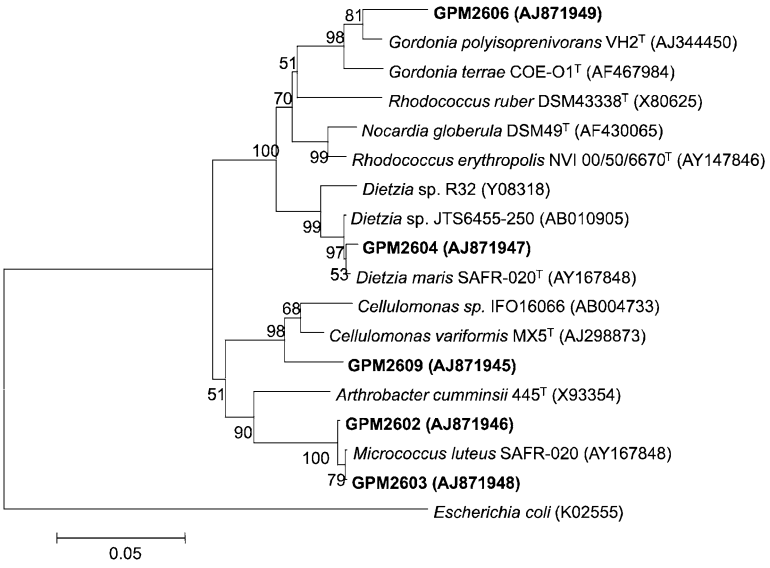
(a)



(b)

Fig. 3. Phylogenetic trees based on 16S rRNA encoding gene, showing the position of isolated strains within the radius of members of respective groups: (a) gamma-proteobacteria, (b) alpha-proteobacteria and (c) actinobacteria. Trees were generated using neighbor-joining analysis. All accession numbers are indicated.

strains with beneficial mutations have a growth advantage in comparison to wild-type and unadapted strains which lower diversity [38,53]. Furthermore, genetic drift may occur on small systems, which probably also contributes to decreasing the diversity on in vitro microcosms, as we observed in the present study.



(c)

Fig. 3. (*continued*)

Table 2
Degradation rate for different hydrocarbons (Py: pyrene; Oc: octane; Ph: phenanthrene; and Fl: fluoranthene) of four pyrene-degrading strains

Strain	Py	Oc	Ph	Fl
GPM2509	16%	100%	0%	11%
GPM2609	55%	0%	100%	15%
GPM2002	21%	5%	18%	0%
GPM2541	27%	10%	50%	0%

Hydrocarbon degradation was measured after 21 days of incubation.

Close inspection of Fig. 2 also reveals diversity differences among the mesocosms: SP1 (9 T-RFs) seems to have lower diversity than SP2 (22 T-RFs) and SP3 (21 T-RFs). This probably reflects the fact that only dominant populations are detected by T-RFLP, as with other molecular methods. The procedure could not detect T-RFs of minor populations in the first sample, since the diversity of communities that grew on all five model hydrocarbons showed similar diversity for the three mesocosms. With regard to specific populations (fragment sizes), the figure also reveals similarities between mesocosm communities and model hydrocarbon communities: fragments with 63/64, 161/163 and 347/348 bp were present on all mesocosms and almost all hydrocarbon enrichments. Such communities are probably highly adapted populations, both to oil and to specific hydrocarbons.

Some populations, on the other hand, although poorly represented on the three mesocosms, were not recovered on most model hydrocarbons (67, 192, 225 and 291 bp). This may be the case for populations that are resistant to oil but without the ability to use PAHs as sources of energy. A final case is that of populations that grew significantly on some specific model hydrocarbons (46 and 352 on Fl; 48, 73, 112 and 292 on Py; 346 on Pr; 339 on Fl and Oc, 344 on Py and Fl and 360 on Pr and Oc), but were not identified on original mesocosms. This is probably the case for populations highly adapted to such hydrocarbons, able to grow under extreme conditions but with less competition.

4.2. Gamma-proteobacteria-associated strains

Strains affiliated with the gamma-proteobacteria group (Fig. 3a) are associated with members of the genera *Marinobacter, Alcanivorax* and *Pseudomonas*, which have already been described as being hydrocarbonoclastic strains [6,10,16,27, 29,49,54], and with the genus *Microbulbifer* [4,21,22,24,61]. Strains affiliated with the *Marinobacter* genus were patched within two branches: one containing *M. hydrocarbonoclasticus* and *M. aquaeolei* and the other with *M. sedimentalis*. Strains GPM2508, GPM2512, GPM2542, GPM2507 and GPM2525 are linked to the first branch, with GPM2508 being the most closely related to the type strains. Strain GPM2541 was related to *M. sedimentalis*.

M. hydrocarbonoclasticus and *M. aquaeolei* were isolated from oil-polluted samples [16] and they can degrade aliphatic or aromatic hydrocarbons. *M. hydrocarbonoclasticus* is capable of using tetradecane, hexane, eicosane, heneicosane, pristane, phenylalcane and phenanthrene as single sources of carbon and energy [2,16], while *M. aquaeolei* is able to degrade pristane, *n*-hexane and crude oil as sole carbon sources [23]. The ability to degrade light aromatic hydrocarbons (benzene, toluene and xylene) has been observed for a bacterial community containing *Marinobacter* species [36]. It is worth emphasizing that strains GPM2507 and GPM2542 used fluoranthene (20%) as the only carbon source and strain GPM2541 degraded pyrene (27%) (Table 1). This is the first time that the capacity of *Marinobacter* species to degrade pyrene and fluoranthene has been described.

Six strains were associated with the *Alcanivorax* genera. Strains GPM2501, GPM2503, GPM2509, GPM2524, GPM2532 and GPM2536 displayed the highest 16S rRNA sequence similarity with *Alcanivorax borkumensis* and *Alcanivorax venusti*. These genera were identified as alkane-degrading bacteria [10,15,56]. It has further been reported that members of the *Alcanivorax* genus are important marine hydrocarbon

degraders, since they are distributed worldwide in coastal waters and sedimentary environments [42]. Although this genus is usually associated with aliphatic hydrocarbon degradation, like pristane, tetradecane, and hexane [10,15,56], here we isolated one strain (GPM2509) that was capable of degrading 16% of pyrene (Table 1).

Within the genus *Pseudomonas* we identified two strains. Strain GPM2534 formed one branch related to *P. stanieri*, while strain GPM2527 was closely related to *P. aeruginosa*. *Pseudomonas* species have been isolated from worldwide contaminated sites, and their ability to grow and degrade different PAHs has been reported in a number of studies: the metabolism of fluoranthene and benzo[a]pyrene by *Pseudomonas* NCIB 9816 [6], the degradation of phenanthrene and fluorene by *P. paucimobilis* and *P. versicularis* [54], and also the ability to degrade a mixture of high molecular weight PAHs by the *Pseudomonads* [24,52]. Specifically, *P. aeruginosa* is usually identified on hydrocarbon-utilizing communities [22,52], while no information on the hydrocarbonoclastic capacity of *P. stanieri* [7] has been previously described. The strains in our sample were found to be able to use fluoranthene, 51% (GPM2534), and octadecane, 67% (GPM2527) (Table 1).

The most striking characteristic within the gamma-proteobacteria group was that of strains of the *Microbulbifer* genus that degrade hydrocarbons. Three strains separated into two branches were found in this genus: GPM2520 and GPM2522 associated with *M. arenaceous*, and GPM2530 affiliated with *M. salipaludis*. The observed degradation capacities of the three strains were, respectively, 5 and 12% of fluoranthene, and 16% of naphthalene. Until now, there have been few isolated strains included in this genus: *M. degradans* [4,19,21], *M. hydrolyticus* [18], *M. elongata*, *M. salipaludis* [57,58] and *M. arenaceous* [50]. These strains are able to flourish under a wide range of conditions and to utilize a variety of complex organic molecules, which is probably an adaptation to changing environmental conditions typical of the salt marshes in which they are isolated [18,50,57,58]. There are two possible explanations for the oil-hydrocarbon degradation potential of isolated members of this group: it may be a characteristic common to the genus but not previously detected because it was not tested, or else our strains acquired this ability through environmental determinism, since this genus is fairly versatile. In this case, gene transfer might be a reasonable explanation, since it is assumed to be commonplace within microbial communities and may play a role in the acclimation of microbial communities to environmental pollutants [30,31,59]. Nevertheless, more tests will have to be performed to verify this hypothesis.

4.3. Alpha-proteobacteria associated strains

Fig. 3b shows that the isolated strains affiliated with the second group, alpha-proteobacteria, form two branches: one with the *Sphingomonas* genus and the other with strains isolated from hydrothermal vents (related to alpha-proteobacteria thiosulfate oxidizing bacteria). Strain GPM2544 is associated with *S. subarctica* and showed the ability to degrade 100% of pristane (Table 1). This is the first time that the ability to degrade branched alkanes has been observed for members of this group, although the *Sphingomonas* genus has already been isolated from contaminated sites [1], and species capable of degrading PAHs [34], dioxins (e.g., *S. paucimobilis*) [35] and polychlorophenols (e.g., *S. subarctica*) [37] have already been described. In general, *n*-alkanes are oxidized by microorganisms, while the alkyl-branched alkanes are less susceptible to biodegradation due to the steric effects that inhibit oxidizing enzymes [44]. Although pristane is highly recalcitrant [32], its oxidation has been reported for different bacteria such as *Brevibacterium* [39], *Corynebacterium* [34], *Rhodococcus* [35] and *Nocardia* [3], and now also for *Sphingomonas*. Most of the isolated strains affiliated with the alpha-proteobacteria were branched (similarity greater than or equal to 97%) with thiosulfate-oxidizing bacterial communities, specifically strains from Galapagos hydrothermal vents (strains AG33, TB66 and NF18). Teske et al. [51], who identified these hydrothermal vent strains, found that the most closely associated validly described genus, to them, was *Roseobacter*. Our isolates did not match directly with *Roseobacter* or other validly described thiosulfate-oxidizing bacteria, but it matched with isolates of the hydrothermal vents themselves. This unexpected phylogenetic link with the microbial population of hydrothermal vents might possibly be explained by the reduced conditions of sediments in the mangroves. However, more details on morphological, chemotaxonomic and physiological properties of such strains are necessary to determine their taxonomy. Several members of the marine alpha group play an important ecological role in the decomposition of sulfur compounds and degradation of complex plant-produced polymers such as lignin and humic substances [51]. It is also interesting to note that these isolated strains were able to degrade polycyclic aromatic hydrocarbons (pyrene, naphthalene, and fluoranthene) (Table 1), an unprecedented characteristic of alpha-proteobacteria thiosulfate oxidizers, which points to the importance of their ecological role.

4.4. Actinobacteria-associated strains

The third group to which our strains are linked, the actinobacteria (Fig. 3c), re-affiliated with the species *Micrococcus luteus* (GPM2602 and GPM2603), *Cellulomonas variformis* (GPM2609), *Dietzia maris* (GPM2604) and *Gordonia polyisoprenivorans* (GPM2606). Strains GPM2603 and GPM2619 showed high hydrocarbonoclastic potential (they degraded 99% of pristane and 55% of pyrene, respectively), and the other strains showed less significant capacity for this: strain GPM2606 degraded 12% of fluoranthene, GPM2604 8% of naphthalene and GPM2602 7% of octadecane. These actinobacteria strains are very important in bioremediation and in pollutant biodegradation studies since they are widely distributed in nature. For example, the *G. alkanivorans* strain (isolated from tar- and phenol-contaminated soil) has the ability to degrade hexadecane [25], *G. polyisoprenivorans* and *G. westfalia* (isolated from foul tire water) are able to solubilize and mineralize natural rubber substrates [27,28] and one strain with 98.8% similarity to *Micrococcus luteus* (isolated from oil-contaminated tropical marine sediments) degraded naph-

Table 3
Comparison of isolates obtained in the present work to previously isolated strains according to the site at which they were isolated and their degradation capacities

Strains	Origin	Degradation capacities	References
Marinobacter genera			
M. aquaeolei	Oil-polluted sediments	Benzene, toluene and xylene	[16]
M. hydrocarbonoclasticus		Tetradecane, hexane, eicosane, heneicosane, Pristine and crude-oil	[23,36]
GPM2507	Oil-contaminated mangrove sediments	Fluoranthene	This work
GPM2541		Pyrene	
Alcanivorax genera	Coastal water and sediment samples	Pristine, tetradecane, hexane	[10,15,42,56]
GPM2509, GPM2501, GPM2503, GPM2524	Mangrove sediments	Pyrene, octane	This work
GPM2532, GPM2536	Oil-contaminated mangrove sediments	Octane	This work
Pseudomonas species	Contaminated sites	Mixture of high molecular weight PAHs	[24,52]
Pseudomonas NCIB 9816		Fluoranthene, benzo[a]pyrene	[6]
P. paucimobilis		Phenanthrene, fluoranthene	[54]
P. versicularis		Phenanthrene, fluoranthene	[54]
GPM2534, GPM2527	Oil contaminated mangrove sediments	Fluoranthene, octadecane	This work
Microbulbifer genera			
GPM2520, GPM2522	Oil-contaminated mangrove sediments	Fluoranthene	This work
GPM2530		Naphthalene	
Sphingomonas genera	Contaminated sites	PAHs	[1]
S. paucimobilis		Dioxins,	[33]
S. subarctica		Polychlorophenols	[35,37]
GPM2544	Oil contaminated mangrove sediments	Pristane	This work

PAHs: Polycyclic aromatic hydrocarbons.

thalene as well as benzene [60]. *D. maris* and *Rhodococcus erythropolis* strains are able to completely oxidize *n*-alkane and *iso*-alkanes [61]. *Dietzia* sp. strain GS-1, isolated from soil samples on a medium enriched with disodium terephthalate, also showed the ability to degrade this compound [45]. The ability to grow and degrade different kinds of materials like rubbers, terephthalate, oil and hydrocarbons indicates that these strains have flexible survival strategies that might be valuable properties for new bioremediation strategies.

4.5. Biodegradation capacities of pyrene-degrading strains

Since pyrene, a four-ringed PAH, is considered to be a compound recalcitrant to degradation, pyrene-degrading strains present a potential for bioremediation processes. We determined the ability of isolated pyrene-degrading strains (GPM2509, GPM2541, GPM2609 and GPM2002) to degrade other hydrocarbon molecules. The four strains tested were able to degrade both aliphatic and aromatic hydrocarbons at different rates. The dioxygenase α-subunit-encoding gene was detected in these strains by hybridization. These results show the biotechnological potential of these strains for bioremediation of environments contaminated with hydrocarbon mixtures. The capacity to degrade hydrocarbons and recalcitrant compounds is not a taxonomic characteristic since it is observed in different species of bacteria, fungi and yeasts [12,20]. In the present study, we observed that species of bacteria belonging to different phylogenetic groups were capable of degrading different types of hydrocarbons. Similarly, the ability of a single strain, to degrade several chemically distinct compounds is notorious. Usually, a hydrocarbon-degrading strain is able to degrade a range of compounds including saturated and aromatic molecules [12]. The gain or loss of this ability is related to genetic mechanisms, linked to enzymatic capacities and also to the instability of genetic elements, and it depends strongly on environmental conditions. The transfer of catabolic genes, by transposition or conjugation events, may play a key role in gaining new degrading abilities, as has been suggested by some authors [21]. This may explain why, among the 64 isolated strains, only 32 were able to significantly degrade the compound used for their isolation. Due to newly imposed conditions during degradation tests, the degrading ability was probably lost, since it was no longer necessary.

In conclusion, results presented here point to the rich microbial diversity of the Guapimirim mangrove. Although mangrove ecosystems have been extensively studied, including their bioremediation capacity, their microbiological potential has not been thoroughly explored. Bacteria isolated from the Guapimirim mangrove showed strong ability to degrade both the aliphatic and aromatic hydrocarbons in which they grew. Table 3 summarizes the degradation capacities of strains isolated in this work and compares them to previously isolated strains. Some of the isolated strains such as *M. hydrocarbonoclasticus, Alcanivorax* sp., *Pseudomonas* sp., *Sphingomonas* sp. and *Gordonia* sp. have already been isolated from hydrocarbon-contaminated sites [5]. However, we also isolated members of genera that had never been previously detected at such sites, nor had their ability to grow or degrade petroleum hydrocarbons been described. For instance, strains related to *Microbulbifer* (GPM2520, GPM2522 and GPM2530) and strains related to isolates from hydrothermal vents (GPM2511, GPM2546, GPM2611, GPM2001, GPM2533, GPM2535 and GPM2545) were shown to significantly degrade hydrocarbons. Furthermore, even for bacterial species already described as

hydrocarbon-degrading bacteria, the strains isolated in this work exhibit new properties important for bioremediation strategies. For example, isolated strains of *Marinobacter* sp. and *Alcanivorax* sp., species known to degrade alkanes, have the ability to degrade PAHs. Similarly, the isolates of *Sphingomonas* sp., species known to degrade aromatic hydrocarbons, are able to use alkane hydrocarbons. These results are consistent with a previous report describing the degradation of both alkane and aromatic compounds by isolates from a mangrove forest [54], indicating the considerable potential of bacterial communities inhabiting mangrove sediments to degrade hydrocarbon compounds. This is not surprising, since mangrove ecosystems are very organically rich environments. In conclusion, most of the isolated strains have interesting properties both from an academic point of view and for further applications. Further analyses are needed to define their potential in bioremediation processes that would include genetic and biochemical studies, so as to elucidate the metabolic pathways involved in PAH degradation.

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