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Differential expression of the catabolic *nahAc* gene and its effect on PAH degradation in *Pseudomonas* strains isolated from contaminated Patagonian coasts

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ABSTRACT

A combination of RT-PCR assays and of Northern blots were used to evaluate the ability of naphthalene, phenanthrene and pyrene to induce *nahAc* from three *Pseudomonas* isolates obtained from oil-contaminated marine sediments. Naphthalene dioxygenase activity based on indigo oxidation correlated with *nahAc* expression in all strains, while variable polycyclic aromatic hydrocarbon (PAH) degradation behaviors were observed. Naphthalene was completely degraded by all strains; however, whereas high levels of *nahAc* transcripts were detected in cultures of *Pseudomonas monteilii* P26 and *Pseudomonas stutzeri* N3 grown with naphthalene, significantly lower levels were detected in those of *Pseudomonas xanthomarina* N12. Phenanthrene was degraded by strain P6 and it strongly induced *nahAc* in this strain. On the other hand, although the strains N12 and N3 removed phenanthrene, the levels of *nahAc* transcripts were very low when these two strains were grown with this substrate. Remarkably, when *P. stutzeri* N3 was exposed to pyrene, an intense band of *nahAc* transcripts was detected by RT-PCR and Northern blot, even though pyrene was degraded to less than 5%. Our results indicate that all the strains from coastal Patagonia displayed the potential to biodegrade PAHs, although in some cases there was no clear correlation between *nahAc* gene expression and the ability to degrade PAHs.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds with toxic, mutagenic and/or carcinogenic properties, consisting of two or more fused benzene rings. Their hydrophobicity, low water solubility and tendency to adsorb to the organic fraction of soils and sediments are largely responsible for their low availability to microorganisms and their persistence in the environment (Hughes et al., 1997). Two- and three-ring PAHs, such as naphthalene, fluorene, phenanthrene and anthracene, are classified as low-molecular-weight (LMW) PAHs and considered to be extremely toxic to aquatic organisms. The presence of four-to seven-ring PAHs (high-molecular-weight, HMW) in contaminated soils causes serious deterioration problems due to their persistence and genotoxicity (Cerniglia, 1992).

Many bacteria have been shown to mineralize PAHs, and are presumed to play a key role in the removal of these contaminants from the environment. The biochemical pathways for bacterial degradation of LMW-PAHs such as naphthalene and phenanthrene have been extensively studied in several *Pseudomonas* strains (Denome et al., 1993; Takizawa et al., 1994; Bosch et al., 1999). However, fewer studies focused on the mineralization of pyrene, a tetracyclic PAH that is considered a model compound for the biodegradation of HMW-PAHs (Chen and White, 2004; Kanaly and Harayama, 2000). Although a number of bacterial isolates have been reported to grow on or mineralize pyrene, most of these are Actinomycetes belonging to the genera *Mycobacterium* and *Rhodococcus* (Walter et al., 1991; Rehmann et al., 1998). In recent reports, the difficulties associated with degrading pyrene have been

Abbreviations: PAH, Polycyclic aromatic hydrocarbon; LMW, Low molecular weight; HMW, High molecular weight.

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demonstrated (Zeng et al., 2010; Song et al., 2011).

Usually, PAHs are found as complex mixtures in the environment. Hence, the degradation capabilities of microorganisms are affected by possible interactions between individual PAHs and their respective bioavailability (Chávez et al., 2004).

The aerobic biochemical pathways involved in the biodegradation of PAHs require the presence of molecular oxygen to initiate the catabolic reactions. The initial step is catalyzed by the naphthalene dioxygenase system (NDO, a three-component class III oxygenase (ferredoxin [NahAb], ferredoxin reductase [NahAa] and terminal dioxygenase [NahAcAd, also NDO]), in which the terminal dioxygenase is a $\alpha_3\beta_3$ hexamer (Kauppi et al., 1998). The naphthalene dioxygenase, which appears to be the most prevalent enzyme in PAH-oxidizing bacteria, is responsible for the formation of *cis*-dihydrodiols from a wide array of aromatic hydrocarbons and heterocycles, including naphthalene and phenanthrene (Gibson and Parales, 2000; Resnick et al., 1996). On the basis of gene sequence analyses of the dioxygenase large subunit (α), Lloyd-Jones et al. (1999) clustered dioxygenases involved in aromatic ring oxidation into three genetically related families: the *nah*-like group, the *dnt*/*ntd* group and the *phn*-type group. Although many of the *Nah*-like encoded NDOs can also catalyze the oxidation of phenanthrene to *cis*-3,4-dihydroxy-3,4-di-hydrophenanthrene, a number of PAH-degrading phenotypes cannot be explained genotypically by comparison with *nah*-like sequences in many bacteria. In the same way, there are numerous environmental factors that can activate or repress gene expression and thereby modulate microbial activities.

In this study, *Pseudomonas monteilii* P26, *Pseudomonas xanthomarina* N12 and *Psudomonas stutzeri* N3, previously isolated from oil-contaminated marine sediments, were used to evaluate the differential expression of the *nahAc* gene when the cells were grown in the presence of naphthalene, phenanthrene or pyrene. All three strains share a large degree of nucleotide identity among their PAH catabolic genes and with those of the naphthalene-degrading archetype *nahAc* (Isaac et al., 2013). In this work, hydrocarbon removal, NDO activity and *nahAc*/16S rRNA gene expression were monitored simultaneously in a set of experiments that were performed to determine how gene expression and enzyme activity patterns may relate to the biodegradation behaviors of indigenous *Pseudomonas* strains of the Patagonian coast.

2. Materials and methods

2.1. Chemicals, bacterial strains and growth conditions

Naphthalene, phenanthrene and pyrene (>99% purity) used in this study were purchased from Sigma–Aldrich Co. (St. Louis, MO, US). All other chemicals were of analytical grade and acquired from standard manufacturers. *P. monteilii* P26, *P. xanthomarina* N12 and *P. stutzeri* N3 were previously isolated from oil-contaminated marine sediment from Patagonian coasts by culturing on minimal medium supplemented with phenanthrene crystals (Isaac et al., 2013). These strains were found to display an NDO activity, on the basis of the dark blue color produced in the indole to indigo conversion assay described by Riva Mercadal et al. (2010).

2.2. Biodegradation assays

To analyze PAH removal by the bacterial strains, a stock solution of naphthalene, phenanthrene or pyrene (25 mmol L^{-1} in acetone) was added to sterilized 25 mL flasks containing 5 mL of the previously described JPP liquid medium (Isaac et al., 2013) to obtain a final concentration of 0.2 mM. Before bacterial inoculation, the acetone was allowed to evaporate. A 250 μL aliquot of an overnight culture of *P. monteilii* P26, *P. xanthomarina* N12 or *P. stutzeri* N3 was added to each flask. Sets of non inoculated flasks were used as controls to determine any abiotic loss of PAH. Cultures were incubated in the dark for 48 h (naphthalene and phenanthrene) or 21 days (pyrene), on an orbital shaker at 30 °C and 180 rpm. Sets of triplicate samples were withdrawn by sacrificing cultures at different times and the content of each flask was serially extracted with 10 mL of acetone and filtered using a 0.22 μm nylon membrane (Microclar, Argentina). The samples were stored at −20 °C until analysis. Other sets of triplicate flasks were sampled for the determination of cell growth by optical density measurement (OD_{600}). The amount of PAH in the extracts was quantified by reverse-phase high performance liquid chromatography (RP-HPLC) according to Isaac et al. (2015). The concentration of PAH in each culture was calculated from a standard curve. Removal was expressed as percentage of PAH removed from each culture compare to the initial amount. Hydrocarbon abiotic loss was considered in all cases.

2.3. Substrate induction assay and RNA preparation

To study the effect of different PAHs on *nahAc* gene expression, all strains were grown in JPP medium supplemented with naphthalene, phenanthrene or pyrene, under the same conditions as those used for the biodegradation assays. Each flask was inoculated with 5% (vol vol^{-1}) of an overnight grown culture (OD_{600} = 0.8) prepared in JPP medium without PAH. Assays were performed in triplicate in all cases and sets of control flasks without PAH addition were also included in the experiments. The cultures were incubated at 30 °C and 180 rpm on an orbital shaker, and induction was allowed until the late exponential growth phase in order to obtain comparable samples for *nahAc* gene expression and NDO activity assays.

Total RNA was extracted from frozen cell pellets collected at the final exponential growth phase by the hot phenol method (Schmitt et al., 1990). RNase-free treatment was performed during the isolation procedure to avoid RNA degradation, using the *RiboPure*™-Bacteria kit (Ambion, Inc), according to the manufacturer's instructions. Following all extractions, RNA samples were stored at −80 °C until further analysis. Prior to amplification, the RNA was treated with DNase using a DNA-free TM kit (Ambion, Austin, TX) in 10 μL reaction mixtures for 20 min, according to the manufacturer's instructions.

2.4. Northern blot analyses

The RNA was quantified by absorption (OD_{260}) and separated by agarose gel electrophoresis under denaturing conditions. After electrophoresis, the RNA was transferred to nylon membranes and hybridized with ^{32}P-labeled random primed probes (Roche, Lewes, East Sussex, United Kingdom). The *nahAc* probe was amplified from the *P. monteilii* P26 genomic DNA using Ac149f/Ac1014r primers (Ferrero et al., 2002), and a single PCR fragment of the predicted size (866 bp) was obtained. The band intensities on Northern blots were measured by densitometry as previously described (Kim et al., 2006). Densitometry quantification of mRNA was performed by the Gel Compare II (version 6.5) software (Applied Maths) and mRNA loading was normalized using the rRNAs bands. Relative expression (%) was calculated as the intensity of the *nahAc* transcripts from each treatment/intensity of *nahAc* transcripts from the control assay × 100.