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

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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.

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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*,

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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).

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.

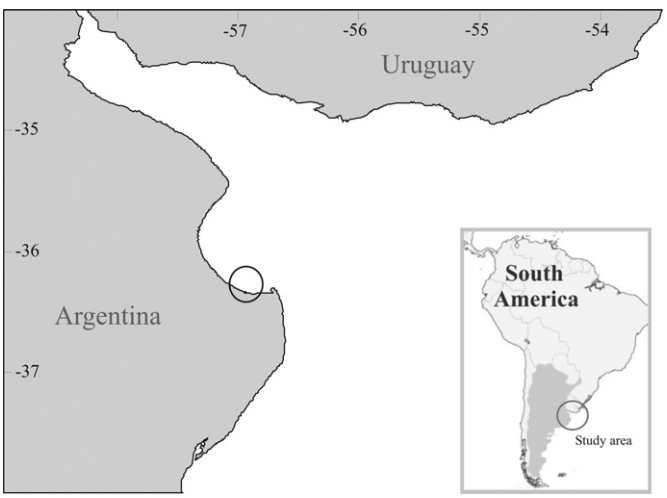

Fig. 1. Map of Samborombón Bay showing the sampling area location (circle).

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