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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.

2.5. Semi-quantitative RT-PCR (SQ-RT-PCR) assays

SQ-RT-PCR assays were performed according to Marone et al. (2001). RT-PCR reactions to amplify *nahAc* and 16S rRNA genes transcripts were performed using the GeneAmp RNA PCR kit (Roche, Branchburg, NJ). The internal housekeeping 16S rRNA gene transcripts (Chang et al., 2009) were amplified together with those of *nahAc*. This serves as a reference point to evaluate changes in *nahAc* gene expression, to detect relative differences in the integrity of individual RNA samples and to indicate the presence of any reverse transcriptase inhibitors (Marlowe et al., 2002). For each sample, a mixture containing 2.5 mM $MgCl_2$; 10 × PCR buffer II; 250 μM each of dGTP, dCTP, dATP and dTTP; 1.25 mM random primers; 1U RNase inhibitor; and 2.5 U reverse transcriptase was added to 4 μg of DNase-treated RNA. The RT reactions were carried out at 25 °C for 10 min, 65 °C for 15 min and 37 °C for 60 min. To check for the presence of residual DNA, controls were run for each RNA sample using the same cDNA synthesis procedure except for the omission of the reverse transcriptase (Liu et al., 2012).

After completion of the RT step, the cDNA stretches of *nahAc* were amplified using Ac149 (5′-CCCCYGGCGACTATGT-3′/Ac635 (5′-GTCCAACCVAYGTGGTA-3′) primers (Ferrero et al., 2002) and those of the 16S rRNA genes using f27 (5′- AGA GTT TGA TCM TGG CTC AG-3′) and r518 (5′-CGT ATT ACC GCG GCT GCT GG-3′) primers (Quillaguamán et al., 2004). To ensure that the transcripts of interest, rather than any contaminating DNA, were amplified by the RT-PCR assays, total RNA extracts were subjected to DNase treatment prior to amplification. Sets of controls of cells grown on JPP medium without PAH addition were also included to determine the basal background levels of the *nahAc* transcripts.

All amplified products were separated by gel electrophoresis on 1.5% agarose gel. Gels were stained with GelRed™ Nucleid Acid Gel Stain and visualized with a Bio-Rad UV transilluminator. RNA samples were checked periodically on gel to evaluate whether RNA degradation might have occurred during storage.

2.6. Determination of whole cells NDO activity

NDO activity was determined from the oxidation of indole to indigo (Ensley et al., 1983). After hydrocarbon exposure, the cells were harvested by centrifugation (16,000 *g*, for 10 min at 4 °C). The pellet was washed twice with phosphate buffer (50 mM, pH 7.0) and re-suspended in 500 μL of the same buffer to an $OD_{600nm} = 1$. The reactions were initiated by adding 10 μL of 100 mM indole in *N,N*-dimethlylformamide. The cell suspensions were incubated at room temperature for 24 h and the reactions were subsequently stopped by centrifugation (16,000 *g*, 10 min at 4 °C). Indigo was extracted with *N,N*-dimethlylformamide, which denatures the proteins and increases the solubility of indigo. NDO activity, as measured from indigo production, was monitored by measuring absorbance at OD_{610} (Jenkins and Dalton, 1985). All determinations were made in triplicate.

2.7. Statistical analysis

All experiments were performed in triplicate and the values shown in figures correspond to mean values with standard deviation. All the results were analyzed using MINITAB 17 (PA, USA). One-way analysis of variance (ANOVA) was used to test the significant differences between responses. When significant differences were found, Tukey's post-test was used to separate the responses into groups. Mean values were considered significantly different at $P < 0.05$.

3. Results

3.1. PAH biodegradation

We assessed the ability of *P. monteilii* P26, *P. xanthomarina* N12 and *P. stutzeri* N3 to degrade naphthalene, phenanthrene and pyrene. All strains removed significant amounts of naphthalene and phenanthrene within 20 h. In all cases, naphthalene (a two-ring PAH) removal was greater than phenanthrene (three-ring PAH). As shown in Fig. 1, naphthalene was completely removed from all cultures after 48 h of incubation. In the case of phenanthrene, *P. stutzeri* N3 removed 100% of it, while *P. monteilii* P26 and *P. xanthomarina* N12 removed respectively 68% and 42% of this PAH. Pyrene (four-ring PAH) was poorly degraded. *P. xanthomarina* N12 and *P. stutzeri* N3 removed less than 5% of it after 21 days of incubation whereas no significant degradation was obtained for *P. monteilii* P26 cultures after the same incubation time. Abiotic loss of naphthalene was around 7–9 % after 48 h of incubation, while the loss of phenantrene and pyrene were negligible.

The growth kinetics in JPP medium containing PAH were similar for all strains. The average growth rate (μ) values for all PAHs were 0.326 ± 0.05 h^{-1} for *P. xanthomarina* N12 and respectively 0.241 ± 0.04 h^{-1} and 0.255 ± 0.03 h^{-1}, for *P. monteilii* P26 and *P. stutzeri* N3. In all cases, the stationary phase was reached within 12 h of incubation. However, while the maximal removal of naphthalene and phenanthrene were reached during the exponential phase of growth, in the case of pyrene the maximal removal was observed after 21 days of incubation.

3.2. Expression of nahAc gene

RT-PCR reactions for both *nahAc* and 16S rRNA transcripts were performed on total RNA extracted from cells grown in the presence of naphthalene, phenanthrene or pyrene at the end of their exponential phase. Visual analysis of the RT-PCR reaction products showed that a basal level of *nahAc* transcription was detected for all three strains grown in the absence of PAH, but the level of transcription was significantly higher in the case of cells grown on any of the three PAHs (Fig. 2A). This observation was confirmed from the Northern blot experiment (Fig. 2B).

Densitometry analysis revealed differential expression patterns for each strain and hydrocarbon exposures. (Fig. 3). The induction abilities followed the order Naph>Phen>Pyr for *P. xanthomarina* N12 and *P. monteilii* P26, and Naph>Pyr>Phen for *P. stutzeri* N3 (Figs. 2 and 3). The levels of expression of 16S rRNA transcripts were the same for all growth conditions, indicating that the cells maintained active cell division during growth on PAHs.

3.3. NDO activity

The conversion of indole to indigo is a NDO-catalyzed reaction that may be used to monitor NDO catalytic activity. This property was found to be strongly induced by naphthalene (Riva Mecadal et al., 2010). Uninduced (without PAH exposure) *P. monteilii* P26, *P. xanthomarina* N12 and *P. stutzeri* N3 produced blue colonies after indole vapour exposure. Therefore, as observed for the PCR-based *nahAc* expression assays, a basal NDO activity was detected in all strains. However, although the response varied depending on the tested PAHs, in general they enhanced the indole-biotransformation reaction (Fig. 4). Similar patterns were obtained for *P. monteilii* P26 and *P. xanthomarina* N12. In both cases, the highest levels of indigo production (105 ± 5 mmol mL^{-1} for *P. monteilii* P26 and 62 ± 2 mmol mL^{-1} for *P. xanthomarina* N12) were observed for naphthalene-induced cells. Indigo formation was 40% lower (64 ± 3 and 37 ± 3 mmol mL^{-1}) for phenanthrene-

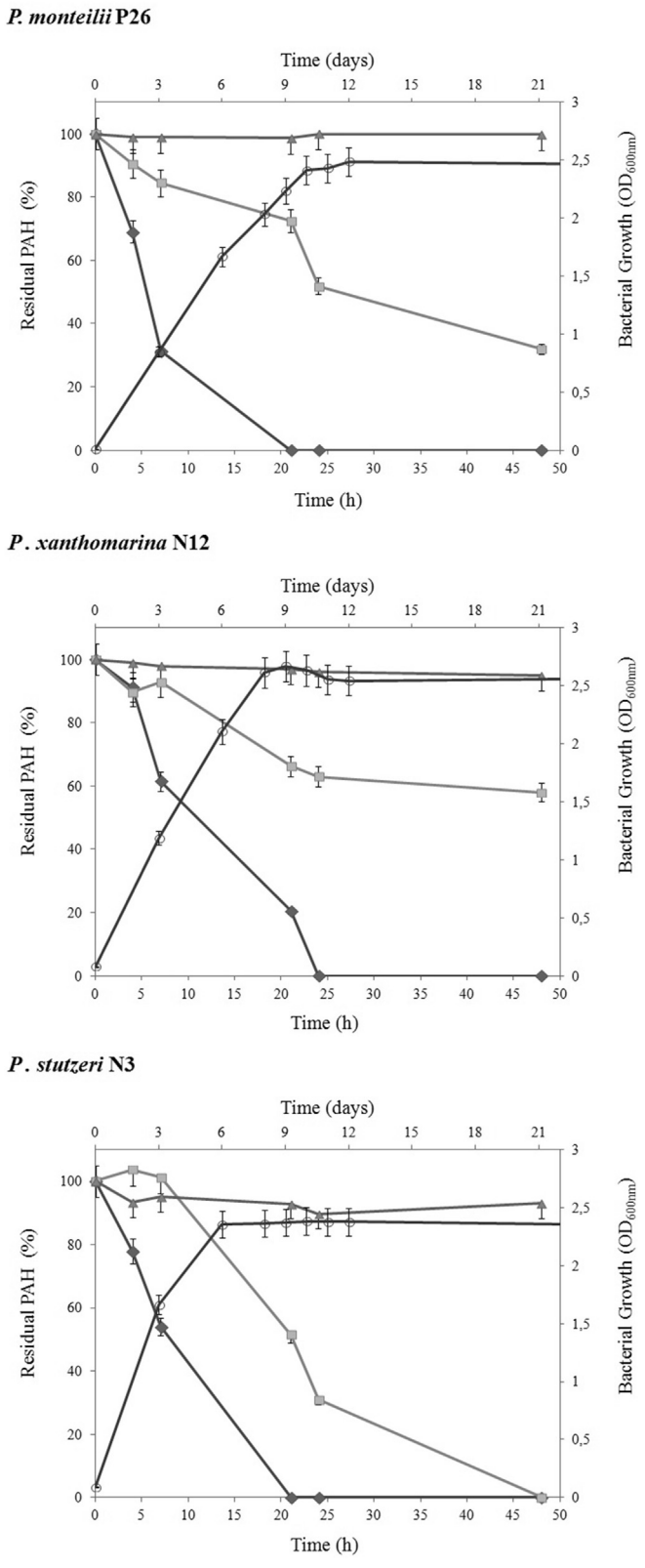

Fig. 1. PAH removal by *Pseudomonas* strains. Data correspond to naphthalene (◆), phenanthrene (■) and pyrene (▲) removal. Principal x-axis correspond to naphthalene and phenantrene removal (48 h of incubation). A second x-axis correspond to pyrene removal (21 days of incubation). A second y-axis shows bacterial growth measured as OD_{600nm} (○). Hydrocarbon abiotic loss was considered in all cases. Strains: *P. monteilii* P26, *P. xanthomarina* N12 and *P. stutzeri* N3. Values are the average ± standard deviation of triplicate measurements.

induced cells than those grown with naphthalene. Finally, the NDO activity was similar to that of the control for both *Pseudomonas* strains when the cells were grown in the presence pyrene. In the case of *P. stutzeri* N3 the maximal indigo formation (123 ± 6 mmol indigo mL^{-1}) was obtained for the naphthalene-induced cells (Fig. 4). However, unlike the other two strains, phenanthrene did not induce *P. stutzeri* N3 NDO activity as seen by the level of indigo production, which did not differ from the control when the cells were grown in the presence of this PAH. On the other hand, 57 ± 3 mmol indigo mL^{-1} were produced when strain N3 was grown in the presence of pyrene, displaying an NDO activity which was three-fold higher than that of the control and similar to those obtained when the other two strains are induced with phenanthrene or naphthalene (Fig. 4).

4. Discussion

The substrate range of the various PAH-degrading *Pseudomonas* may vary significantly, in spite of the genetic similarity of their catabolic genes (Yang et al., 1994). Gene regulation for the catabolism of PAHs has been extensively studied in the archetype plasmid NAH7 of *Pseudomonas putida* G7 (Harayama et al., 1987). However, the mechanisms involved in the regulation of non-archetype PAH degrading genes have been less frequently investigated, and in some cases, they remain unknown except by inferred analogy to the *nah* system. Moreover, it has been suggested that induction responses may vary among species and operons (Foght, 2004).

In this study, different *Pseudomonas* strains isolated from oil-contaminated intertidal sediments (Isaac et al., 2013) were used to evaluate the effect of naphthalene, phenanthrene and pyrene on the expression level of *nahAc*. Hydrocarbon removal, NDO activity and *nahAc* gene expression were monitored simultaneously to determine how gene expression and enzyme activity patterns are related to the biodegradation behavior of these indigenous strains. In all cases, *nahAc* expression profiles were consistent with the whole-cell NDO activity measured by monitoring indol oxidation, a NDO-catalyzed reaction. Results showed that NDO was present and active in all strains, even though the expression levels were quite low for some strains depending on the growth conditions. Naphthalene and phenanthrene induced *nahAc* gene expression differentially in the three strains, and though at different levels, the three of them were shown to remove both PAHs. Remarkably, when the *P. stutzeri* N3 cells were grown in the presence phenanthrene, although 100% of the substrate was removed from the culture medium, the *nahAc* gene expression and NDO activity levels were less than 5% above those of the control. Moreover, data showed that *nahAc* of this strain was induced by pyrene, and compared to the other two strains, it removed the highest amount of this PAH after 21 days.

On the other hand, we must emphasize that many other dioxygenases than NDO may catalyze the oxidation of this three-ring PAH. A study has revealed the presence of multiple PAH-degrading pathways in *Mycobacterium* sp. strain PYR-1, which were suggested to be due to the presence of different dioxygenases or to a relaxed specificity of the same dioxygenase for the initial attack (Moody et al., 2001). Another novel route for the metabolism of phenanthrene was also proposed by Prabhu and Phale (2003) wherein *Pseudomonas* sp. strain PP2 initiated phenanthrene degradation by a double hydroxylation, resulting in the formation of 3,4-dihydroxyphenanthrene. Furthermore, analysis of the phenanthrene catabolic pathway of *Pseudomonas* sp. DLC-P11 revealed that this strain degraded this PAH via 1-hydroxy-2-naphthoic acid, 1-naphthol and o-phthalic acid, when it was used as the sole source of carbon and energy. These results show that phenantrene may be metabolized through pathways involving an

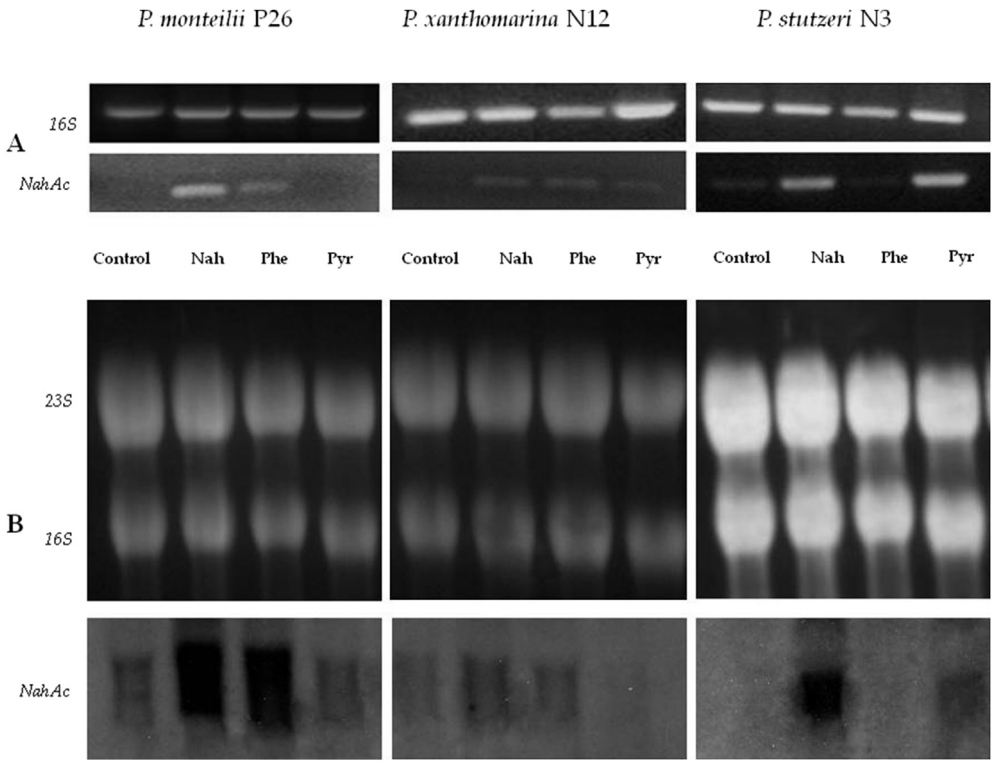

Fig. 2. Differential expression patterns of the catabolic *nahAc* gene. (A) RT-PCR detection of 16S rRNA and *nahAc* mRNA transcripts in all strains and conditions. (B) Northern blot analyses of 16S rRNA and *nahAc* genes. RNA (10 μg) of each strain and under each set of conditions was loaded on each lane. The ethidium bromide-stained gels of the autoradiogram are shown in the upper part of the panel.

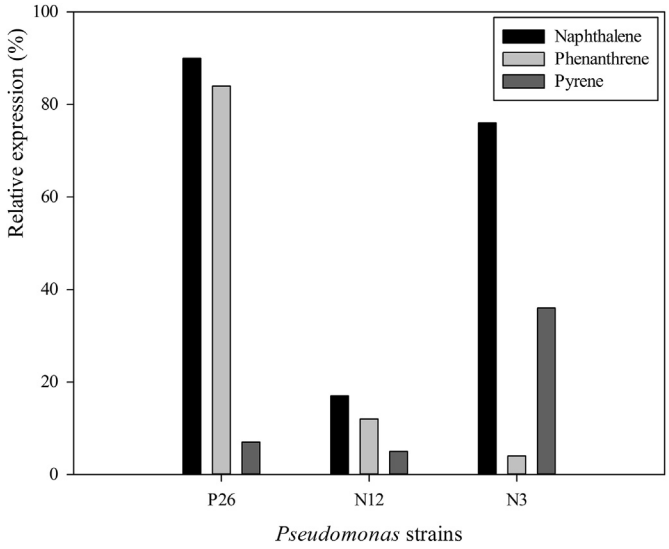

Fig. 3. Relative expression (%) of the *nahAc* gene in *Pseudomonas* strains, based on Northern blot analysis. Relative expression (%) was calculated as the intensity of the *nahAc* transcript from each treatment / intensity of *nahAc* transcript from the control assay × 100.

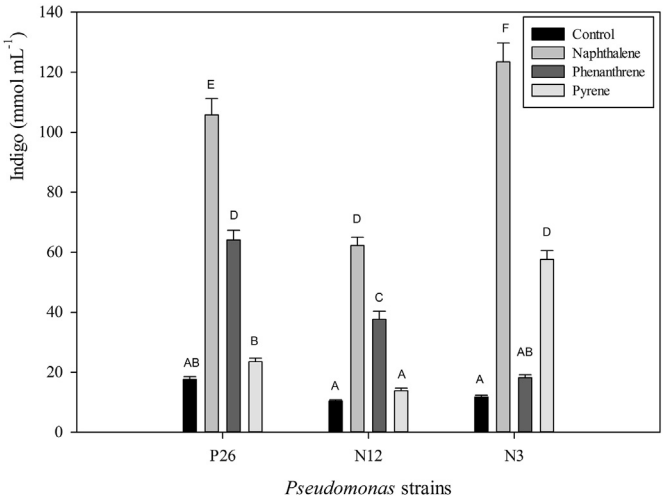

Fig. 4. Indigo formation (mmol L^{-1}) after indol oxidation by NDO activity in whole cells of *Pseudomonas* strains after naphthalene, phenanthrene and pyrene exposure. Mean values that do not share a same letter are significantly different (ANOVA followed by multiple comparisons, $p < 0.05$).

initial attack at different sites of the molecule (Labana et al., 2007).

On the other hand, while pyrene significantly induced the expression of *nahAc* gene and NDO activity in *P. stutzeri* N3, the removal of this hydrocarbon was very low (<5%) even after 21 days of incubation. Although we cannot exclude the possibility that NDO may be involved in pyrene degradation in *P. stutzeri* N3, care must be taking when inferring the degradation pathway of high molecular weight PAHs such as pyrene only on the basis of the *nah* system genes regulation. According to Cerniglia (1993), the main reason for the prolonged persistence of PAHs in the environment is their low water solubility. However, although in our study, this key property may have hampered pyrene biodegradation, it did not prevent *nahAc* gene induction in *P. stutzeri* N3. Approaches aimed at increasing the apparent solubility of this PAH, such as the addition of synthetic surfactants or production of biosurfactants, should be carried out to enhance this transformation. In addition, more

genotypic and physiological studies are necessary to elucidate the regulation of pyrene catabolism in *P. stutzeri* N3, considering that degradation of HMW-PAHs is unusual in *Pseudomonas* strains (Das and Mukherjee, 2007; Obayori et al., 2008; Ma et al., 2013; Ghosh et al., 2014).

5. Conclusions

In this work we evaluated the effect of naphthalene, phenanthrene and pyrene on the expression levels of the *nahAc* gene in different *Pseudomonas* strains isolated from oil-contaminated marine sediments. *P. stutzeri* N3, *P. monteilii* P26 and *P. xanthomarina* N12 exhibited different catabolic gene expression and biodegradation behaviors. Our results suggest that *nahAc* is involved in naphthalene and phenanthrene degradation in these *Pseudomonas* strains. NDO activity may also be involved in pyrene degradation in *P. stutzeri* N3, however this conclusion has to be confirmed because its removal was very slow. Further experiments will therefore be needed to investigate the catabolism of pyrene.

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