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First Characterization of PAH-degrading bacteria from Río de la Plata and high-resolution melting: an encouraging step toward bioremediation

Silvina A. Izzo[a], Silvina Quintana[b,c], Mariela Espinosa[d], Paola A. Babay[d] and Silvia R. Peressutti[a]

[a]Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP), Mar del Plata, Buenos Aires, Argentina; [b]Área de Biología Molecular de Fares Taie, Instituto de Análisis, Rivadavia, Mar del Plata, Argentina; [c]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina; [d]Comisión Nacional de Energía Atómica, Buenos Aires, Argentina

ABSTRACT
The Río de la Plata, one of the most important estuarine environments in South America that sustains valuable fisheries, is affected by PAH contamination associated with oil industry and port activities. A total of 95 bacteria with potential to degrade phenanthrene were obtained from water samples using traditional culture methods. PCR-RFLP analysis of 16S rDNA partial fragments was used as a screening tool for reducing the number of isolates during diversity studies, obtaining 42 strains with different fingerprint patterns. Phylogenetic analysis indicated that they were affiliated to 19 different genera of Gamma- and Alpha-Proteobacteria, and Actinobacteria. Some of them showed an efficient phenanthrene degradation by HPLC (between 83% and 97%) and surfactant production (between 40% and 55%). They could be an alternative for microbial selection in the degradation of PAHs in this estuarine system. In order to detect and monitor PAH-degrading bacteria in this highly productive area, rDNA amplicons of the 33 isolates, produced by PCR real time, were tested by the high-resolution melting (HRM) technique. After analyzing the generated melting curves, it was possible to accurately distinguish nine patterns corresponding to eight different genera. HRM analysis allowed a differentiation at the species level for genera Pseudomonas, Halomonas and Vibrio. The implementation of this method as a fast and sensitive scanning approach to identify PAH-degrading bacteria, avoiding the sequencing step, would mean an advance in bioremediation technologies.

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KEYWORDS
Polycyclic aromatic hydrocarbon; microorganisms; biodegradation; restriction fragment length polymorphism; high-resolution melting

Introduction

The Río de la Plata, located at 35°S on the Atlantic coast of South America, is one of the most relevant estuarine environments in the continent. This system, as well as the adjacent marine waters, represents a highly productive area in both the ecological and the economic aspects, with great importance on coastal fisheries [1]. Despite the large water flow and dilution power of the estuary, industrial and port activities produce a significant impact on this ecosystem, with the input of various contaminants [2]. Among these, polycyclic aromatic hydrocarbons (PAHs) are serious pollutants due to their toxicity, mutagenicity, carcinogenicity and potential to bio-accumulate [3,4].

Due to their chemical stability and high recalcitrance properties, the evaluation of different strategies for degradation of PAHs is a matter of global concern. Biodegradation is the main process for the successful removal of these compounds from contaminated environments [5]. The increasing pollution of coastal and marine ecosystems owing to anthropogenic activities has prompted the development of effective bioremediation tools [6]. This is an efficient and low-cost treatment, which uses the metabolic ability of naturally occurring microorganisms to convert the compounds into harmless or less toxic products, reducing the input of chemicals, energy and time [7,8]. One of the main benefits of this biotechnology is its compatibility with the biogeochemical cycles and recycling routes of marine environments. Thus, bioremediation is a sustainable and environmentally eco-friendly approach for the cleanup of contaminated ecosystems [9]. In spite of the difficulties of the level and the scale in marine areas, it may be a cost-effective and feasible treatment for the restoration of certain contaminated estuaries, shorelines and marine sites [10].

Microorganisms isolated from PAH-polluted ecosystems have shown high biodegradation rates in bioremediation treatments. In this way, different researches have focused on isolation and description of PAH degraders, and an important number of bacteria have already been identified and characterized [11]. Some of them showed high biodegradation potential and ability to

CONTACT Silvia R. Peressutti silviap_ar@yahoo.com Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP), Paseo Victoria Ocampo N°1, 7600 Mar del Plata, Buenos Aires, Argentina

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enhance hydrocarbon bioavailability like biosurfactant production [12]. The optimal conditions for PAH microbial degradation may vary; therefore, using the most-suitable bacteria for each environment leads to successful biodegradation efforts [13]. Obtaining and characterizing bacterial isolates capable of PAH degradation is a preliminary step toward understanding the microbiology and fate of PAHs in marine environments, as well as for a proper management of the contaminated sites through bioremediation technologies [14]. In spite of the abundant information, mainly in contaminated soil or marine sediments [15,16], there are no researches on indigenous PAH-degrading bacterial communities in the Río de la Plata waters.

Furthermore, to carry out an effective bioremediation system, it is important to monitor PAH-degrading microorganisms along this process. In this sense, it could be an advantage to cut short the time associated with bacterial identification. PAH-degrading bacteria detection through a fast and efficient technique of diagnosis is a significant way to promote the implementation of these treatments. The increasing deterioration of the environment has encouraged a considerable need for rapid and cost-effective analytical methods. Conventional identification by 16S rDNA gene sequencing has the limitations of the PCR method [17]; added to this, sequencing by Sanger includes the cost of nucleotide sequence analysis, and due to the requisite computational and bioinformatic steps the results do not manifest immediately afterwards [18]. The high-resolution melting (HRM) technique represents a powerful and robust technology for detecting DNA sequence variants and offers considerable time and cost savings compared to other screening methods [19]. After real-time PCR amplification, HRM analysis generates DNA melt curve profiles that are both specific and sensitive enough to distinguish nucleic acid species based on small sequence differences, enabling to identify microorganisms to the genus or species level and to greatly decrease the burden of sequencing [20]. This study aims to isolate, identify and assess the PAH biodegradation potential of indigenous bacteria from the Río de la Plata waters, using cultivation and molecular approaches. Likewise, we performed a sensitive scanning method based on HRM with the purpose of obtaining a rapid identification of PAH-degrading bacteria, during bioremediation treatments.

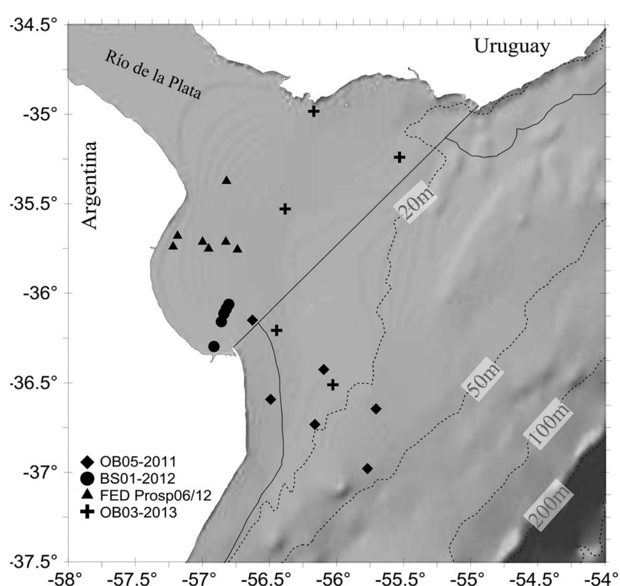


Figure 1. Localization of sample stations along the Río de la Plata estuary and adjacent marine waters.

Materials and methods

Culture media and bacterial growth

Phenanthrene (Phe) was selected as the model PAH compound in the present study. A stock solution 30 mg ml^{-1} of Phe (Sigma, Argentina, purity ≥ 96%) was prepared in ethanol and used for all the assays. For the enrichment of Phe-degrading microorganisms, an MSM-Phe liquid medium-containing mineral salts medium (MSM) according to Schlegel et al. [21], NaCl 2% (w/v) and Phe (150 mg l^{-1}) as the sole carbon and energy source, was used. Cultures were incubated aerobically on an orbital shaker at 25°C and 150 rpm. Bacterial growth was evaluated by optical density at 600 nm (OD_{600}). For the isolation of Phe-degrading bacteria, MSM-Phe-agarose agar plates were prepared as follows: MSM medium with no carbon source plus 1.2% agar was plated on Petri dishes. The surface of these plates was covered with a layer of agarose 1% (w/v) with Phe at a final concentration of 340 mg l^{-1} [22]. Rich medium (NA), containing 1% tryptone, 0.5% yeast extract, 2% NaCl and 1.2% agar, was used for identification of colony morphology and Gram staining. Plates were incubated at 28°C for 3–7 days.

Sampling and isolation of PAH-degrading isolates

Surface water samples were collected in sterile plastic containers along the Río de la Plata estuary and adjacent marine waters (34°30′–37°30′S, 58°–59°W), Argentina, during four INIDEP research cruises (Figure 1). For enrichment cultures water sub-samples (10 ml) were inoculated into 500-ml flasks containing 100 ml of MSM-Phe liquid medium. Two-milliliter aliquots were withdrawn from each culture every week for a month, transferred to the fresh sterile medium, and incubated as described

above. Finally, cultures were plated on MSM-Phe-agarose. Colonies of candidate Phe-degrading strains were picked up and further purified by repetitive streaking on fresh MSM-Phe-agarose plates. The pure cultures of the final isolates selected were preserved as 10% dimethyl sulfoxide stocks at − 80°C.

Identification of bacterial isolates and phylogenetic analysis

Isolated strains were initially characterized according to their colony features such as color, shape and size (diameter) on both MSM-Phe-agarose and NA plates, and morphology following the Gram staining.

Molecular identification was carried out by phylogenetic analysis following the sequencing of 16S rDNA. Genomic DNA from each isolate was extracted according to Wilson [23], and its quality was checked in a 0.8% agarose gel electrophoresis after staining with ethidium bromide. PCR amplifications were performed by using the universal primers F27 (5′-AGAGTTTGATCMTGGCTCAG-3′) and R1492 (5′-TACGGYTACCTTGTTACG ACTT-3′) [24], in 25 µl-reactions containing 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 0.2 µM dNTPs, 0.5 µM of each primer, 1 U of T-Free DNA polymerase (Inbio-Highway, Tandil, Argentina) and 10 ng of DNA. The program used for the amplification was: 5 min at 94°C, 40 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C; and a final elongation of 15 min at 72°C [25], in a thermocycler (Life Express, TC-96/T/H.a).

The isolates were screened by restriction fragment length polymorphism (RFLP) analysis. PCR products were digested with 5 U of the restriction endonuclease HaeIII (Promega, Madison, WI) and resultant restriction fragments were analyzed by electrophoresis on 2% agarose gel and visualized under UV, by staining with ethidium bromide. The representative isolates of each RFLP pattern were identified by 16S rDNA gene sequencing. 16S rDNA-PCR products were purified with DNA PuriPrep-GP Kit (Inbio-Highway, Tandil, Argentina) and sequenced commercially at INTA (Castelar, Argentina) by using the primers F63 (5′-CAGGCCTAACACAT GCAAGTC-3′) and F530 (5′-GTGCCAGCMGCCGCGG-3′). Both retrieved nucleotide sequences were edited using the FinchTV 1.4.0 program (Geopiza Inc.) and overlapped with the BioEdit software [26]. Each obtained consensus sequence was screened against EzTaxon data base [27] using the BLASTn tool [28]. Sequences were analyzed phylogenetically with the MEGA 5.2 program [29]. Phylogenetic trees were constructed through the neighbor-joining (NJ) algorithm, from a distance matrix calculated following Kimura's two-parameter model. Stability among the clades was assessed with the 1000-replication bootstrap analysis. Sequences were deposited at the GenBank database under accession numbers KY020297 to KY020338.

Phenanthrene degradation

Biodegradation assays were conducted in two independent experiments, inoculating an exponential phase culture of each bacterial strain in 50 ml of MSM-Phe liquid medium, and non-inoculated flasks were used as abiotic controls. Cultures were incubated aerobically on an orbital shaker at 28°C and 150 rpm for 9 days. To measure residual Phe concentrations, 3-ml aliquots were withdrawn from the cultures after 9 incubation days and subsequently extracted with 6 ml of acetonitrile. Tubes were incubated on an orbital shaker for 1 h at 25°C and 150 rpm. After that, the extracts of each culture were centrifuged (2500 g, 10 min) and the supernatants analyzed by reverse-phase HPLC according to NIOSH [30].

Chromatographic measurements were carried out with an ACCELA 600 HPLC instrument (Thermo Scientific, USA), consisting of a quaternary pump, an autosampler and a photodiode-array detector. Column oven temperature was set at 50°C and quantification wavelength was 254 nm. Separation was performed using a 3 µm particle C-18 column of 250 × 4.6 mm (Inertsil ODS-3; GL Science, Japan). Isocratic elution with 80% acetonitrile/20% water was performed at a flow rate of 0.9 ml min^{-1}.

Biosurfactant production assays

Emulsification assay was carried out as described in [31], from biodegradation assays cultures. Briefly, 3 ml of hexadecane and 3 ml of cell-free culture supernatant were vortexed at high speed for 2 min and incubated at 25°C for 24 h. An emulsification index value (E_{24}) was calculated as (height of emulsion layer/height of total mixture) x 100). Fresh MSM-Phe media without substrate and containing 0.75% of sodium dodecyl sulfate solution were used as negative and positive controls, respectively.

HRM analysis

Real-time PCR amplifications of the 16S rDNA gene were performed on a Rotor-Gene Q thermocycler (Qiagen, Hilden, Germany) in a final volume of 20 µl, using EvaGreen as an intercalating fluorescent dye (KAPA FAST, Biosystems, Woburn, USA). In an attempt to identify and differentiate Phe-degrading bacteria, generic primers p201 (5′-GAGGAAGGIGIGGAIGACGT-3′) and p1370 (5′-AGICCCGIGAACGTATTC AC-3′) were used [32]. Primers were synthesized at Operon (Huntsville,

Alabama, USA). Different PCR amplification conditions were tested by changing variables such as annealing temperature, primer concentration, number of cycles, DNA concentration and final reaction volume, in order to produce suitable fluorescence levels for HRM analysis. During the validation process, the PCR products were run on agarose gels to check the size of the amplicons. The selected cycling program consisted of an initial denaturation of 2 min at 95°C, and 45 cycles of 10 s at 94°C, 15 s at 60°C and 15 s at 72°C. This program was followed by an HRM melting curve comprising temperature increment steps of 0.1°C, from 75°C to 95°C, during which the fluorescence was read at the end of each increment. The results were analyzed using the Rotor-Gene Q software, version 1.7.94 (Qiagen).

Results and discussion

Isolation, screening of the phe-degrading bacteria and molecular characterization

The Río de la Plata waters are exposed to a broad diversity of contaminants owing to the high industrial activity in that zone and the lack of adequate disposal managements. Related to oil refineries and chemical industries, PAHs are common pollutants in this area. With the purpose of isolating the PAH-degrading bacteria, samples of contaminated waters were inoculated into mineral medium-containing Phe as the only substrate source. This tricyclic aromatic hydrocarbon has often been used as the model substrate in studies on the environmental degradation of PAHs, since it is widely distributed throughout the environment and its structure is found in carcinogenic PAHs such as benzo[a]pyrene or benz[a]anthracene [33]. From these enrichments, and after several transfers to fresh medium, aliquots were inoculated on MSM-Phe-agarose plates, obtaining significant colony diversity. Taking into account the formation of clear zones around the colonies and their differences in morphology, a total of 95 bacteria with the potential to degrade Phe were detected.

In order to avoid repeated identifications, screening by the RFLP method was performed. PCR-RFLP plays an important role in the rapid analysis of PAH-degrading bacteria in 16S rDNA libraries [34,35]. This method allows identifying the same bacterial species according to the profile of the DNA restriction fragments, making it possible to reduce the number of bacteria assayed by subsequent sequencing and phylogenetic analysis, and lowering the cost and the time of the assays. The restriction analysis of the 16S rDNA-PCR products revealed that the 95 isolates produced 42 different fingerprint patterns, suggesting that the Phe-degrading isolates were diverse. Figure 2 shows the RFLP pattern of 10 strains isolated from the FED Prosp 06/12 research campaign (see Figure 1). As an example, two pairs of strains with a similar pattern (FED 03B/08A and FED F02A/F02B) were detected. In this case, one strain of each pair (FED 03B and FED F02A) was selected for subsequent analysis, reducing the number of sequencing reactions. Thus, on the basis of microbial morphology and the results of RFLP analysis, 42 strains of Phe-degrading bacteria were isolated from the Río de la Plata waters. Gram stain results showed that 28 strains were Gram negatives and 13 Gram positives.

Partial sequencing of about 1400 nucleotides' length of 16S rDNA gene and BLAST searching in the EzTaxon-e database showed that isolates matched with the reference sequences with similarities higher than 98% in all cases. Phylogenetic analysis indicated that the strains were affiliated to 19 different genera of γ- and α-*Proteobacteria*, and *Actinobacteria* with bootstrap value more than 70 (Figure 3). Bacteria belonging to *Gammaproteobacteria* accounted for the highest proportion (54%), including the genera *Pseudoalteromonas*, *Vibrio*, *Marinomonas*, *Psychrobacter*, *Acinetobacter, Cobetia, Halomonas*, *Pseudomonas* and *Stenotrophomonas* (Figure 3(A)). Within the clade *Alpha-proteobacteria* (15%), the isolates were affiliated to *Thalassospira*, *Celeribacter*, *Ochrobactrum and Rhizobium* (Figure 3(B)). Finally, *Actinobacteria* phylum (31% of the isolates) was represented by *Rhodococcus*, *Mycobactertium*, *Dietzia, Micrococcus* and *Microbacterium* (Figure 3(C)). The reference sequences that resulted closely affiliated to the isolates corresponded to bacteria isolated from marine environments or species previously characterized as PAH-degrading bacteria.

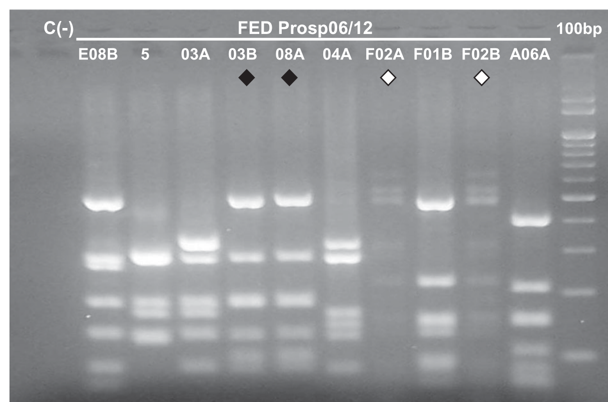


Figure 2. Representative agarose gel of RFLP analysis of the 16S rDNA gene in 10 Ph-degrading strains (FED Prosp06/12 campaign). Resultant restriction fragments of PCR products digested with the restriction endonuclease HaeIII are shown. FED 03B and 08A strains (◆), as well as FED F02A and F02B (◇) showed the same pattern.

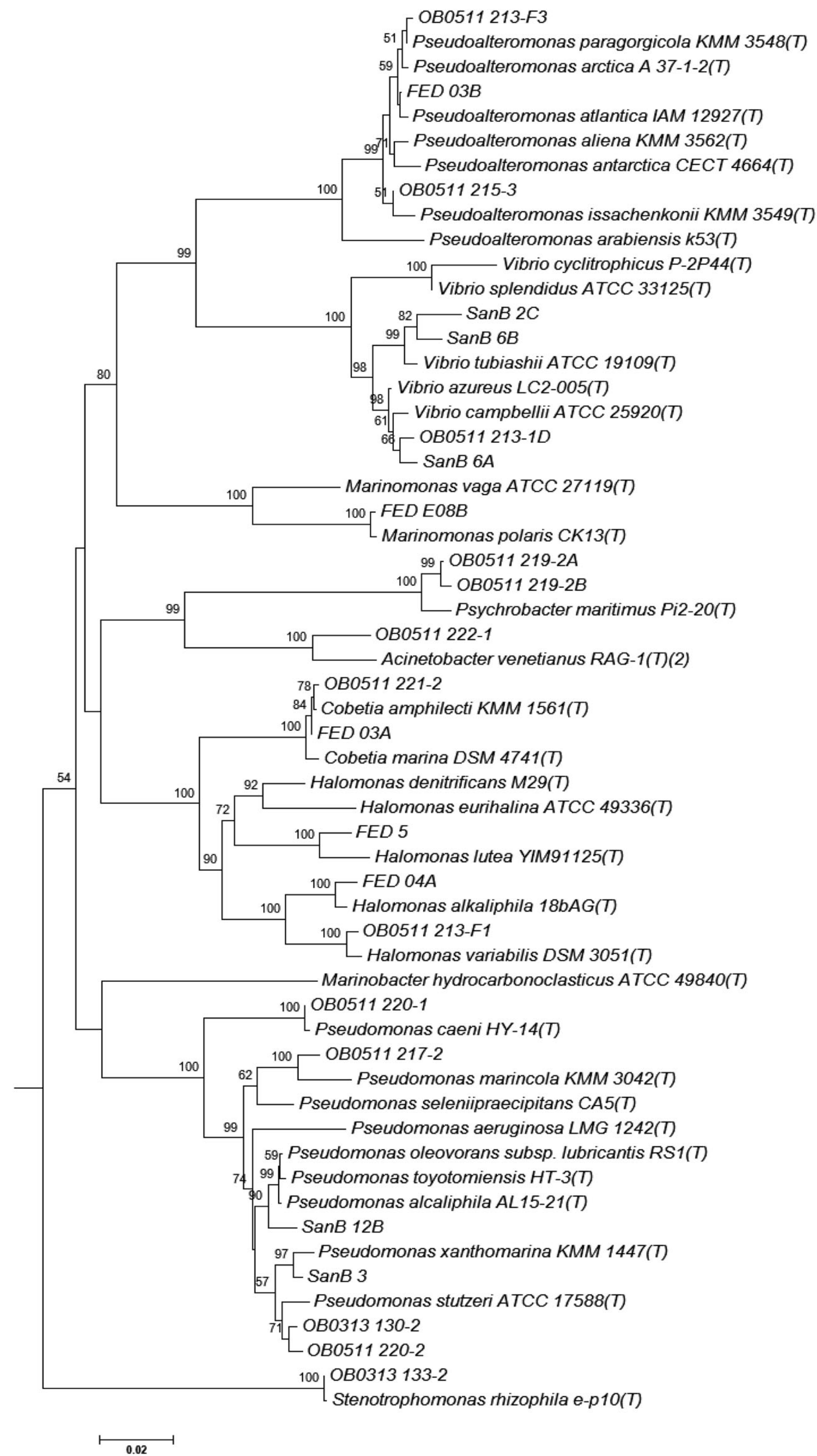
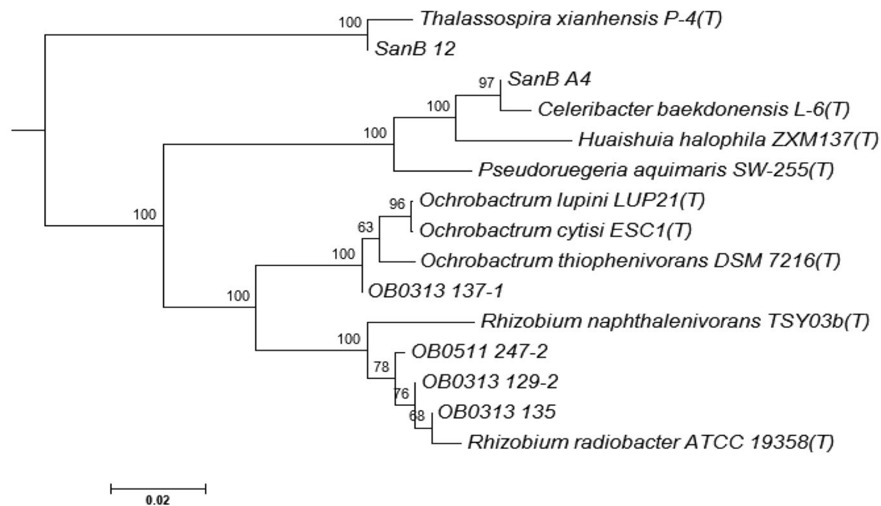
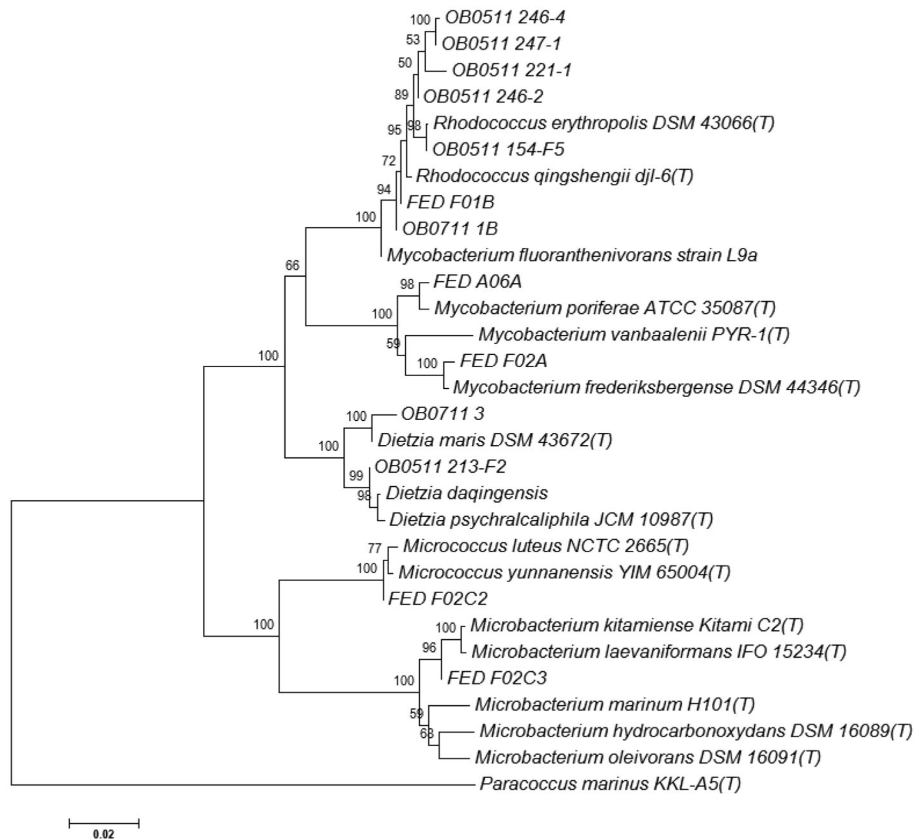


Figure 3. Phylogenetic tree of the 42 isolates, belonging to *Gammaproteobacteria* (A), *Alpha-proteobacteria* (B) and *Actinobacteria* (C), and related taxa from the EzTaxon-e database. The dendrogram was based on an approximately 1400 bp segment of the 16S rDNA gene sequence and constructed by the neighbor-joining algorithm. Only bootstrap values higher than 50% out of 1000 replication are shown.

Most of the genera found in this research have been earlier described as dominant members of PAH-degrading bacteria in marine environments, especially *Pseudoalteromonas* [36], *Vibrio* [37], *Marinomonas* [3], *Marinobacter* [38], *Psychrobacter* [39], *Halomonas* [40], *Pseudomonas* [41], *Thalassospira* [42], *Rhodococcus* [43],



(B) *Alphaproteobacteria*



(C) *Actinobacteria*

Figure 3. *Continued*

Mycobactertium [44] and *Dietzia* [3]. In the Río de la Plata environment, we have previously characterized four strains identified as *Pseudomonas* sp. and *Sphingomonas* sp., with high Phe-degradation efficiency and capability of surfactant production, but they were isolated from sediment samples [12]. Therefore, our work is the first report describing diverse Phe-degrading bacteria isolated from marine waters of this highly productive area.

Phenantrene bioremediation and biosurfactant production

Phe bioremediation by each isolated strain was monitored by HPLC assay. As is shown in Figure 4, 32 of these bacterial strains were able to degrade more than 50 and up to 97% of Phe after 9 days of incubation. Particularly, 15 strains (SanB 2C; OB0511 222-1; FED 04A; OB0511 213-F1; OB0511 217-2; SanB 12B; SanB 3; OB0313 133-2; OB0511 247-2; OB0313 129-2; OB0313 135; OB0511 247-1; OB0511 154-F5; OB0711 3, OB0511

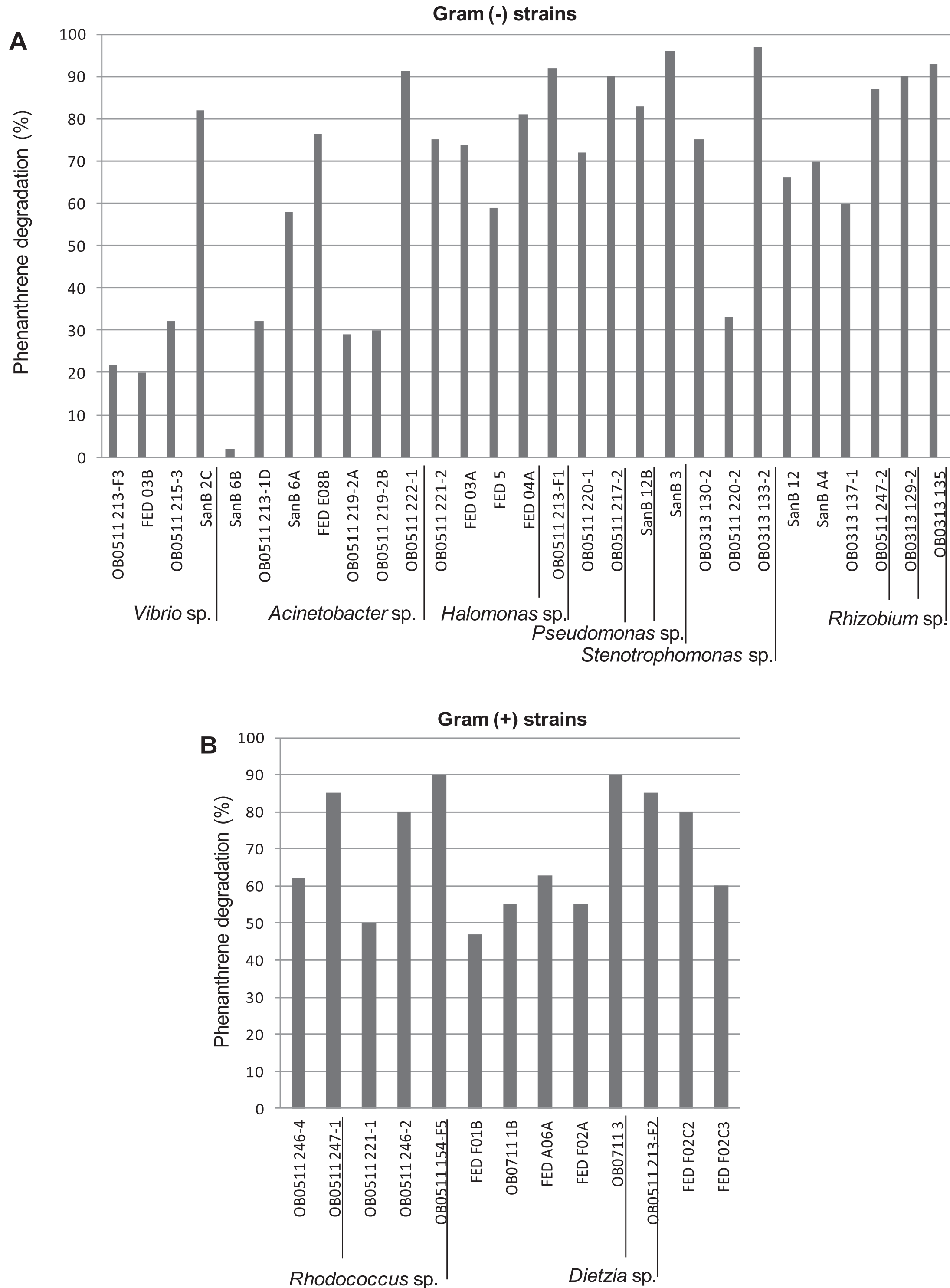


Figure 4. Phenanthrene bioremediation determined by HPLC for Gram negative (A) and Gram positive (B) strains. Isolates were inoculated in MSM-Phe and 3-ml samples were withdrawn after 9 incubation days, extracted and analyzed as indicated in Materials and Methods. Taxa related to efficient degrading strains are pointed out.

213-F2) related to species of the genera *Vibrio* sp., *Acinetobacter* sp., *Halomonas* sp., *Pseudomonas* sp., *Stenotrophomonas* sp., *Rhizobium* sp., *Rhodococcus* sp. and *Dietzia* sp., demonstrated efficient bioremediation because they were able to metabolize between 83% and 97% of the added phenanthrene (150 mg l^{-1}) in 9 days.

Previous studies have reported that some degrading strains recovered from enrichment cultures were not detected in natural contaminated sites by using culture-independent methods, suggesting that they may be in low proportion in the original environment and their growth is stimulated through the selective enrichment procedure [45]. The indigenous bacteria isolated in this study, which have catabolic abilities to degrade PAHs, could be stimulated *in situ* to increase their biomass and the degradation activity into the native microbial community [14].

In addition, although pure cultures of several PAH-degrading bacteria can utilize PAHs as the carbon source, degradation results may improve if a mixed bacterial consortium is used. There are synergistic effects of mixed microbial populations due to their cooperative metabolic activities that enhance degradation efficiency [46,47]. In this aspect, further analysis of mixed cultures could be performed in order to improve degradation capability of the autochthone strains obtained in this study, even if they show low degradation activity in pure culture.

On the other hand, biosurfactant production is an important physiological feature of microorganisms in contaminated environments. They are amphipathic molecules which are secreted to the medium, enhancing the bioavailability of lipophilic compounds such as hydrocarbons [48,49]. The 15 strains found to display high Phe-degradation efficiency were subsequently assayed for surfactant production through the analysis of hexadecane emulsification abilities of cell-free culture media. Culture supernatants from the strains *Vibrio* sp. SanB 2C; *Acinetobacter* sp. OB0511 222-1; *Pseudomonas* sp. OB0511 217-2; SanB 12B and SanB 3; *Stenotrophomonas* sp. OB0313 133-2; *Rhodococcus* sp. OB0511 247-1 and OB0511 154-F5; and *Dietzia* sp. OB0711 3 exhibited relatively high emulsifying indexes (E24), between 40 and 55% (Table 1). The capability of these isolates for production of surface-active agents was meant to be an advantage for PAH seawater treatments.

Table 1. Emulsifying activity from isolates after 72 h of incubation

Strain	Emulsification Index (%)
Vibrio sp. SanB 2C	40
Acinetobacter sp. OB0511 222-1	45
Halomonas sp. FED 04A	23
Halomona sp. OB0511 213-F1	10
Pseudomonas sp. OB0511 217-2	52
Pseudomonas sp. SanB 12B	55
Pseudomonas sp.; SanB 3	50
Stenotrophomonas sp. OB0313 133-2	45
Rhizobium sp. OB0511 247-2;	0
Rhizobium sp. OB0313 129-2	0
Rhizobium sp. OB0313 135	0
Rhodococcus sp. OB0511 247-1	48
Rhodococcus sp. OB0511 154-F5	52
Dietzia sp. OB0711 3	45
Dietzia sp. OB0511 213-F2	15

High-resolution melting analysis

HRM analysis of ribosomal genes or other highly conserved genes has been used extensively to identify microorganisms to the genus or species level in clinical research [20,50,51], and in a few works to compare bacterial community composition in soils treated with herbicides [52] and pesticides [18]. In this study, 33 of the rDNA fragments identified phylogenetically, showing high degradation rates, were subsequently tested by HRM analysis in order to identify and monitor PAH-degrading bacteria in this productive area.

After analyzing the generated melting curves, it was possible to accurately distinguish nine patterns corresponding to eight different genera (Figure 5), including *Pseudoalteromonas* sp. strains (OB0511 213-F3, FED 03B and OB0511 215-3); 2 patterns for *Vibrio* sp. (SanB 2C, SanB 6B, OB0511 213-1D and SanB 6A); *Pseudomonas* sp. (OB0511 220-1, OB0511 217-2, SanB 12B, SanB 3, OB0313 130-2 and OB0511 220-2); *Celeribacter* sp. (SanB A4); *Rhizobium* sp. (OB0511 247-2, OB0313 129-2 and OB0313 135); *Rhodococcus* sp. (OB0511 246-4, OB0511 247-1, OB0511 221-1, OB0511 246-2, OB0511 154-F5, FED F01B and OB0711 1B); *Mycobactertium* sp. (FED A06A, FED F02A) and *Dietzia* sp. (OB0711 3, OB0511 213-F2) *(data not shown)*. Interestingly, these patterns matched the clades in the dendrogram, which was performed with the strains previously identified by conventional amplification of 16S rDNA and sequencing, as described above. Strains showing a similar HRM profile are closely related to the NJ tree with similarities higher than 99%, suggesting that they could be the same species. Two genera were not detected by HRM, *Acinetobacter* sp. (OB0511 222-1) and *Stenotrophomonas* sp. (OB0313 133-2).

In addition, the HRM analysis allowed a differentiation at the species level for genera *Pseudomonas* (Figure 6(A)), *Halomonas* (Figure 6(B)) and *Vibrio* (Figure 5). These results are also in agreement with phylogenetic analysis shown in Figure 4. We detected 4 patterns for *Pseudomonas* which matched 4 nodes in the dendrogram, corresponding to individual or closely related species as *P. caeni* (OB0511 220-1); *P. marincola* (OB0511 217-2);

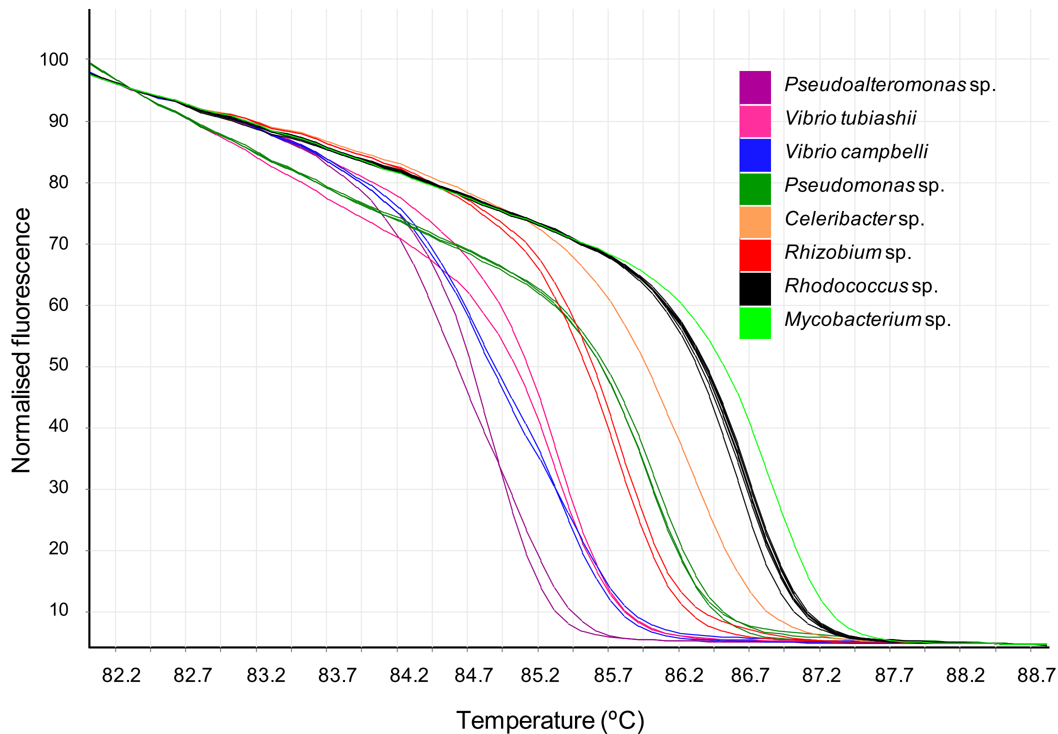


Figure 5. Normalized, HRM from 29 phenanthrene-degrading bacteria. Characteristic HRM patterns are observed for genera *Pseudoalteromonas* sp., *Vibrio* sp., *Pseudomonas* sp., *Celeribacter* sp., *Rhizobium* sp., *Rhodococcus* sp. and *Mycobacterium* sp.

P. oleovarans/toyotomiensis/alcaliphila (SanB 12B) *and P. xanthomarina/stutzseri* (SanB 3, OB0313 130-2 and OB0511 220-2). Both *P. oleovarans, P. toyotomiensis* and *P. alcaliphila; like P. xanthomarina* and *P. stutzseri* are located in the same 16S rDNA phylogenetic branch [53,54]. *In the case of Halomonas genera, species H. lutea* (FED 5), *H. alcaliphila* (FED 04A) and *H. variabilis* (OB0511 213 F1) showed characteristic HRM curve shapes. In Figure 5 we also differentiated 2 species of genus *Vibrio* by their melting profiles.

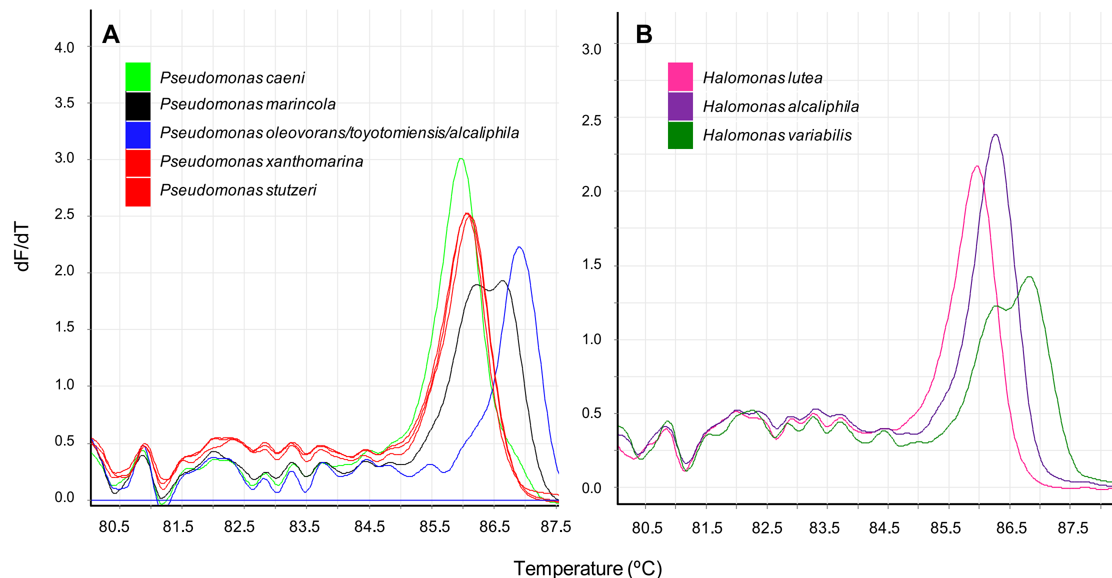


Figure 6. High-resolution melting curves of *Pseudomonas* sp. and *Halomonas* sp. A: Different *Pseudomonas* species (*P. caeni*, *P. marincola*, *P. oleovarans/ toyotomiensis/alcaliphila*, *P.xanthomarina* and *P. stutzseri*) show characteristic melting temperature and HRM curve shapes. B: Different *Halomonas* species (*H. lutea*, *H. alcaliphila* and *H. variabilis*) also show characteristic melting temperature and HRM curve shapes.

Even though HRM has been mainly used for the identification of a large number of bacteria from the 16S rDNA gene, all of these were in the field of current clinical research and diagnostics of disease [55,56]. It is the first time, to our knowledge, that HRM has been carried out to identify PAH-degrading bacteria from seawater samples. HRM melt curves do not provide phylogenetic information themselves [18]. Yet, we propose a strategy whereby having a background of bacterial taxonomic affiliations by conventional methods, the obtained melting curves could be used as a screening technique for the rapid and highly specific identification of PAH-degrading bacteria. This approach could be applied for monitoring PAH degraders during bioremediation treatments in contaminated environments like seawater or marine sediments.

Conclusions

The present study contributed to the microbial resource and knowledge of indigenous degrading bacteria which could be applied in eco-sustainable bioremediation treatments in this important estuarine environment. We found a variety of degraders and some of them, showing high Phe degradation capacity and surfactant production, could be an alternative for microbial selection in the degradation of PAHs in this system. Likewise, bacterial adaptability to specific environmental conditions and non-pathogenicity should also be considered during these approaches.

On the other hand, we reported in this work the implementation of HRM as a rapid method to identify PAH-degrading bacteria along a remediation process. Obtaining specific melting curves as a screening method, which avoids the sequencing step, represents an encouraging step for the application of bioremediation. Nevertheless, pilot scale experiments are necessary to make this technology a reliable option to mitigate the ecological damage caused by PAH pollution in marine environments.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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