International Biodeterioration & Biodegradation 84 (2013) 161–167

Contents lists available at SciVerse ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Phenanthrene degradation and strategies to improve its bioavailability to microorganisms isolated from brackish sediments

Andrea Pedetta [a,1], Karina Pouyte [c,1], María K. Herrera Seitz [a], Paola A. Babay [b], Mariela Espinosa [b], Marcela Costagliola [c], Claudia A. Studdert [a], Silvia R. Peressutti [c,*]

[a] Instituto de Investigaciones Biológicas, Universidad Nacional de Mar del Plata, Casilla de Correo 1245, 7600 Mar del Plata, Buenos Aires, Argentina
[b] CNEA Comisión Nacional de Energía Atómica, Centro Atómico Constituyentes, Av. Gral Paz 1499, 1650 San Martín, Buenos Aires, Argentina
[c] Instituto Nacional de Investigación y Desarrollo Pesquero, Casilla de Correo 175, 7600 Mar del Plata, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 31 January 2012
Received in revised form
26 April 2012
Accepted 30 April 2012
Available online 28 July 2012

Keywords:
Polycyclic aromatic hydrocarbon
Phenanthrene
Biosurfactant
Chemotaxis
Bioremediation

ABSTRACT

Samborombón Bay, Argentina, is a highly productive area exposed to chronic contamination, including polycyclic aromatic hydrocarbons. Four phenanthrene-degrading strains were isolated from sediments collected in this area. Analysis of partial 16S rRNA sequencing and a BLAST search indicated that three of the strains belong to genus *Pseudomonas* and one to *Sphingomonas*. All the strains were able to grow in 150 mg l^{-1} phenanthrene as the sole carbon and energy source, with high degradation efficiency (75–100% in 72–168 h). Growth in sodium salicylate indicated that the *Pseudomonas* strains used this pathway to degrade phenanthrene.

Strategies that may enhance substrate bioavailability, such as surfactant production and chemotactic responses, were tested. Two *Pseudomonas* strains showed significant production of surface-active compounds, and a strong chemotactic response toward phenanthrene. Together with the ability to consume the supplied phenanthrene to completion, these characteristics make the mentioned strains good candidates for bioremediation strategies intended to clean up polluted areas.

1. Introduction

Anthropogenic inputs of polycyclic aromatic hydrocarbons (PAHs) from oil spills, ship traffic, and discharge of industrial effluents have caused significant accumulation in marine sediments (Mount et al., 2003). During recent years there has been increasing concern about PAHs contamination in marine environments owing to these substances' persistence, toxicity, mutagenicity, and carcinogenicity (Dibble et al., 1990; Menzi et al., 1992; Haeseler et al., 1999). These pollutants are not only toxic to fishes and other aquatic organisms (White and Triplett, 2002) but they have also been found to bioaccumulate in marine organisms with possible transfer to humans via seafood (Morales-Caselles et al., 2008).

Microbial degradation is a major process for the successful removal and elimination of PAHs from the environment since it is an efficient, inexpensive, and environmentally safe cleaning method. Although hydrocarbon-degrading microorganisms can be found in contaminated sites, bioremediation processes are usually limited by the low solubility and availability of these compounds.

Bioavailability is determined by the rate at which the substrate reaches the cell relative to the rate of uptake and metabolism (Bosma et al., 1997). Some PAH-degrading bacteria display strategies to improve hydrocarbon accessibility, such as close attachment to the PAH source and high affinity uptake systems (Harms and Bosma, 1997). In recent years, biosurfactant production has been pointed out as an advantageous feature (Moran et al., 2000; Olivera et al., 2009). Biosurfactants are amphipathic molecules that increase bioavailability either by increasing the apparent hydrocarbon solubility of the substrate in the aqueous phase or by expanding the contact surface due to emulsification (Rosenberg and Ron, 1999). Lately, bacterial chemotaxis, which allows microorganisms to direct their movement according to a chemical gradient, has also been shown to have a key role in biodegradation processes (Pandey and Jain, 2002; Parales, 2004; Paul et al., 2006).

Phenanthrene (PHE) is a highly reactive compound that belongs to the low-molecular-weight aromatic hydrocarbons group, having three aromatic rings per molecule (Mrozik et al., 2003). Its low water solubility (1.29 mg l^{-1}) makes its biodegradation difficult; however, there are several reports of microorganisms capable of degrading it. Microorganisms of the genera *Pseudomonas*,

* Corresponding author. Tel.: +54 223 4862586; fax: +54 223 4861830.
E-mail address: silviap_ar@inidep.edu.ar (S.R. Peressutti).
[1] Both should be considered as first author.

0964-8305/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ibiod.2012.04.018

Burkholderia, *Sphingomonas*, *Acinetobacter*, *Rhodococcus*, and *Mycobacterium* have been identified as PAH-degraders, and complete PAH mineralization has been demonstrated for both low- and high-molecular-weight PAHs (Mrozik et al., 2003; Johnsen et al., 2005). In addition, several consortia of PHE-degrading bacteria have been characterized (Kim et al., 2009; Sorensen et al., 2010).

Samborombón Bay is a C-shaped bay located in the southern end of the Río de la Plata estuary, on the Atlantic coast of South America (Fig. 1). It is one of the most important estuarine environments in the continent. This is a highly productive area that sustains valuable artisanal and coastal fisheries (Mianzan et al., 2001; Jaureguizar et al., 2003). However, this area is exposed to PAH pollution, associated mainly with the chemical industry, oil refining, and port activities (Colombo et al., 2005). Autochthonous bacteria isolated from this polluted ecosystem can provide a useful tool for applied microbiology, and to our knowledge there are no reports on indigenous PAH-degraders isolated from Samborombón Bay sediments.

Therefore, the present study aims to: (1) select and identify autochthonous microorganisms capable of degrading phenanthrene (PHE); and (2) characterize their biodegradation capacity, emulsifying activity, and chemotaxis ability in order to know the potential of these microorganisms for future bioremediation processes.

2. Materials and methods

2.1. Culture media and bacterial growth

A stock solution 30 mg ml^{-1} of phenanthrene (Sigma, Argentina, purity ≥96%) was prepared in ethanol and used for all the assays. A mineral salts medium/phenanthrene liquid medium for the isolation of PHE-degrading microorganisms was prepared with mineral salts medium (MSM) (Schlegel et al., 1961) modified by addition of NaCl 2% (w/v) and phenanthrene at a final concentration of 150 mg l^{-1} (0.84 mM) as the sole carbon and energy source. 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}). Mineral salts medium/phenanthrene 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 phenanthrene at a final concentration of 340 mg l^{-1} (Bogardt and Hemmingsen, 1992).

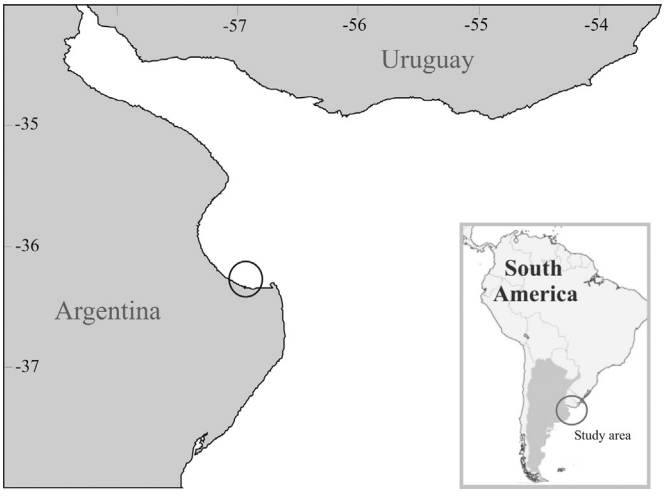

Fig. 1. Map of Samborombón Bay showing the sampling area location (circle).

Rich medium (NA), containing 1% tryptone, 0.5% yeast extract, 2% NaCl, and 1.2% agar, was used for identification of colony morphology and CFU determination. Plates were incubated at 28 °C for 3–7 days.

Growth with phenanthrene as the sole carbon and energy source was tested in MSM–PHE liquid medium inoculated with an exponential phase culture of each bacterial strain. At different time intervals growth was measured by OD_{600} and total CFU ml^{-1} was determined on NA medium. Control cultures lacking a carbon source were performed for each isolate. MSM-NaSal medium contained MSM plus 0.5% w/v sodium salicylate as the sole carbon and energy source.

2.2. Sampling and isolation of PAH-degrading isolates

Surface sediment samples (0–3 cm) were collected at four stations in Samborombón Bay (S 36° 17′ - W 56° 46′), Argentina (Fig. 1). Sub-samples (10 g) were inoculated into 500-ml flasks containing 100 ml of MSM–PHE culture medium. Two-milliliter aliquots were withdrawn from each culture every week for a month, transferred to fresh sterile medium, and incubated as described above. Finally, cultures were plated on MSM–PHE agar. Colonies of candidate phenanthrene-degrading strains were picked up and further purified by repetitive streaking on fresh MSM–PHE agar plates. The pure cultures of the final isolates selected were preserved under refrigeration on nutrient agar slants or as 10% dimethyl sulfoxide (DMSO) stocks at −80 °C.

2.3. Sample preparation for scanning electron microscopy (SEM)

Cells were grown overnight in liquid MSM–PHE medium. Six microliters of culture were loaded on a glass coverslip and then left for 2 h in glutaraldehyde 2.5% in phosphate buffer 0.1 M. After three washes of 15 min each in the same buffer, 5-min washes with increasing ethanol concentrations (30%, 50%, 70%, 80%, 90%, 95%) were made. Finally, samples were covered overnight with hexamethyldisilazane (HMDS, Sigma, Argentina) and then processed for SEM.

2.4. Molecular characterization of the bacterial isolates

The identification of the isolates was based on 16S ribosomal RNA (rRNA) gene sequence analysis. Single colonies of each isolate were resuspended in 50 μl sterile water and boiled for 5 min. The resulting suspension (5–10 μl) was used as a template for polymerase chain reaction (PCR) using primers F43Eco (5′-CGGAATTCCAGGCCTAACACATGCAAGTC-3′) and R1387Eco (5′-CGGAATTCGGGCGGWGTGTACAAGGC-3′), as described by Marchesi et al. (1998). PCR reactions contained: 0.25 μmol l^{-1} of each primer (F43Eco and R1387Eco), 0.5 mmol l^{-1} dNTPs, 0.5 U Taq DNA polymerase (P-BL, Quilmes, Argentina), 1× Taq DNA polymerase buffer, and 3 mmol l^{-1} $MgCl_2$. Amplifications were carried out using the following temperatures: (94 °C 10 min) × 1; (95 °C 1 min, 55 °C 1 min, 72 °C 90 s) × 30, (72 °C 5 min) × 1. PCR products were electrophoresed on 0.8% (w/v) agarose gels containing SyberSafe (Invitrogen, Argentina). The separated bands were visualized under a blue light transilluminator (SafeImager, Invitrogen, Argentina). The DNA fragments of the expected size (1.3 kbp) were eluted, purified, and sequenced using primers F43Eco and R1387Eco (INTA Castelar, Argentina). The resulting sequenced fragment was ~900 kbp in length. The nucleotide sequences of the amplified fragments were compared against sequences contained within Public Databases (http://rdp.cme.msu.

edu and NCBI/BLAST). All the sequences are available at GenBank database.

2.5. Phenanthrene degradation

2.5.1. HPLC

To measure residual phenanthrene concentrations, 3-ml aliquots were periodically withdrawn from the cultures 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 were analyzed by reverse-phase HPLC according to NIOSH (1998). Phenanthrene degradation was conducted in two independent experiments in MSM–PHE liquid medium (150 mg l^{-1}, w/v) and non-inoculated flasks were used as abiotic controls.

The instrumental system consisted of a binary pump (Thermo Spectra series P200, USA), a column heater (Eppendorf, USA) set at 50 °C, a UV–VIS detector (Thermo UV 100, USA) set at a detection wavelength of 254 nm, and a fluorescence detector (Linear Fluor LC305, USA) set at an excitation wavelength of 340 nm and an emission wavelength of 426 nm. Data were acquired and processed with EZChrom software (Chromatography Data System Version 6.8, USA). Separations were performed using a 3-μm particle octadecyl silica column of 250 × 4.6 mm (Inertsil ODS-3; GL Science, Japan). Linear gradient elution was carried out from 50% acetonitrile/50% deionized water to 90% acetonitrile/10% deionized water over 30 min, followed by isocratic hold at 90% acetonitrile for 10 min. The flow rate was 1 ml min^{-1}.

2.5.2. CO_2 production

A CO_2 production test was performed as described by Hanstveit (1992). The CO_2 produced by the cells was trapped into vials containing 2 ml of 1 M NaOH that were put into flasks containing MSM–PHE. Vials were replaced daily with new vials containing fresh NaOH solution during the course of the experiment. The flasks were tightly sealed with wrapped rubber stoppers, and daily CO_2 production was indirectly calculated based on the results of titration of the remaining NaOH.

2.6. Biosurfactant production assays

A droplet collapse assay was carried out as described in Jain et al. (1991). Briefly, 1 μl of methylene blue [0.1% (w/v)] was added to 20 μl of cell-free medium from saturated cultures grown in MSM–PHE. The resulting mixture was spotted onto a piece of Parafilm sheet (Pechiney Plastic Packaging, USA) and photographed after 5 min. The methylene blue was added solely for visualization purposes and does not influence droplet collapse activity. Fresh MSM–PHE medium containing either no addition or 1% SDS were used as negative and positive controls, respectively.

Emulsification ability of the culture supernatant was determined by vortexing 2 ml of hexadecane and 2 ml of aqueous supernatant at high speed for 2 min and incubating at 25 °C for 24 h. An emulsification index value (E_{24}) was calculated as: (height of emulsion layer/height of total mixture) × 100 (Iyer et al., 2006).

2.7. Chemotaxis assays

Agarose-in-plug assays were carried out as described by Yu and Alam (1997) with slight modifications. Plugs contained 2% low-melting-temperature agarose (NuSieve GTG Agarose; FMC Bioproducts, Rockland, ME, USA) in motility buffer (10 mM potassium phosphate pH 7, 0.1 mM EDTA) and no addition (negative control), 2% (w/v) casamino acids (positive control), or 0.01% w/v phenanthrene. Drops (10 μl) of the melted agarose mixtures were placed on a microscope slide, and a coverslip supported by two plastic strips was then placed on top to form a chamber. Cells grown in MSM–PHE were harvested in log phase (OD_{600} values between 0.3 and 0.7) by centrifugation at 1000 × *g*, washed twice, and resuspended in motility buffer to an OD_{600} of approximately 0.6–0.8. Cell motility was checked under a phase-contrast microscope, and highly motile cell suspensions were flooded into the chamber surrounding the agarose plugs. Each chamber was photographed 2–5 min after the addition of cells.

Swimming plate assays were carried out as described by Lanfranconi et al. (2003). Semi-solid agar plates were prepared with MSM medium with the addition of 2% (w/v) NaCl, 100 mg l^{-1} (w/v) phenanthrene, and 0.25% agar. Cultures of the analyzed strains were grown in MSM–PHE and adjusted to an OD_{600} of 1.0. Two microliters of these cultures were spotted into the center of the semi-solid agar plates and incubated at 28 °C.

3. Results

3.1. Isolation and identification of PHE-degrading microorganisms

Samborombón Bay sediments are under chronic exposure to a wide variety of pollutants due to the high industrial activity in that area and the lack of appropriate disposal treatment. Associated with the oil refineries and other chemical industries, PAHs are regular contaminants in these sediments. In order to identify phenanthrene-degraders, samples of hydrocarbon-contaminated sediment obtained from the Samborombón Bay were inoculated into mineral medium supplemented with phenanthrene as the only source of carbon and energy. After four weekly transfers to fresh MSM–PHE medium, aliquots of each sample were plated on MSM–PHE agar plates, where considerable colony diversity was observed. Based on differences in the morphology of the colonies, four of them were selected and isolated. Phenanthrene biodegradation was confirmed in liquid media.

Analysis of partial 16S rRNA sequencing and BLAST searching indicated that three of these isolates belong to genus *Pseudomonas* (similarity values ranged between 97 and 98%) and one of them to *Sphingomonas* (98% similarity). The partial 16S rRNA gene sequences of all four strains were deposited in the GenBank under the accession numbers JQ083664 (PH-a, *Pseudomonas* sp.), JQ083665 (PH-b, *Sphingomonas* sp.), JQ083666 (PH-c, *Pseudomonas* sp.), and JQ083667 (PH-d, *Pseudomonas* sp.).

Phenanthrene degradation was confirmed for each isolated strain both in liquid and solid media and the morphology of colonies was analyzed in rich medium (Fig. 2a) and in MSM–PHE agar plates (Fig. 2a, inset). Creamy colorless colonies were observed for the three *Pseudomonas* strains. By contrast, *Sphingomonas* sp. developed brilliant yellow-colored colonies, which is in accordance with previously described *Sphingomonas* strains (Takeuchi et al., 2001). When observed by scanning electron microscopy, all the isolates showed a rod-shaped structure, as expected for *Pseudomonas* sp. and *Sphingomonas* sp. strains (Fig. 2), with approx 2 μm length and 0.5 μm width. For strains PH-b and PH-c, the presence of an extracellular filamentous material was observed (Fig. 2, black arrows).

3.2. Bacterial growth and phenanthrene degradation in liquid medium

Due to the low water solubility of phenanthrene, addition of 150 mg l^{-1} to the medium resulted in saturation; therefore, at the beginning of the experiment phenanthrene was present mainly in the form of crystals that disappeared during growth. When growth

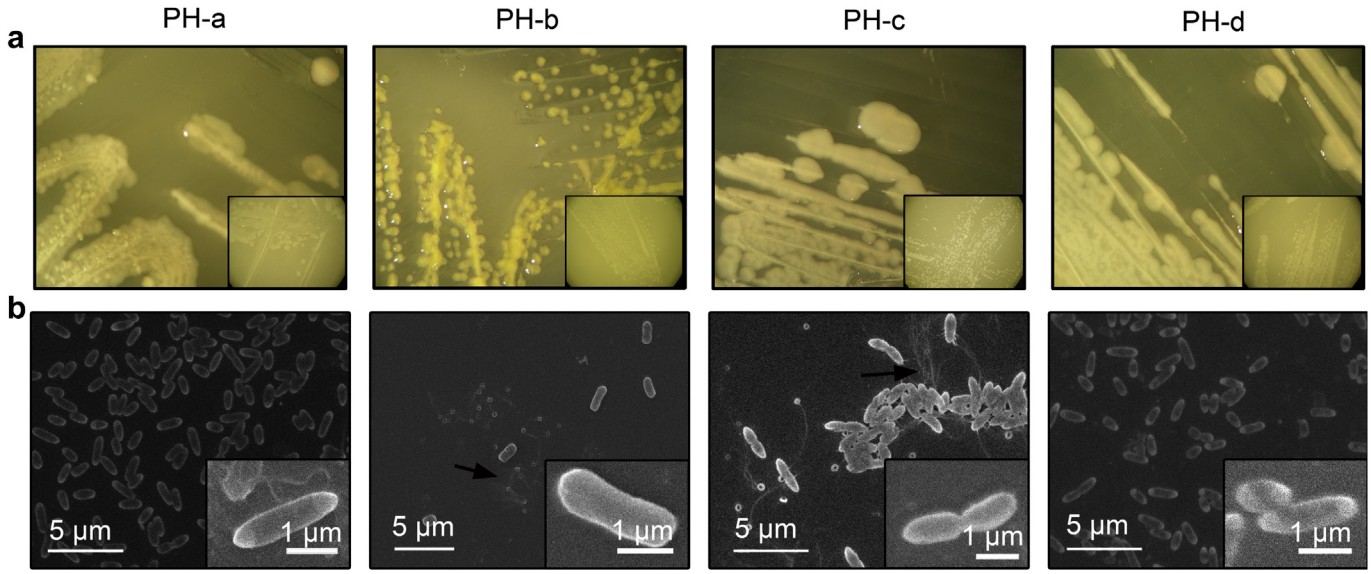

Fig. 2. Morphology of phenanthrene-degrading bacteria isolated from Samborombón Bay. (a.) Morphology of colonies of the four isolated strains grown on rich medium, or on minimal medium with phenanthrene as the sole carbon source (inset). PH-a, *Pseudomonas* sp; PH-b, *Sphingomonas* sp.; PH-c *Pseudomonas* sp.; PH-d, *Pseudomonas* sp. (b.) Cells of isolated strains were grown on MSM–PHE and then prepared as described in material and methods for SEM. 5000× (inset 10,000×). Black arrows point to filamentous extracellular material.

in MSM–PHE was followed for each isolate (Fig. 3), maximal OD values ranging from 2 to 4, depending on strain, were reached in all the cases after 96 h of incubation (Fig. 3a). The microbial growth did not show an extended lag phase for any of the isolates. After 72–96 h, the biomass reached a stationary phase for the four isolates as determined by OD measurement. Similar results were obtained when growth was analyzed by bacterial counts. Strains PH-a and PH-b increased from 10^3 to 10^8 CFU ml^{-1}, while strains PH-c and PH-d increased from 10^3 to 10^9 CFU ml^{-1}. Phenanthrene concentrations higher than 150 mg l^{-1} were assayed (500–1000 mg l^{-1}) and growth was slower but not completely inhibited at any concentration tested (data not shown).

Phenanthrene biodegradation was analyzed by HPLC at an initial concentration of 150 mg l^{-1} (Fig. 3b). A sudden drop in phenanthrene level was observed for strains PH-b, PH-c, and PH-d, followed by a slower decrease as the assay proceeded. For PH-a, instead, a constant degradation slope from the beginning of the experiment was observed. Complete PHE degradation was observed for strains PH-d, PH-c, and PH-b after 72, 96, and 168 h, respectively, whereas for PH-a degradation reached 75%. The abiotic controls showed negligible phenathrene elimination by evaporation. Degradation rates were as follows: PH-a, 15 mg l^{-1} day^{-1}; PH-b, 30 mg l^{-1} day^{-1}; PH-c, 37 mg l^{-1} day^{-1}; and PH-d, 48 mg l^{-1} day^{-1}. These biodegradation rates were concomitant with the cell abundances and optical densities mentioned earlier.

The biochemical pathways used for phenanthrene degradation have been characterized in several microorganisms. In one of the primary pathways used for phenanthrene degradation, the upper part of the pathway renders 1-hydroxy-2-naphtoic acid. This compound is further metabolized by one of two main routes, the phthalic acid pathway, described by Kiyohara et al. (1976), or the naphthalene pathway, in which salicylic acid is an intermediary metabolite (Evans et al., 1965). In order to address whether the strains isolated during this study used one of the two major known phenanthrene degradation pathways, each strain was tested for growth in minimal medium containing sodium salicylate (MSM-NaSal) or phthalic acid (MSM-phthalate) as the only carbon source. The three *Pseudomonas* strains PH-a, PH-c, and PH-d seem to use

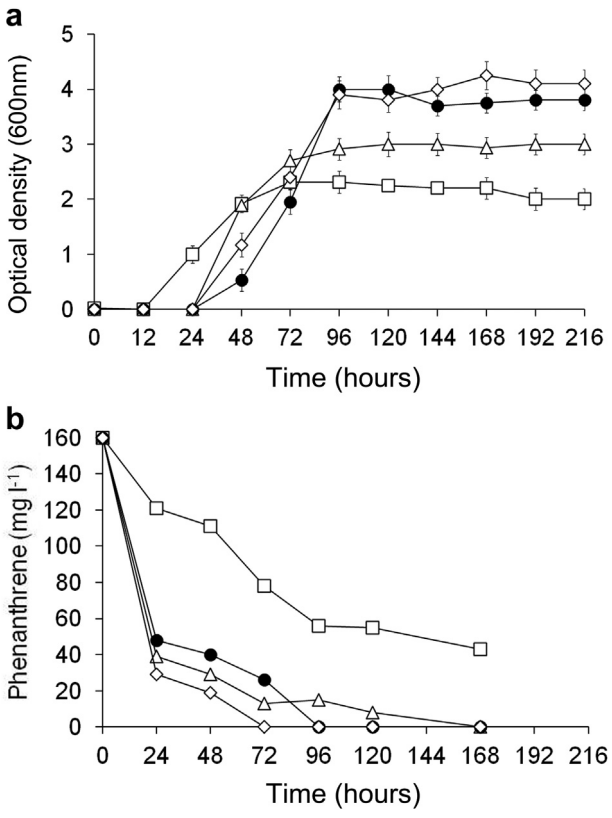

Fig. 3. Growth and degradation of phenanthrene by isolated strains. (a.) Each strain was inoculated in MSM–PHE (150 mg l^{-1}) and growth was followed by OD_{600} determination. PH-a (□), PH-b (△), PH-c (•), PH-d (◆). Values are means ± standard deviations for three replicates. (b.) Phenanthrene degradation was determined by HPLC. Isolated strains were inoculated in MSM–PHE and 3 ml-samples were withdrawn at different times, extracted and analyzed as indicated in Materials and Methods. PH-a (□), PH-b (△), PH-c (•), PH-d (◆). The results are representative of two independent experiments.

the naphthalene pathway, since they were able to grow at the expense of salicylate, but not phthalate. PH-b, in contrast, was not able to grow using either sodium salicylate or phthalicacid.

In order to get information about the extent of phenanthrene mineralization, CO_2 present in the gaseous phase produced during 7 days was measured. Strains PH-c and PH-d yielded 1.2 mM ± 0.05 mmol l^{-1} CO_2, whereas PH-a and PH-b reached 0.8 ± 0.07 and 0.3 ± 0.03 mmol l^{-1} CO_2, respectively, indicating that the four strains yielded considerable CO_2 production when grown on PHE compared to control cultures with no carbon source. These preliminary results suggest that PHE is being at least partially mineralized to CO_2 and H_2O. The remaining substrate may be transformed to biomass and metabolic products. The appearance of a brownish color in the culture medium after growing cells in MSM–PHE indicates accumulation of intermediates of phenanthrene metabolism.

3.3. Surfactant production

One possible mechanism to enhance the bioavailability of non-polar substrates is the production of surface-active agents. Surfactant production by the strains isolated in this study was determined through the analysis of the droplet collapsing and hexadecane emulsification abilities of cell-free culture media. Cell-free medium from saturated cultures of all three *Pseudomonas* strains (PH-a, PH-c, and PH-d) indicated the presence of surface-active compounds, both in the droplet collapse (Fig. 4b) and in the hexadecane emulsification assays (Fig. 4a). Culture supernatants from the PH-d and PH-c strains showed relatively high emulsifying indexes (E_{24} = 50 and 38, respectively) and a droplet collapsing activity similar to that of 1% SDS, while surfactant production by the strain PH-a was somehow lower, as indicated by a reduced droplet-collapsing activity and a lower emulsifying index (E_{24} = 10). On the other hand, the *Sphingomonas* strain (PH-b) did not show emulsifying activity in any of the assays (Fig. 4a,b).

3.4. Motility and chemotaxis

Bacterial chemotaxis, the ability of cells to detect chemical gradients and direct their movement in consequence, helps microorganisms find optimal conditions for their growth and survival. There have been previous descriptions of how chemotaxis can be an advantage for degradation of aromatic compounds (Parales and Harwood, 2002; Paul et al., 2006).

Exponential phase cultures of the isolated strains on MSM–PHE were observed under the phase contrast microscope to assess their motility. Although all four strains were motile, motility observed for strains PH-a and b was variable and in some cases it was lost after harvesting cells by centrifugation. Thus, chemotaxis assays were restricted to the highly motile *Pseudomonas* strains PH-c and PH-d. When inoculated into semi-solid phenanthrene-containing agar plates, both PH-c and PH-d were able to spread on the plate well beyond the inoculation point after two days of incubation (Fig. 5a), and all over the plate after longer incubation (not shown). This behavior is consistent with a chemotaxis response to the gradient that would form as a result of phenanthrene consumption by the cells, since this hydrocarbon represented the only carbon source in the medium.

The agarose-in-plug assay was then used in order to assess the chemotactic response toward phenanthrene in a more direct way. In this assay, cells accumulate around attractant-containing plugs. Since the chemotactic response to amino acids by *Pseudomonas* and related organisms has been well described (Taguchi et al., 1997; Lanfranconi et al., 2003), a casamino-acids-containing plug was included as a positive control in this assay. Cells of both PH-c and PH-d strains clearly accumulated around the casamino-acids-containing plug, as expected (Fig. 5b, top-right plugs). Likewise, the accumulation of cells around phenanthrene-containing plugs was observed in both strains, clearly showing a chemotactic response toward this hydrocarbon. Moreover, both strains showed a response toward phenanthrene that was comparable to that toward the strong attractant amino acids (Fig. 5b, top-left plugs). No accumulation of cells was detected around the control plug without attractant (Fig. 5b, bottom plugs).

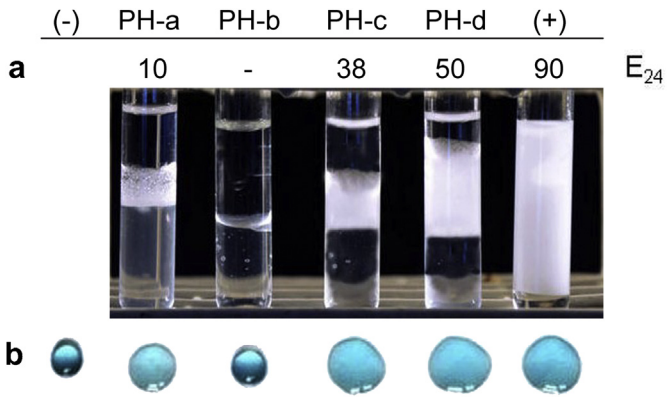

Fig. 4. Production of surface-active compounds. (a.) Emulsifying activity from isolates after 72 h incubation. Two ml of hexadecane plus two ml of the indicated cell-free culture supernatants were vigorously vortexed and then left to stand during 24 h. Emulsification index (E_{24}) was calculated as described in Materials and Methods. A 1% SDS solution in MSM was used as positive control (+). (b.) Droplet collapse assay. Droplets (20 μl) of the indicated cell-free supernatants were colored by the addition of methylene blue, spotted on a piece of Parafilm sheet and photographed after standing during 5 min. Methylene blue was added to each sample to aid in visualization. MSM medium containing no addition (−) or 1% SDS (+) were used as negative and positive controls, respectively.

4. Discussion

Our study focuses on phenanthrene-degrading isolates from Samborombón Bay sediments, in the Río de la Plata estuary, exposed to continuous hydrocarbon pollution. We have isolated and preliminarily characterized three strains belonging to genus *Pseudomonas* sp. and one to *Sphingomonas* sp. *Pseudomonas* is one of the most studied genera and has been reported as a degrader of a wide range of organic pollutants including PAHs and other recalcitrant xenobiotics (Mulet et al., 2011). *Sphingomonas* has also been described as a phenanthrene-degrader in more recent works (Chen et al., 2008; Coppotelli et al., 2010).

In this work bacterial growth was observed in a wide range of phenanthrene concentrations, as seen in previous work (Tam et al., 2002). However, when 500 and 1000 mg l^{-1} PHE were added in the media, the growth rate was diminished, probably indicating some toxic effect on the cells. Because of that, 150 mg l^{-1} was chosen as the optimal concentration for this study and high bacterial biomass was reached in the cultures, mainly for strains PH-c and PH-d.

In the biodegradation experiments, during the initial stage of incubation a sudden drop in phenanthrene concentration measured in the culture medium was observed for strains PH-b, PH-c, and PH-d. A similar trend was also seen by Tian et al. (2002). This initial drop might be explained due to dynamic assimilation by the cells or adsorption to the cell wall. Furthermore, this largely insoluble hydrocarbon was completely utilized by *Pseudomonas* sp. strains PH-c and PH-d during the first 72 and 96 h, respectively, and by *Sphingomonas* sp. strain PH-b after 168 h. This fact may indicate that real degradation could have occurred, at least partially, after the early assimilation or adsorption, and so it should

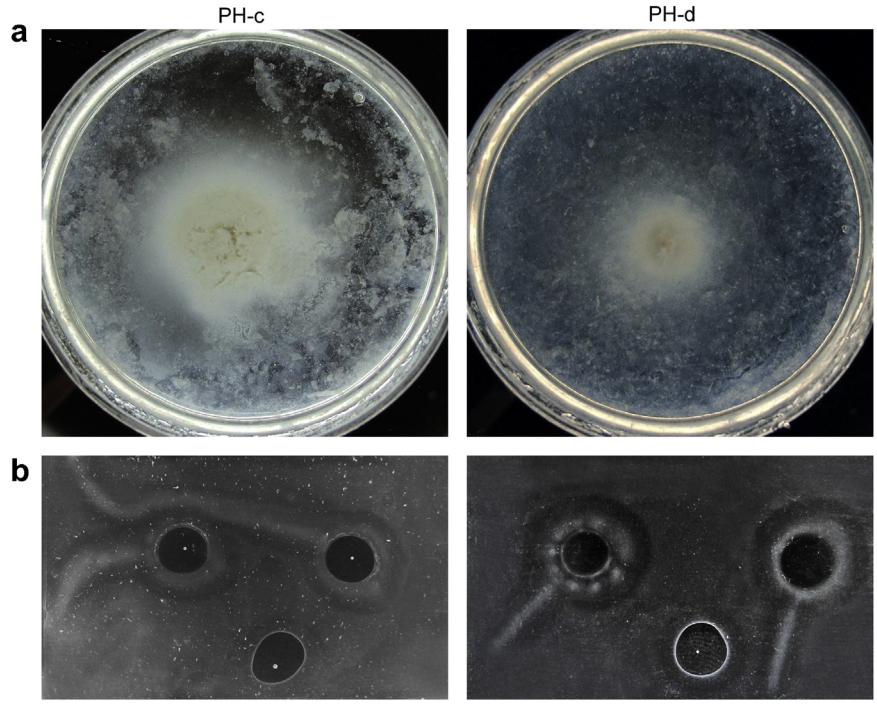

Fig. 5. Chemotaxis assays. (a) Swimming in semi-solid agar plates. 2 μl of the indicated cultures were inoculated in the center of plates containing 100 mg l^{-1} (w/v) phenanthrene. Plates were photographed after 48 h incubation at 28 °C. (b) Agarose-in-plug method. Cells from the indicated cultures were harvested, washed and resuspended in motility buffer, and then flooded into a chamber containing three agarose plugs. The chambers were photographed 2–5 min after the addition of cells. Top left plug: 2% (w/v) casamino acids in motility buffer; top right plug: 0.01% (w/v) phenanthrene in motility buffer; bottom plug: motility buffer without any attractant. Notice that cells accumulate both around the attractant containing plugs and also along a tail presumably originated in a plug-washing effect that took place as the chamber was filled with the cell suspension.

be taken into account during the discussion of degradation kinetics. The rates of phenanthrene degradation used as the sole carbon and energy source were similar to, if not higher than, rates reported elsewhere (Reddy et al., 2010), especially for strains PH-c and PH-d. Remarkably, the addition of simpler carbons sources was not necessary to obtain good PAH biodegradation rates, unlike what has been reported for other strains (Yuan et al., 2000).

Different pathways can be involved in phenanthrene degradation and many intermediate metabolites are produced, accumulated, and consumed as it proceeds (Resnick et al., 1996; Mrozik et al., 2003), which makes it a complex process. Growth of *Pseudomonas* strains in sodium salicylate, as well as the associated CO_2 production, would indicate the complete phenanthrene degradation through the salicylic acid metabolic pathway. More in depth studies would be needed to address whether a complete mineralization occurs or not and to identify intermediate products in case of a partial mineralization. Likewise, the high OD and cell number values, and the brownish color in the cultures, evidenced that phenanthrene was transformed into biomass and intermediate metabolic products. On the other hand, *Sphingomonas* sp. strain (PH-b) did not grow on salicylate as the only carbon and energy source. In opposition to previous reports for *Sphingomonas* sp. (Xia et al., 2005; Coppotelli et al., 2010), this result indicates that an alternative pathway might be used in PHE degradation.

The maintenance of high biomass values (OD_{600} approximately 4.0), even after phenanthrene disappearance for strains PH-b, PH-c, and PH-d and decreasing down to 43 mg l^{-1} for strain PH-a, indicates accumulation of intermediate metabolites, which did not seem to have an inhibitory effect on strains growth, unlike other authors' reports of increasing toxicity owing to the presence of phenanthrene metabolites in the cultures (Yuan et al., 2001). Even though most of these studies focused on the identification of novel pathways or toxic end products (Pagnout et al., 2006), the effect of intermediate accumulation on the extent of biodegradation and mineralization remains unclear.

Many PAH-degrading microorganisms have developed mechanisms to increase the availability of low aqueous solubility PAH. Biosurfactant production increases the dissolution flux of the substrate and reduces the distance between cell and substrate via structures that bind on the cell surface (Olivera et al., 2009). In this work, both the drop collapse and emulsification tests confirmed the presence of surfactant in *Pseudomonas* strains. A significant production of surfactant activity was detected for the strains PH-a, PH-c, and PH-d grown in phenanthrene, suggesting that the surfactant secreted to the medium would be involved in PHE degradation. The extracellular filamentous material observed by SEM in some of the cultures when grown in phenanthrene as sole carbon source might also be related to phenanthrene utilization by increasing the substrate surface area, as has been suggested previously (Rocha et al., 2011).

In addition, chemotaxis toward PHE would be an advantageous feature since it improves the accessibility of degrading-bacteria to a low solubility pollutant such as phenanthrene. Chemotactic responses to phenanthrene were unequivocally demonstrated in this study for strains PH-c and PH-d (Fig. 5). Remarkably, response to PHE was as evident as the response toward casamino acids (Fig. 5b), usually stronger than the one to hydrocarbon compounds (Lanfranconi et al., 2003). Besides, these responses seem to be sensitive to the phenanthrene concentration and are not so clear at high PHE concentrations (data not shown).

Pseudomonas strains PH-c and PH-d displayed both high surfactant production and a strong chemotactic response toward phenanthrene, which could explain their ability to completely degrade phenanthrene and at a higher rate than the other two isolates. However, to assess the dependence of degradation rate on the mentioned characteristics will require further analysis.

To conclude, we showed that these strains carried out efficient phenanthrene degradation and some of them exhibited physiological properties that might have ecological significance. Due to the recognized importance of coastal fisheries communities in Samborombón Bay, decontamination of brackish sediments by bioremediation approaches represents a great challenge. The strains characterized in this study can be an alternative for microbial selection in the degradation of PAHs. Based on these results, their biodegradation potential should be further examined and optimized for bioremediation purposes. More studies on PAH metabolism of the strains, physiological features, and the effects of different environmental factors on biodegradation are essential.

Acknowledgments

This research was supported by the National Institute for Fisheries Research and Development (INIDEP) and by a grant from the Agencia Nacional de Promoción Científica y Tecnológica, Secretaría de Ciencia y Técnica Argentina (PICT 2010-2533). We gratefully acknowledge the contribution of Andrés Jaureguizar, who provided the samples.

References

Bogardt, A.H., Hemmingsen, B.B., 1992. Enumeration of phenanthrene-degrading bacteria by an overlayer technique and its use in evaluation of petroleum-contaminated sites. Applied and Environmental Microbiology 58, 2579–2582.

Bosma, T.N.P., Middeldorp, P.J.M., Schraa, G., Zender, A.J.B., 1997. Mass transfer limitation of biotransformation: quantifying bioavailability. Environmental Science and Technology 31, 248–252.

Colombo, J.C., Barreda, A., Bilos, C., Cappelletti, N., Demichelis, S., Lombardi, P., Migoya, M.C., Skorupka, C., Suarez, G., 2005. Oil spill in the Rio de la Plata estuary, Argentina: 1. Biogeochemical assessment of waters, sediments, soils and biota. Environmental Pollution 134, 277–289.

Coppotelli, B.M., Ibarrolaza, A., Dias, R.L., Del Panno, M.T., Berthe-Corti, L., Morelli, I.S., 2010. Study of the degradation activity and the strategies to promote the bioavailability of phenanthrene by *Sphingomonas paucimobilis* strain 20006FA. Microbial Ecology 59, 266–276.

Chen, J., Wong, M.H., Wong, Y.S., Tam, N.F., 2008. Multi-factors on biodegradation kinetics of polycyclic aromatic hydrocarbons (PAHs) by *Sphingomonas* sp. a bacterial strain isolated from mangrove sediment. Marine Pollution Bulletin 57, 695–702.

Dibble, A.S., Cheng, C., Biggar, C.A.H., 1990. Polycyclic aromatic hydrocarbon carcinogens. In: Pariza, M.W., Aeschbacher, H.U., Fenton, J.S., Sato, S., Wiley-Liss, N.Y. (Eds.), Mutagens and Carcinogens in the Diet. Wiley-Liss, New York, pp. 109–127.

Evans, W.C., Fernley, H.N., Griffiths, E., 1965. Oxidative metabolism of phenanthrene and anthracene by soil Pseudomonads. The ring-fission mechanism. Biochemical Journal 95, 819–831.

Haeseler, F., Blanchet, D., Druelle, V., Vandecasteele, J.P., 1999. Ecotoxicological assessment of soils of former manufactured gas plant sites: bioremediation potential and pollutant mobility. Environmental Science Technology 33, 4379–4384.

Hanstveit, A.O., 1992. Biodegradability of petroleum waxes and beeswax in an adapted CO_2 evolution test. Chemosphere 25, 605–620.

Harms, H., Bosma, T.N.P., 1997. Mass transfer limitation of microbial growth and pollutant degradation. Journal of Industrial Microbiology 18, 97–105.

Iyer, A., Mody, K., Jha, B., 2006. Emulsifying properties of a marine bacterial exopolysaccharide. Enzyme and Microbial Technology 38, 220–222.

Jain, D.K., Collins-Thompson, D.L., Lee, H., Trevors, J.T., 1991. A drop-collapsing test for screening surfactant-producing microorganisms. Journal of Microbiological Methods 13, 271–279.

Jaureguizar, A., Menni, R., Bremec, C., Mianzan, H., Lasta, C., 2003. Fish assemblages and environmental patterns in the Río de la Plata estuary. Estuarine Coastal and Shelf Science 56, 921–933.

Johnsen, A.R., Wick, L.Y., Harms, H., 2005. Principles of microbial PAH-degradation in soil. Environmental Pollution 133, 71–84.

Kim, Y.M., Ahn, C.K., Woo, S.H., Jung, G.Y., Park, J.M., 2009. Synergic degradation of phenanthrene by consortia of newly isolated bacterial strains. Journal of Biotechnology 144, 293–298.

Kiyohara, H., Nagao, K., Nomi, R., 1976. Degradation of phenantrene through o-phthalate by an *Aeromonas* sp. Agricultural and Biological Chemistry 40, 1075–1082.

Lanfranconi, M.P., Alvarez, H.M., Studdert, C.A., 2003. A strain isolated from gas oil-contaminated soil displays chemotaxis towards gas oil and hexadecane. Environmental Microbiology 5, 1002–1008.

Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J., Dymock, D., Wade, W.G., 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Applied and Environmental Microbiology 64, 795–799.

Menzi, C.A., Potocki, B.B., Santondonato, J., 1992. Exposure to carcinogenic PAHs in the environment. Environmental Science Technology 26, 1278–1284.

Mianzan, H.W., Lasta, C., Acha, E.M., Guerrero, R., Machi, G., Bremec, C., 2001. The Río de la Plata Estuary, Argentina, Uruguay. In: Seeliger, U., de Lacerda, L.D., Kjerve, B. (Eds.), Ecological Studies: Coastal Marine Ecosystems of Latin America. Springer, Berlin, pp. 185–204.

Morales-Caselles, C., Rica, A., Abbondanzi, F., Campisi, T., Iacondini, A., Riba, I., DelValls, A., 2008. Assessing sediment quality in Spanish ports using a green algae bioassay. Ciencias Marinas 34, 329–337.

Moran, A.C., Olivera, N., Commendatore, M., Esteves, J.L., Sineriz, F., 2000. Enhancement of hydrocarbon waste biodegradation by addition of a biosurfactant from *Bacillus subtilis* O9. Biodegradation 11, 65–71.

Mount, D.R., Ingersoll, C.G., McGrath, J.A., 2003. Approaches to developing sediment quality guidelines for PAHs. In: Douben, P.E.T. (Ed.), PAHs: an Ecotoxicological Perspective. John Wiley and Sons Ltd., Chichester.

Mrozik, A., Piotrowska-Seget, Z., Labuzek, S., 2003. Bacterial degradation and bioremediation of polycyclic aromatic hydrocarbons. Polish Journal of Environmental Studies 12, 15–25.

Mulet, M., David, Z., Nogales, B., Bosch, R., Lalucat, J., Garcia-Valdes, E., 2011. Pseudomonas diversity in crude-oil-contaminated intertidal sand samples obtained after the Prestige oil spill. Applied and Environmental Microbiology 77, 1076–1085.

NIOSH, 1998. Polynuclear Aromatic Hydrocarbons by HPLC, Method 5506. Manual of Analytical Methods (NMAM), fourth ed., Issue 3, pp. 1–9.

Olivera, N.L., Nievas, M.L., Lozada, M., Del Prado, G., Dionisi, H.M., Sineriz, F., 2009. Isolation and characterization of biosurfactant-producing *Alcanivorax* strains: hydrocarbon accession strategies and alkane hydroxylase gene analysis. Research in Microbiology 160, 19–26.

Pagnout, C., Rast, C., Veber, A.M., Poupin, P., Ferard, J.F., 2006. Ecotoxicological assessment of PAHs and their dead-end metabolites after degradation by *Mycobacterium* sp. strain SNP11. Ecotoxicology and Environmental Safety 65, 151–158.

Pandey, G., Jain, R.K., 2002. Bacterial chemotaxis toward environmental pollutants: role in bioremediation. Applied and Environmental Microbiology 68, 5789–5795.

Parales, R.E., 2004. Nitrobenzoates and aminobenzoates are chemoattractants for *Pseudomonas* strains. Applied and Environmental Microbiology 70, 285–292.

Parales, R.E., Harwood, C.S., 2002. Bacterial chemotaxis to pollutants and plant-derived aromatic molecules. Current Opinion in Microbiology 5, 266–273.

Paul, D., Singh, R., Jain, R.K., 2006. Chemotaxis of *Ralstonia* sp. SJ98 towards p-nitrophenol in soil. Environmental Microbiology 8, 1797–1804.

Reddy, M.S., Naresh, B., Leela, T., Prashanthi, M., Madhusudhan, N.C., Dhanasri, G., Devi, P., 2010. Biodegradation of phenanthrene with biosurfactant production by a new strain of *Brevibacillus* sp. Bioresource Technology 101, 7980–7983.

Resnick, S.M., Lee, K., Gibson, D.T., 1996. Diverse reactions catalyzed by naphthalene dioxygenase from *Pseudomonas* sp strain NCIB 9816. Journal of Industrial Microbiology and Biotechnology 17, 438–457.

Rocha, C.A., Pedregosa, A.M., Laborda, F., 2011. Biosurfactant-mediated biodegradation of straight and methyl-branched alkanes by *Pseudomonas aeruginosa* ATCC 55925. AMB Express 1, 9.

Rosenberg, E., Ron, E.Z., 1999. High- and low-molecular-mass microbial surfactants. Applied Microbiology and Biotechnology 52, 154–162.

Schlegel, H.G., Kaltwasser, H., Gottschalk, G., 1961. A submersion method for culture of hydrogen-oxidizing bacteria: growth physiological studies. Archives of Microbiology 38, 209–222.

Sorensen, S.R., Johnsen, A.R., Jensen, A., Jacobsen, C.S., 2010. Presence of psychrotolerant phenanthrene-mineralizing bacterial populations in contaminated soils from the Greenland High Arctic. FEMS Microbiology Letters 305, 148–154.

Taguchi, K., Fukutomi, H., Kuroda, A., Kato, J., Ohtake, H., 1997. Genetic identification of chemotactic transducers for amino acids in *Pseudomonas aeruginosa*. Microbiology 143, 3223–3229.

Takeuchi, M., Hamana, K., Hiraishi, A., 2001. Proposal of the genus *Sphingomonas* sensu stricto and three new genera, *Sphingobium, Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses. International Journal of Systematic and Evolutionary Microbiology 51, 1405–1417.

Tam, N.F., Guo, C.L., Yau, W.Y., Wong, Y.S., 2002. Preliminary study on biodegradation of phenanthrene by bacteria isolated from mangrove sediments in Hong Kong. Marine Pollution Bulletin 45, 316–324.

Tian, L., Ma, P., Zhong, J., 2002. Kinetics and key enzyme activities of phenanthrene degradation by *Pseudomonas mendocina*. Process Biochemistry 37, 1431–1437.

White, J.C., Triplett, T., 2002. Polycyclic aromatic hydrocarbons (PAHs) in the sediments and fish of the Mill River, New Haven, Connecticut, USA. Bulletin of Environmental Contamination and Toxicology 68, 104–110.

Xia, Y., Min, H., Rao, G., Lv, Z.M., Liu, J., Ye, Y.F., Duan, X.J., 2005. Isolation and characterization of phenanthrene-degrading *Sphingomonas paucimobilis* strain ZX4. Biodegradation 16, 393–402.

Yu, H.S., Alam, M., 1997. An agarose-in-plug bridge method to study chemotaxis in the Archaeon *Halobacterium salinarum*. FEMS Microbiology Letters 156, 265–269.

Yuan, S.Y., Wei, S.H., Chang, B.V., 2000. Biodegradation of polycyclic aromatic hydrocarbons by a mixture culture. Chemosphere 41, 1463–1468.

Yuan, S.Y., Chang, J.S., Yen, J.H., Chang, B.V., 2001. Biodegradation of phenanthrene in river sediment. Chemosphere 43, 273–278.