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Insights in the regulation of the degradation of PAHs in *Novosphingobium* sp. HR1a and utilization of this regulatory system as a tool for the detection of PAHs

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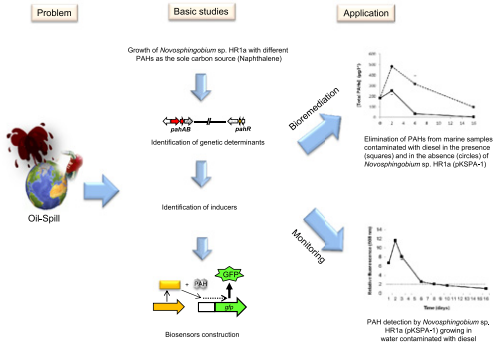
HIGHLIGHTS

- *Novosphingobium* sp. HR1a degrades several polycyclic aromatic hydrocarbons (PAHs).
- The dioxygenase and regulatory genes of the PAH degradation have been identified.
- The regulatory circuits of PAH degradation in this strain have been described.
- Biosensors for PAH detection were constructed and tested with environmental samples.
- These biosensors are excellent tools for bioremediation and monitoring contaminants.

GRAPHICAL ABSTRACT



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ABSTRACT

Novosphingobium sp. HR1a is able to grow using diverse polycyclic aromatic hydrocarbons (PAHs) as the sole carbon sources. We have identified two transposons that contain genes encoding several ring-hydroxylating dioxygenases and we have demonstrated the crucial role of one of these dioxygenases in the PAH metabolism in this strain; a mutant in the large subunit of this dioxygenase was unable to growth with 2-, 3-, or 4-rings aromatic hydrocarbons. Using a construction of *lacZ* gene fused with the pathway promoter, we determined that the expression of the dioxygenase gene was specifically induced in the presence of some PAHs and intermediates of their metabolic pathway. In silico analysis of the ORFs within the transposons and construction of the corresponding knock-out mutants allowed us to identify the main regulatory protein involved in PAH degradation in *Novosphingobium* sp. HR1a. To our knowledge this is the first time that a regulatory protein controlling the degradation pathway of high-molecular weight PAHs has been investigated. A deeper knowledge of the regulatory circuits that control the expression of PAH degradation has allowed us to design two biosensors for monitoring environments contaminated with oil-derived mixtures. *Novosphingobium* sp. HR1a (pKSR-1), the biosensor based on the promoter of the regulatory protein *PahR*, was more sensitive and faster in the detection of aromatic contaminants in environmental samples than *Novosphingobium* sp. HR1a (pKSA-1), the biosensor that is based on the PAHs-dioxygenase promoter (P_{pahA}). *Novosphingobium* sp. HR1a (pKSR-1) was able to detect PAHs in the range of μg l^{-1} (ppb).

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1. Introduction

Pollution causes severe alterations in the environment, damaging not only the ecology of the site, but also the economy of the area (Junoy et al., 2005). Aromatic hydrocarbons are among the most abundant contaminants in the environment; low molecular weight hydrocarbons are relatively soluble in water, making them bioavailable for their degradation (Blum et al., 2009). However, aromatic compounds with two or more fused aromatic rings, called polycyclic aromatic hydrocarbons (PAHs), are poorly soluble in water, therefore they are less bioavailable for degrading microbes and, consequently, they are more persistent in the environment. Furthermore, PAHs can be adsorbed to particulate materials, thus accumulating in these materials and becoming a reservoir for contaminants (Simpson et al., 1996; Morales-Caselles et al., 2006), producing the so-called 'chronic toxic effects' (Aske et al., 2002; Ghazali et al., 2004). Because of their toxic effects damage to DNA exerting carcinogenic effects, interference with the growth rate and reproductive success of animals, increase sensitivity to pathogens and damage to the immune system of animals (Paolo et al., 1997; Wilcke, 2000, Laffon et al., 2006; Tang et al., 2014), 16 PAHs have been listed as priority pollutants to be eliminated in water, and 24 were also listed in soils, sediments and hazardous solids (US EPA, last updated 2006).

Many microorganisms, including bacteria, fungi, and algae have been described as being able to degrade PAHs; these microorganisms have been isolated or identified from very diverse environments: edaphic and aquatic as well as from aerobic and anaerobic sites (reviewed by Haritash and Kaushik, 2009; Bacosa et al., 2013). Especially interesting are strains included among the *Sphingomonadaceae* and *Actinobacteriaceae*, because of their capacity to metabolize high molecular weight PAHs that are the most recalcitrant and dangerous PAHs (Sohn et al., 2004; Suzuki and Hiraishi, 2007; Yuan et al., 2009; Bacosa and Inoue, 2015; reviewed by Vila et al., 2015; Rodriguez-Conde et al., 2016).

The biochemical pathways for the bacterial degradation of PAHs, such as naphthalene (Resnick et al., 1996; Annweiler et al., 2000), phenanthrene (Menn et al., 1993; Kiyohara et al., 1994; Pinyakong et al., 2003a), anthracene and acenaphthene (Dean-Ross et al., 2001; Pinyakong et al., 2004), have been thoroughly investigated. Aerobic biodegradation of PAHs requires the presence of molecular oxygen to initiate the enzymatic attack of the aromatic rings. The initial step consists of the oxidation of one of the aromatic rings mediated by a multicomponent dioxygenase to yield *cis*-dihydrodiols. In some strains, such as *Sphingomonas*, *Mycobacterium* and *Novosphingobium*, this first enzyme has a broad substrate specificity, catalyzing the dihydroxylation of various PAHs ranging in size from two to five fused rings (Demanèche et al., 2004: Jouanneau and Meyer, 2006; Kim et al., 2006; Schuler et al., 2009; Lyu et al., 2014). The dihydroxylated intermediates are cleaved by intradiol or extradiol ring-cleaving dioxygenases through either an *ortho*-cleavage pathway or a *meta*-cleavage pathway, leading to the central intermediate salicylic acid, which is metabolized through either catechol or gentisate to tricarboxylic acid (TCA) cycle intermediates (Cerniglia, 1992; Eaton and Chapman, 1992; Gibson and Parales, 2000).

Members of the sphingomonad group have been described as degraders of a wide range of natural and xenobiotic compounds, such as biphenyls, naphthalenes, fluorene, phenanthrenes, pyrene, diphenylethers, furans, dioxins, carbazole, estradiol, polyethylenglycols, chlorinated phenols and various herbicides and pesticides (Basta et al., 2005) and are among the most abundant PAH-degrading isolates in soil (Leys et al., 2004). Genes for the catabolism of low-medium molecular weight (2, 3-rings) PAHs have been studied in many *Sphingomonadaceae* (Pinyakong et al., 2003b). These degradative genes are encoded in the chromosome and/or in large plasmids (Kim et al., 1996; Feng et al., 1997; Romine et al., 1999; Ogram et al., 2000; Basta et al., 2004; Segura et al., 2014), but, contrary to what happens in other organisms (i.e. *Pseudomonas* or *Burkholderia*), these genes are not organized in operons. This has posed a problem for the assignment of function to certain genes and in fact, in many organisms there are only predictions about the function of certain genes related to the degradation of aromatic compounds (Romine et al., 1999).

In a previous study, we isolated *Novosphingobium* sp. HR1a from the rhizosphere of plants growing close to an industrial area on the basis of its capacity to utilize phenanthrene as a carbon source (Rodriguez-Conde, personal communication). The objective of this study was to characterize in depth the genetic determinants of PAH degradation in *Novosphingobium* sp. HR1a with the idea of utilizing these genetic elements for the construction of devices to monitor the presence of contaminants in different environments. We have identified the PAH hydroxylating dioxygenase genes (named *pahAB*), an enzyme that is essential for PAH degradation and whose expression is induced by several PAHs. We have also identified *pahR*, the main gene involved in the regulation of the PAH hydroxylating dioxygenase, which is to the best of our knowledge, the first time that a regulator involved in medium- and high-molecular weight PAH degradation in *Sphingomonadaceae* has been identified. Because of the wide variety of PAHs that *Novosphingobium* sp. HR1a is able to detect and metabolize, we have taken advantage of this capacity to construct two biosensors based on different genetic elements of the PAH-degradation system of this strain.

2. Material and methods

2.1. Growth on PAHs and PAH degradation

Bacteria were grown overnight in M9 minimal medium plus the corresponding carbon source as indicated in each case. The final concentration of the different carbon sources used was 5 mM for glucose and 5 mM for monocyclic aromatics except for salicylate that was toxic at this concentration, therefore 1 mM of salicylate was added every day during the first 3 days of growth on this substrate. PAHs were used at saturating concentrations. To prepare media saturated with the different PAHs used as substrate, flasks containing 25 ml of M9 minimal medium and 2.5 mg of PAHs were prepared the day before the inoculation to allow the maximum dissolution of the PAHs (the solubility of PAHs in water is low).

Flasks containing 25 ml of media with the different carbon sources were inoculated to an initial $OD_{660\ nm}$ of 0.1 and they were cultured at 30 °C in an orbital shaker (200 strokes/min). Samples were taken at different times to measure culture turbidity. For PAH quantification flasks with 100 ml of M9 minimal medium saturated with the corresponding hydrocarbon were inoculated with *Novosphingobium* sp. HR1a as above and 20 ml samples were taken at different times for analysis.

2.2. Preparation of environmental samples for PAH quantification

Environmental samples were prepared as described by Hernández-Sánchez et al. (2016). Diesel was prepared as a 0.02% (v/v) solution and petrol as a 0.004% (v/v) solution in water. Crude North Sea oil (1 g) or *Prestige* spill oil (4 g) was added to 40 ml of water and shaken overnight in closed bottles in the dark. The resulting saturated water solution was used for GC/MS analysis. No previous preparation was needed for Messina, Gela, Motril and Salobreña samples, which were directly added to the GC-MS vials. For Gela and Messina edaphic sediment samples, 5 g of sediment were added to 5 ml of water and shaken overnight in a closed bottle. Samples were analyzed after centrifugation to avoid any material in suspension (Hernández-Sánchez et al., 2016).

2.3. HPLC and GC/MS analysis

High-performance liquid chromatography (HPLC) was used for the detection of phenanthrene in bacterial cultures as previously described (Pinyakong et al., 2000; Rodriguez-Conde et al., 2016).

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GC/MS samples were analyzed by solid-phase micro-extraction coupled to a gas chromatograph with a mass-spectrometry detector (SPME-GC/MS). PAHs were measured with a 65 μm PDMS/DVB fiber (Supelco, Bellefonte PA, USA), conditioned at 250 °C for 0.5 h prior use. The fiber was immersed directly into 20 ml magnetic crimped vials filled with 18 ml of each sample for 60 min at 60 °C and stirred at 500 rpm, immediately drawn back into the needle and transferred without delay (<5 s) into the injection port of the GC (CTC Analytics CombiPal autosampler system). A desorption time of 10 min at 300 °C (splitless time 4 min) was used. The analyses were performed on a Varian Model 450GC coupled to a 240 MS detector. The chromatographic column was Thermo TG-5SILMS 30 m × 0.25 mm × 0.25 μm film. Helium was the carrier gas at 1 ml/min. The GC oven program was: initial temperature 50 °C held for 1 min, ramped at 11 °C/min to 350 °C and held for 8 min. The interface was kept at 280 °C, the ion trap at 240 °C. The mass spectra were acquired in two modes. Samples with major concentrations were analyzed by Electron Impact (70 eV energy) TIC Full Scan/SIM mode (100–300 *m/z* mass scan range) and samples with minor concentrations by Electron Impact Ms./Ms. mode (selected parent and daughter ions not showed). The compounds were quantified by using calibration curves from the corresponding analytical standards. The most probable metabolite structures were based on their retention times, fragment ions of the standard and by using NIST library spectra included in the MS Workstation software 6.9.1.

Monocyclic aromatic hydrocarbons were measured with different conditions: the fiber was immersed into 20 ml vials filled with 10 ml of sample (Head Space SPME mode) at 30 °C for 4 min. Desorption time 3 min (splitless time 3 min) at 150 °C. The GC oven program was: initial 45 °C (3.5 min), ramped at 38 °C/min to 80 °C, held for 4 min, ramped at 75 °C/min to 150 °C and held 6 min. The mass spectra were obtained by Electron Impact Full Scan and SIM mode (45–300 *m/z* mass scan range).

Degradation metabolites were measured by DI-SPME mode using 65 μm PDMS/DVB and 85 μm polyacrylate fibers (conditioned previously as indicated by manufacturer). 10 ml magnetic crimped vial filled with 9 ml of bacterial cultures. The GC conditions were the same as those used with PAHs with 280 °C of desorption temperature. Mass spectra were acquired in the range 45–600 *m/z*.

2.4. DNA techniques

Preparation of chromosomal DNA, digestion with restriction enzymes, electrophoresis and Southern blotting were done using standard methods (Ausubel et al., 1995). PCR digoxigenin labeling was used to synthesize probes for hybridization following the manufacturer's instructions (Roche Laboratories). Electroporation of *Sphingomonadaceae* cells was done as described previously (Enderle and Farwell, 1998).

2.5. Genome sequencing

For genome sequencing, *Novosphingobium* sp. HR1a genomic DNA was purified using the Wizard Genomic DNA purification kit, sequenced using 454 technology (Macrogen), and assembled into 208 contigs (25× coverage). These contigs were ordered by comparison (BLASTn) with the sequences from other available sphingomonads genomes (*Sphingobium* sp. KK22, id. NZ_BATN00000000.1; *Sphingobium* sp. Ant17, id. NZ_JEMV00000000.1; *Novosphingobium pentaromativorans* US6-1, id. NZ_CP009291.1; *Novosphingobium aromaticivorans* DSM 12444, id. NC_007794.1; and *Novosphingobium* sp. PP1Y, id. NC_015580.1).

Genomic DNA was automatically annotated using a program pipeline based on Glimmer 3.0 for gene prediction (Delcher et al., 1999), and BLAST and RPS-BLAST for functional assignment of open reading frames (ORFs) based on sequence similarity to sequences deposited in the NR, SwissProt, COG, Pfam, Smart, and PRK databases (Altschul et al., 1997). Automatic annotations were manually curated and deposited in Rapid Annotation using Subsystem Technology (RAST) base data (*Novosphingobium* sp. HR1a id. 6666666.39990) http://rast.nmpdr.org (Aziz et al., 2008).

Genome comparisons to determine protein homology, common genes and genetic islands were performed using the RAST software and BLASTp. Phylogenetic studies of complete genomes were performed using the platform Composition Vector Tree (http://tlife.fudan.edu.cn/cvtree/) (Qi et al., 2004) and for individual proteins using the platform Phylogeny.fr (http://www.phylogeny.fr/) (Dereeper et al., 2008).

2.6. RNA preparation, RT-PCR and RACE

Novosphingobium sp. HR1a was grown overnight in M9 minimal medium plus glucose and naphthalene. Cells were then diluted 100-fold in fresh medium until the culture reached a turbidity of approximately 1.0 at 660 nm. Cells were harvested by centrifugation (5000 × *g* for 10 min) and processed for RNA isolation according to the method of Marqués et al. (1993).

For Reverse Transcription Polymerase Chain Reaction (RT-PCR), 1 μg of RNA was analyzed using the Titan OneTube RT-PCR system according to the manufacturer's instructions (Roche Laboratories). Oligonucleotides used for testing the contiguity in the mRNA of the genes are shown in Suppl. Table 1.

5′RACE was done with the 5′RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (InvitrogenTM), using 5 μg of RNA and the oligonucleotides listed in Suppl. Table 1.

2.7. Construction of the phnA and phnR mutant strains

For the construction of a *phnA* mutant in *Novosphingobium* HR1a DNA fragments upstream and downstream of the *phnA* gene were amplified using oligonucleotides phnAKpnI and phnABamHI (amplicon of 620 bp) and phnA2B and phnA2Sal (amplicon of 710 bp). The 620 bp amplicon was cloned into pMBL-T and tranformed into *E. coli*. The resulting plasmid was digested with *Bam*HI and *Sal*I to linearize the plasmid, once the correct orientation of the fragment was confirmed. The 710 bp amplicon was digested with the same enzymes (restriction sites are included in the primer sequences) and ligated to the previous plasmid. The resulting plasmid, pPhn8, was digested with *Bam*HI and the Ω-Km fragment of pHP45ΩKm (Prentki and Krisch, 1984), previously digested with the same restriction enzyme, was ligated with pPhn8 that had been linearized with *Bam*Hl. For the construction of the *pahR* mutant a 301 bp fragment of *Novosphingobium* sp. HR1a genome was amplified using the oligonucleotides 1952-F and 1952-R (Suppl. Table 1). This fragment was cloned into pMBL-T plasmid to construct the pPAHR1 plasmid. The resulting plasmid was cut with *Bam*HI and the Ω-Km cassette of plasmid pHP45ΩKm (Prentki and Krisch, 1984), previously digested with *Bam*HI, was ligated into this plasmid. The resulting plasmids were transferred to *Novosphingobium* sp. HR1a by electroporation. Transformants that integrated the plasmid into the host chromosome via homologous recombination were selected on LB plus kanamycin plates and checked by Southern blot hybridization (not shown).

2.8. β-Galactosidase assays

The intergenic region between *ORF1924* (permease) and *ORF1925* (*pahA* gene) in *Novosphingobium* sp. HR1a was amplified with primers Prom1 and Prom1R (Suppl. Table 1) incorporating an *Eco*RI site in the primer designed to meet the 5′ end and a *Pst*I site in the primer designed to meet the 3′ end. Upon amplification, DNA was digested with *Eco*RI and *Pst*I and ligated into the low-copy-number pMP220 vector (Spaink et al., 1987) previously cut with the same enzymes to produce plasmid pHR1a (*phnA* promoter). The plasmid was sequenced to verify the promoter sequence and was electroporated into *Novosphingobium*

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sp. HR1a. Transformants were selected in LB plates plus tetracycline; individual colonies were grown in liquid media and the plasmid was extracted and digested with *Eco*RI and *Pst*I to verify the correct incorporation of the plasmid.

A similar protocol was used to construct pPR1952, pPR1935 and pPR1939, plasmids that contain the upstream DNA sequence of the ORF1952 (236 bp amplified using the primers Pr1952-F and Pr1952-R), ORF7394 (200 pb amplified using the primers Pr7394-F and Pr7394-R) and pPR7935 (200 pb amplified with primers Pr7395-F, and Pr7395-R), respectively.

Selected strains were grown overnight on M9 minimal media plus glucose and then diluted to an OD of 0.1 in fresh M9 minimal medium plus glucose. Decanate and salicylate were added at a final concentration of 1 mM; benzoate, protocatechuate and *O*-phthalate were added at a final concentration of 5 mM. Biphenyl, phenanthrene naphthalene, 1-hydroxy-2-naphthoic acid, anthracene, chrysene and pyrene powder was used to obtained saturated M9 minimal media solutions of each compound. Because of the toxicity and volatility of toluene and ethylbenzene, these compounds were added in a curve glass bar with open ends to allow evaporation into the flasks to reach saturated concentration in the media.

Environmental samples were tested as follows: 5% (v/v) of Mesina, Gela, Motril and Salobreña water solutions (prepared as in 2.2) and 5% (v/v) of saturated water of *Prestige* spill oil or crude oil (prepared as in 2.2) were added directly to the M9 minimal media plus glucose. Soil from the Institute garden (Zaidin soil), Messina harbor sediment (Messina sediment), Gela refinery sediment (Gela sediment) were added directly at a concentration of 0.5% (w/v).

β-Galactosidase assays (Miller, 1972) were done 5 and 24 h after inoculation with the corresponding strain. For each condition, at least three independent experiments were run and β-galactosidase activity was assayed in duplicate.

2.9. Bioreporter construction

For the construction of the *Novosphingobium* sp. HR1a (pKSPA-1) bioreporter, plasmid pHR1a was digested with *Eco*RI and *Pst*I, and the promoter fragment was cloned into pSEVA637 (Silva-Rocha et al., 2013), previously digested with the same restriction enzymes.

For the construction of *Novosphingobium* sp. HR1a (pKSR1), the P_{pahR} promoter fragment was excised from plasmid pPR1952 after digestion with *Eco*RI and *Pst*I. The DNA fragment was ligated into pSEVA637 previously cut with the same enzymes to obtain pKSR1.

For the construction of *Novosphingobium* sp. HR1a (pKSPA-R), we amplified the DNA fragment containing P_{pahR}:*pahR* using the genome of *Novosphingobium* sp. HR1a as template and the primers Pr1956HR1a-EcoR1 and 1956HR1a-*Not*I. The fragment was cut with *Eco*RI and *Not*I and cloned into pSEVA637 previously cut with the same restriction enzymes as in pKSR1.

pKSPA-1, pKSR-1 and pKSPA-R were introduced into *Novosphingobium* sp. HR1a by electroporation and cells with the plasmid were selected on LB plates plus gentamycin (30 μg ml^{-1}).

2.10. Bioreporter assays

Bioreporter assays were done as described in Hernández-Sánchez et al. (2016). Briefly, different dilutions of saturated solutions of PAHs, monocyclic aromatic compounds or environmental samples were added to 10 ml of cultures of *Novosphingobium* sp. HR1a in M9 minimal or ONR7a media (Dyksterhouse et al., 1995) with an initial $OD_{660\ nm}$. After 7.5 h or 24 h, the $OD_{660\ nm}$ was measured and adjusted to 0.1/ml. Fluorescence was then directly measured in a LPS-220B fluorometer (Photon Technology International). λ_{ex} was 485 nm and λ_{em} 510 nm. Fluorescence values are given as fluorescence induction ratio (fluorescence emitted in conditions of induction/fluorescence emitted by the control culture without inducer). Error bars mean the standard deviation of three experimental repeats.

3. Results

3.1. Novosphingobium sp. HR1a was able to grow with a broad range of PAHs as the sole carbon source

Phenotipic characterization of *Novosphingobium* sp. HR1a demonstrated that this strain was able to grow using different PAHs as the carbon source: Fig. 1 shows that the strain was able to grow with compounds with 2-aromatic rings (naphthalene and biphenyl) and PAHs of 3- (anthracene and phenanthrene) and 4-aromatic rings (chrysene and pyrene) and in possible intermediates of the degradation pathway (salicylate, protocatechuate and *o*-phthalate). The number of colony forming units (CFUs) increased from 4.4×10^6 to 8.5×10^8, 2.3×10^8 and 1.6×10^8 in phenanthrene, anthracene and pyrene respectively after 6 days of incubation (Suppl. Table 2). In addition, the culture medium turned into a reddish color in the presence of anthracene, into a light yellowish color in the presence of naphthalene or pyrene; and black in the presence of biphenyl (not shown). These data indicated that *Novosphingobium* sp. HR1a was, at least, able to productively metabolize different PAHs.

To further analyze this phenotype we analyzed the decrease of PAHs in the culture media by HPLC and/or GC-MS as well as the accumulation of some metabolic intermediates along the growth curve.

Naphthalene was not detectable in the culture media 3 days after inoculation with *Novosphingobium* sp. HR1a. In controls flasks without bacteria, the naphthalene concentration remained constant at 31.6 mg l^{-1} (saturation limit) indicating that losses due to evaporation during cultivation were not responsible for the disappearance of the naphthalene. No accumulation of known intermediates was observed at any time, probably because the yellow compound(s) is not detected with the methodology used in this work.

Although *Novosphingobium* sp. HR1a was able to grow on biphenyl, 2- and 3-hydroxy- and 2-3-dihydroxy-derivatives of this molecule were accumulated in the culture medium (not shown); further degradation of these compounds was verified by the observation of benzoic-acid derivatives six days after inoculation with this strain.

Phenanthrene (2.5 mg) was not detected in culture media 6 days after inoculation with *Novosphingobium* sp. HR1a (Fig. 2). The intermediate compound 1-hydroxy-2-naphthoic acid was transiently accumulated. Interestingly, the concentration of phenanthrene dissolved in the medium increased in the first 48 h when bacteria were present in the culture, whilst the concentration of soluble phenanthrene in control flasks without bacteria remained constant (1.78 ± 0.5 mg l^{-1}), suggesting a solubilization effect produced by the presence of the bacteria.

Approximately 1/6 of the initial anthracene concentration remained in the cultures with *Novosphingobium* sp. HR1a six days after inoculation. Naphthalenediol and 2-hydroxy-1-naphthalenecarboxyaldehyde, were detected in the culture, indicating at least partial metabolization of this molecule (data not shown).

The solubilization effect observed in cultures with phenanthrene was also evident in cultures with chrysene and pyrene, as higher concentrations of these compounds were detected in the culture media after six days of inoculation than in the non-inoculated flasks (not shown). Partial mineralization of these compounds was evidenced by the detection in both cultures of the corresponding dihydro-derivatives six days after inoculation. Additionally, a compound with the chemical formula $C_{13}H_8O_2$ was detected in cultures with chrysene, and phenanthrenone, phenalene and $C_{14}H_8O_2$ in cultures with pyrene (data not shown).

All these results show that *Novosphingobium* sp. HR1a was able to metabolize low, medium and high molecular weight PAHs, and that this metabolization allowed the strain to grow using these compounds as the sole carbon source.

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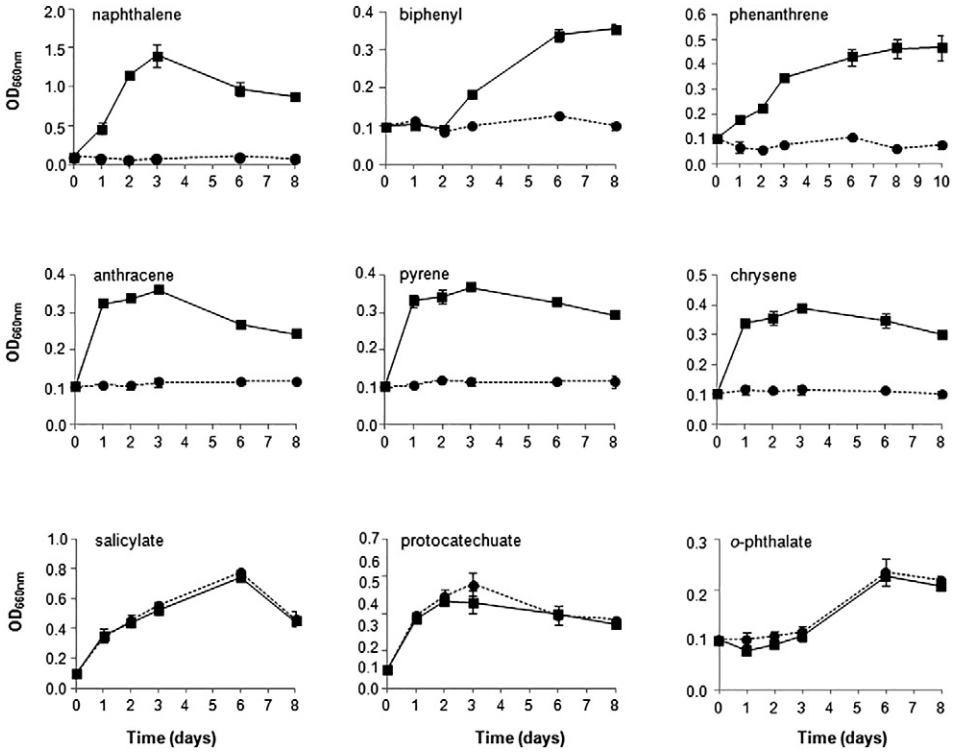

Fig. 1. Growth of the wild-type and *pahA* knock-out mutant strain with different carbon sources. Growth of *Novosphingobium* sp. HR1a (squares and continuous line), and *Novosphingobium* sp. HR1a *pahA* knock-out mutant (circles and discontinuous line) using different PAHs, biphenyl and possible intermediates of the PAHs degradation pathway as carbon source.

3.2. The pahA gene product is the key dioxygenase in PAH degradation/transformation in Novosphingobium sp. HR1a

Under aerobic conditions, the first step in the degradation of PAHs involves the activity of a multicomponent ring-hydroxylating oxygenase, which consists of a terminal dioxygenase, a ferredoxin and a ferredoxin reductase. The terminal dioxygenase is composed of two subunits; the α-subunit comprises a catalytic domain including a mononuclear iron center that serves as a substrate-binding site and a Rieske domain that holds a [2Fe-2S] cluster acting as an electron transfer entity for the mononuclear iron center, and the small or β-subunit (Harayama et al., 1992). The analysis of the complete genome sequence of *Novosphingobium* sp. HR1a revealed the presence of multiple genes encoding dioxygenases; however only 9 of them encoded genes for an α- subunit contiguous to a β-subunits. Amino-acid sequence comparison among the α-subunits of these dioxygenases and dioxygenases in the databases whose function has been experimentally determined, allowed us to identify ORF1925 as the dioxygenase with the highest homology with dioxygenases classified as unspecific PAHs dioxygenases present in the *Sphingomonas* group (Suppl. Fig. S1).

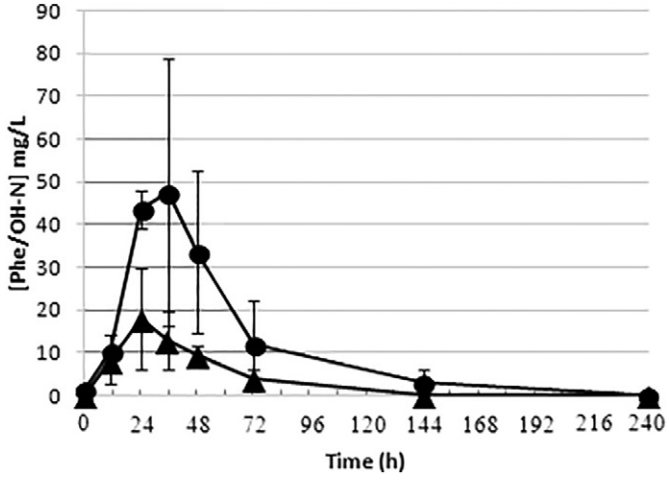

Fig. 2. Phenanthrene (Phe) consumption (circles) and 1-hydroxy-2-naphthoic acid (OH-N) formation (triangles), when *Novosphingobium* sp. HR1a was cultivated in M9 minimal medium with phenanthrene as the sole carbon source.

Novosphingobium sp. HR1a ORF1925 is encoded in a genomic region that contains two consecutive transposons (Fig. 3). The first transposon contained 21 ORFs that are flanked by two transposases (ORF1921 and ORF1943). Interestingly, whilst the first genes of the transposon presented a high identity of amino-acid sequence (>90%) with genes present on the chromosome or in plasmids of other members of the *Sphingomonadaceae* family, this identity decreases to 80–50% in the last 8 genes (ORF1935 [MarR family regulator]; ORF1936 [peroxidase], ORF1937 [thiosterase], ORF1938 [ubiquinone biosynthesis monooxygenase], ORF1939 [MarR family regulator] and ORFs 1940–1942 [multidrug ABC transport system]). The second transposon contained four dioxygenases; ORF1945-ORF1947 and ORF1967-ORF1968 are related to dioxygenases for monoaromatic compounds, ORF1970- ORF1971 are related to *p*-cumate and anthranilate dioxygenases and ORF1974–1975 is closely related to the salicylate 5-hydrolase of *Pseudomonas putida* (Suppl. Fig. S1). This transposon is flanked by transposases ORF1944 and ORF1994. All the genes within this transposon were highly homologous to those found in the chromosomes or plasmids of other *Sphingomonadaceae* (>90% identity in amino-acid sequence).

To demonstrate that *Novosphingobium* sp. HR1a ORF1925 (the large subunit of the PAH dioxygenase) was involved in PAH degradation, we constructed a knockout mutant in this *gene*. As shown in Fig. 1, this mutant was unable to grow on minimal media with naphthalene, phenanthrene, anthracene, chrysene, biphenyl or pyrene as the sole carbon source. Accordingly, the culture media did not display any color shift and no degradation compounds were detected by either HPLC or GC-

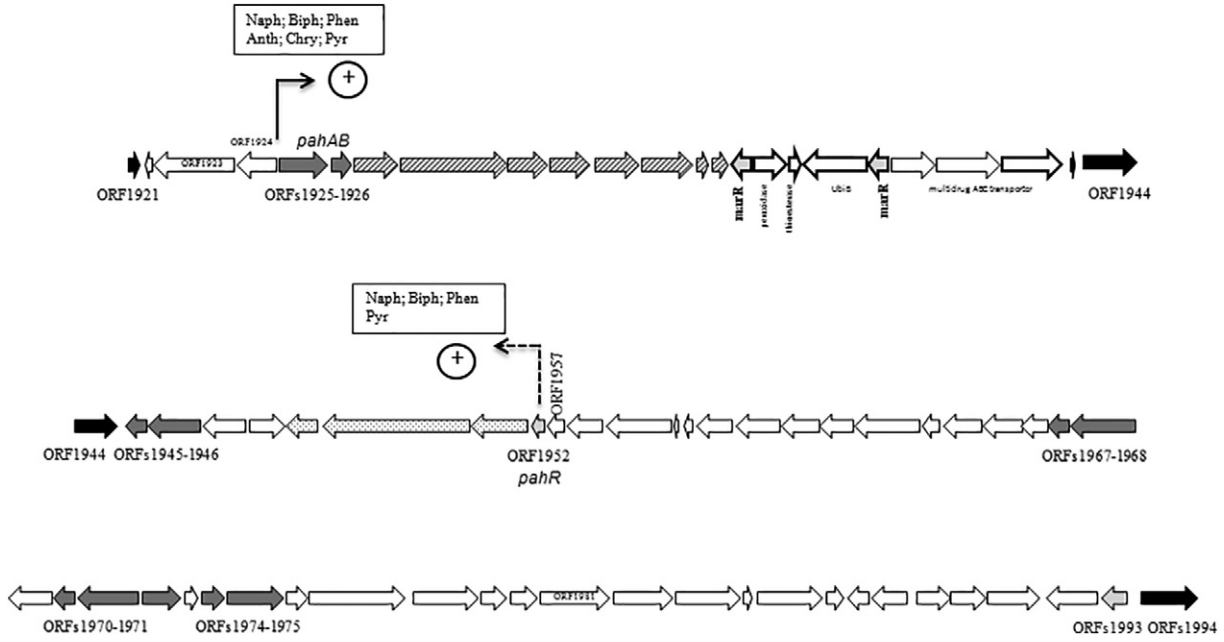

Fig. 3. Organization of the genetic region of *Novosphingobium* sp. HR1A involved in PAH degradation. ORFs are presented as block arrows. Black arrows, genes with functions related to transposases; dark gray, genes that code for dioxygenases; light gray, genes that code for putative regulators. Stripped arrows, genes that constitute a single operon together with *pahAB*. Dotted arrows, genes that are co-transcribed with *pahR*. Complete list of the ORFs contained in the transposon can be found in Supp. Mat. Table S3.

MS. No differences were observed between the growth rate of the wild-type and mutant strains in rich medium (LB) or in minimal medium containing glucose as sole carbon source (not shown), suggesting that no general growth defects were responsible for the lack of growth with the PAHs. Furthermore, growth with different central metabolites in the degradation of aromatic compounds, such as salicylate, protocatechuate or *o*-phthalate, was not affected by the mutation in the PAH dioxygenase gene (Fig. 1). All these results indicate that ORF1925 was directly involved in the initial steps in the degradation/transformation of these PAHs and therefore it was named *pahA*.

RT-PCR analysis revealed that *pahA*, *pahB* (which encodes the small subunit of the dioxygenase) and the subsequent eight genes (which encoded genes with putative functions: alcohol dehydrogenase; pyruvate phosphate dikinase; dihydrodipicolinate synthase; ketopantoate reductase; tartrate dehydrogenase; monooxygenase; hypothetical protein; and BphX-like protein) are co-transcribed (not shown).

3.3. Characterization of the promoter region of pahA

The 239 pb intergenic region between *orf1924* that codes for a putative permease and the *pahA* gene (Fig. 4) was analyzed for the presence of promoters. Using 5′RACE we identified *pahA* transcription start site (tss) in the A located at position 51 upstream of *pahA* start codon (Fig. 4). Upstream of the tss of *pahA* we found a putative RNA polymerase binding site with conserved −35 (**CTGTCA**) and a poorly conserved −10 (**TCAGGT**) consensus sequence separated by 16 nucleotides (Fig. 4).

Upstream of the tss (−110/−117 and −131/−138) we were able to identify two perfect inverted repeats that may constitute binding sites for regulators. All these DNA features are highly conserved in other members of the *Sphingomonadaceae* family (Suppl. Fig. S2).

This intergenic region was cloned with $P_{pahA\text{-HR1a}}$ promoter towards the promoterless *lacZ* gene in the broad host range vector pMP220 (Spaink et al., 1987), yielding pHR1a that was introduced into *Novosphingobium* sp. HR1a and into its *pahA* mutant. $P_{pahA\text{-HR1a}}$ was induced 5-fold by naphthalene, 2.5-fold by phenantrene and anthracene and also 2- and 1.5-fold by high molecular weight PAHs, such as pyrene and chrysene, respectively (Fig. 5A). Induction was also observed in the presence of some intermediates of the PAH-degradation pathway, such as 1-hydroxy-2-naphthoic acid (Fig. 5A) and salicylic acid, which was the best inducer (7-fold induction). Other monocyclic aromatic compounds, possible intermediates in the PAH-degradation pathways (*o*-phtalate and protocatechuate), and several organic solvents (benzene, toluene, ethylbenzene) were unable to induce expression of *pahA* (not shown). However, *p*-xylene, but not *o*- and *m*-xylene was able to induce the expression of the P_{pahA} promoter (2.5-fold). The reasons for the specific induction by *p*-xylene are unknown; in fact we had expected induction with *o*-xylene which is structurally more similar to salicylic acid than the other isomers. Induction of *pahA* expression was observed at concentrations as low as 100 ng l^{-1} of phenanthrene and 360 ng l^{-1} of naphthalene (Fig. 5B).

Induction of the expression of P_{pahA} was not significantly affected in the *pahA* mutant in the presence of phenanthrene, naphthalene or salicylate (data not shown), suggesting that these compounds, and not their degradation products are the inducers of the dioxygenase.

3.4. phnA expression is induced by environmental samples from hydrocarbon-polluted sites

To evaluate the biotechnological properties of *Novosphingobium* sp. HR1a, we tested if the PAH-degradation genes were expressed in the presence of different oil-contaminated samples collected from a series of polluted sites. When *Novosphingobium* sp. HR1a (pHR1a) was exposed to water from the Messina harbor (Italy), water saturated with heavy fuel oil coming from the *Prestige* oil spill or with Dansk crude, or sediments from either Messina harbor or from a refinery near Gela (Italy), we observed an increase of β-galactosidase expression from the P_{pahA} promoter (Fig. 6). No increase was observed in the presence of seawater from Motril harbor (Spain) or in non-contaminated samples such as soil from our Institute garden (EEZ) or from Salobreña beach (Fig. 6). GC-MS analysis of the PAH content of these samples confirmed the presence of different PAHs in these samples (Table 1). These results indicate that genes related to PAH degradation were expressed when exposed to environmental samples, and suggest that biodegradation of these contaminants was not inhibited by other compounds in the samples.

The P_{pahA} expression was also induced in the presence of commercial petrol and diesel (Fig. 6B). *Novosphingobium* sp. HR1a was not able to

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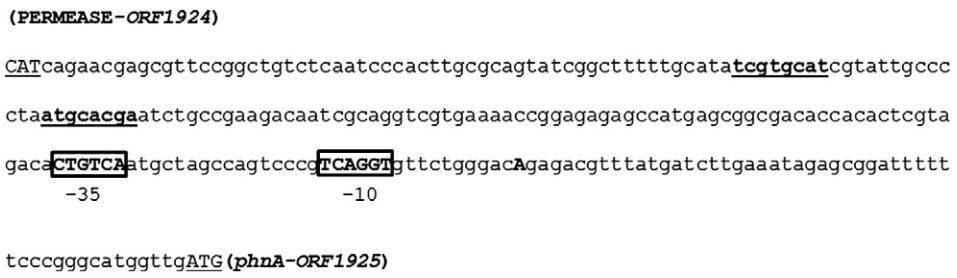

Fig. 4. *pahA* regulatory region; the palindromic sequences are underlined; the boxes indicate a putative −35/−10 s70-RNA polymerase binding site. The transcription start site is the bolded (A).

grow with petrol concentrations higher than 0.1% (v/v); however there was a high expression of the β-galactosidase activity when 0.05% (v/v) petrol was added to the culture.

3.5. Novosphingobium sp. HR1a (pKSPA-1) is able to degrade PAHs and monitor their biodegradation simultaneously

Because PAHs are among the most abundant contaminants in oil spills in the sea, we decided to measure the growth and PAH disappearance in an ONR7a artificial marine medium supplemented with 0.5% (v/v) of diesel. To easily monitor PAH biodegradation we constructed a bioreporter in which the P_{pahA} promoter controlled the expression of the *gfp* gene (green fluorescence protein) resulting in plasmid pKSPA-1, and we introduced it into *Novosphingobium* sp. HR1a. The resultant strain *Novosphingobium* sp. HR1a (pKSPA-1) was able to detect phenanthrene, naphthalene, 2-methyl naphthalene and salicylate as pure compounds in the artificial marine medium. The detection and saturation limits of the biosensor are shown in Table 1. Detection limits of the biosensor in marine media ONR7 were higher for all the compounds tested (less sensitivity) than those of the biosensor in M9 minimal media, probably because the high salinity of the medium was causing stress to *Novosphingobium* sp. HR1a (this strain grew more slowly in the ONR7a marine medium than in M9 medium).

When tested in an ONR7a marine medium artificially contaminated with diesel, the biosensor pKSPA-1 gave positive results at diesel concentrations as low as 0.1% (v/v) (Fig. 7A) and the optimum response time was 24 h (not shown). The amount of PAHs in the sample at this diesel concentration was 40 μg ml^{-1} (ppb).

The biosensor was used in a degradation experiment in an ONR7a marine medium supplemented with 0.5% v/v of diesel. No bacterial growth was detected in the contaminated medium in the absence of the biosensor in the 16 days of the experiment. In flasks with uncontaminated water and inoculated with *Novosphingobium* sp. HR1a (pHR1a) the number of CFUs decreased with time and no viable cells were detected after 10 days; however, when diesel was in the medium the number of CFUs increased during the first 3 days and showed a slow decrease until day 16 (Fig. 7B). GC-MS analysis of the PAHs present in the samples showed a decrease in PAH content during the six first days, with this decrease being significantly higher in the presence of *Novosphingobium* sp. HR1a (pKSPA-1) than in the sterile control (Fig. 7C). The amount of PAHs 6 days after inoculation was 32.5 μg l^{-1}, and slowly decreased down to 5.4 μg l^{-1} at day 16. Accordingly, the biosensor was able to give positive signals up to 8 days after inoculation

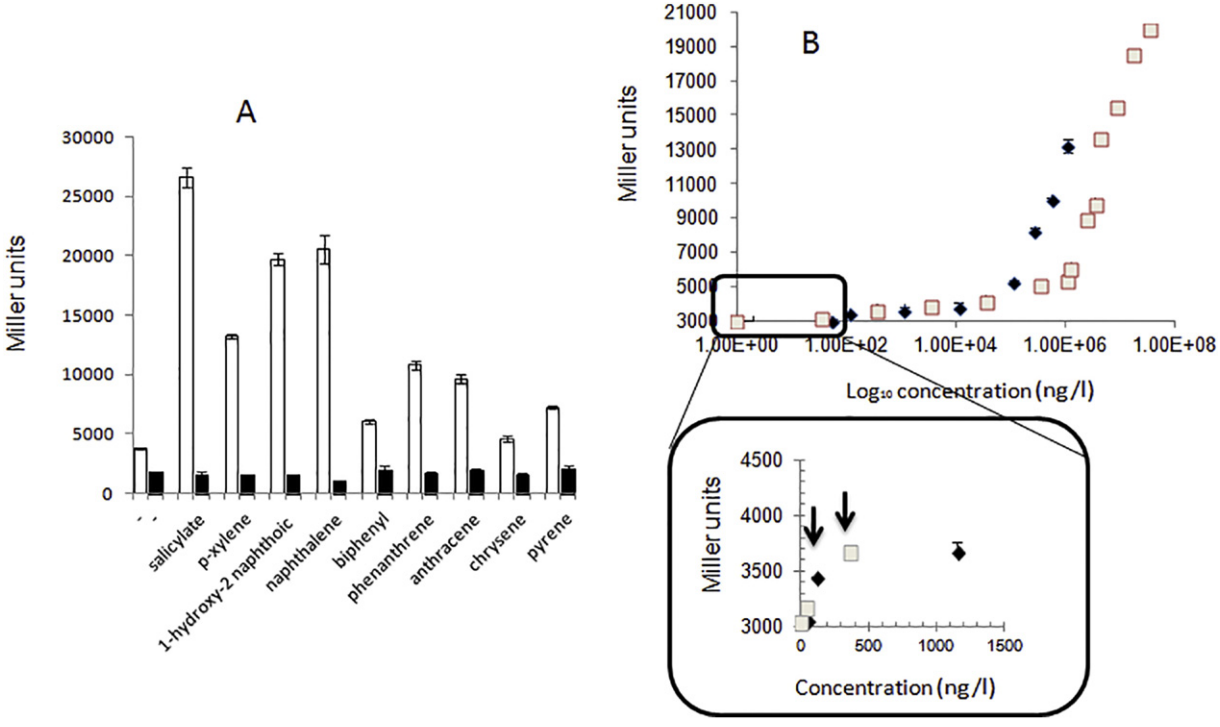

Fig. 5. Expression of P_{pahA} in the presence of aromatic compounds. A) The expression of P_{pahA} in *Novosphingobium* sp. HR1a (white bars), and its *pahR* mutant (black bars) in the absence (-) and presence of different aromatic compounds. B) Expression from the P_{pahA} promoter at different naphthalene (squares) and phenanthrene (diamonds) concentrations. In both experiments cells were grown on minimal media M9 plus glucose and the corresponding aromatics added as indicated in M&M.

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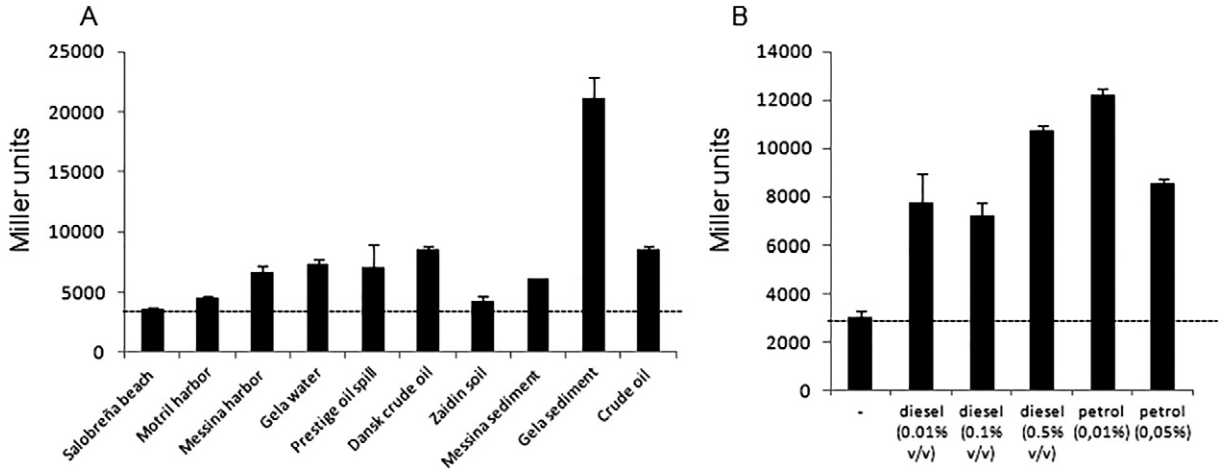


Fig. 6. Expression from P_{pahA} in the presence of contaminated samples. β-galactosidase expression from P_{pahA} promoter in *Novosphingobium* sp. HRa1 exposed to A) different environmental samples: water from Salobreña beach, Motril and Messina harbors, and Gela water were used at 5% (v/v). Saturated solutions of the *Prestige* oil spill, and Dansk crude were also used at 5% (v/v). Sediments from Messina harbor, Gela refinery and soil from the Estación Experimental del Zaidín (EEZ) garden were used at 5% (w/v). B) Water artificially contaminated with petrol and diesel.

(Fig. 7D). These results indicate that the limit of detection of the biosensor when growing with diesel as the sole carbon source is very close to the detection limit determined in the laboratory under optimum nutrition conditions (with glucose in the medium).

3.6. pahR regulates the expression of pahA and the degradation of PAHs in Novosphingobium sp. HR1a

Two ORFs (*orf1935* and *orf1939*), with homology with regulatory proteins of the MarR family were encoded in the vicinity of the *pahA* gene in *Novosphingobium* sp. HR1a. To determine if any of them was controlling the expression of *pahA*, we constructed mutants in these two *orfs*. When plasmid pHR1a was introduced into the mutant strains and β-galactosidase assays were performed, no differences in the induction level of P_{pahA} promoter were observed when compared with the wild-type. Furthermore, the mutant strains grew similarly to the wild-type strain in naphthalene, phenanthrene, anthracene, chrysene or pyrene as the sole carbon source (data not shown). These results indicate that these regulators were not directly involved in *pahA* expression.

We thus investigated other regulator genes present in *Novosphingobium* sp. HR1a PAH degradation cluster (Fig. 3). One of the transposons in the cluster included an ORF with homology with NtrC family regulators (*orf1952*). We constructed a knock-out mutant in *orf1952* and assayed its growth and induction phenotype as described above. The knock-out mutant in *orf1952* was unable to grow with naphthalene, phenanthrene, anthracene, chrysene or pyrene as the sole carbon source. When plasmid pHR1a was introduced and assayed in the knockout mutant strain no induction of β-galactosidase activity was observed in the presence of naphthalene, biphenyl, phenanthrene, anthracene, chrysene or pyrene (Fig. 5A). This result suggests that *orf1952*, now annotated as *pahR*, is involved in the regulation of the expression of the PAH-degradation dioxygenase gene *pahA*.

Table 1
PAH quantification by GC-MS in the environmental samples and in artificially contaminated waters: PAHs values are given in mg l^{-1}.

	Total PAHs	2 rings	3 rings	>3 rings	Naphthalene
Sea water (5% v/v)					
Salobreña	0.226	0.031	0.003	0.192	0.031
Motril	0.285	0.091	0.009	0.184	–
Messina	0.523	0.454	0.025	0.044	0.422
Gela	0.585	0.473	0.029	0.083	0.441
Edaphic samples (5% v/v)					
Messina sediment	0.2066	0.0434	0.094	0.0692	0.038
Gela	2.5186	2.424	0.148	0.0138	2.34
Petrol and derivatives					
Petrol (0.05%)	76.7745	75.6225	0.963	0.189	74.655
Diesel (0.5%)	350.37	321.57	18.774	10.026	287.712
Prestige crude oil (5% water saturated)	9.691	9.378	0.227	0.086	9.187
Dansk crude (5% water saturated)	64.909	64.423	0.402	0.084	64.862

3.7. pahR is induced by phenantrene, anthracene and pyrene

RT-PCR demonstrated that *pahR* was co-transcribed with three other *orfs* with homology to ferredoxin reductase, pyruvate carboxylase and a second ferredoxin reductase, respectively (Fig. 3). The 234 bp intergenic region between *pahR* and *orf*1957 was cloned in pMP220 with P_{pahR} promoter oriented towards the *lacZ* gene and β-galactosidase assays were carried out in the presence of different PAHs. Induction of the enzymatic activity was detected in the presence of phenanthrene, naphthalene and pyrene, with phenanthrene being the best inducer; there was no induction in the presence of anthracene and chrysene (Fig. 8A). When this construction was introduced into the *pahR* mutant no induction of the β-galactosidase activity was observed, suggesting that this regulator is controlling its own expression in the presence of PAHs (not shown).

Using 5′RACE we identified the transcription start site (tss) in the G located at position 24 upstream of *pahR* start codon (Fig. 8B). Upstream of the tss of *pahA* we identified a consensus sequence −12/−24 for σ^{54}-RNA polymerase binding site (GG-N_{10}-GC) (Fig. 8B). Upstream from this sequence, between positions −99 and −124, two perfect inverted repeats may constitute the upstream activating sequences (UAS) for binding of an NtrC family regulator and agrees with *PahR*, a regulator belonging to this family, controlling P_{pahR}.

3.8. The biosensor Novosphingobium sp. HR1a (pKSPA-R), based on P_{pahR}, is more sensitive and faster than the biosensor based on P_{pahA}

Given the similarity in the inducer profile between the P_{pahA} and P_{pahR} promoters, we constructed a biosensor in which the promoter sequence of *pahR* was fused to the *gfp* (in plasmid pSEVA637) and we analyzed its performance. The new biosensor, *Novosphingobium* sp. HR1a (pKSR-1) was less sensitive to PAHs than *Novosphingobium* sp. HR1a (pKSPA-1) (data not shown).

To improve the biosensor, we constructed *Novosphingobium* sp. HR1a (pKSPA-R); in this biosensor in addition to the elements of pKSR-1, a copy of the *pahR* regulator was introduced in the bioreporter under the control of the P_{pahR} promoter. This biosensor presented a

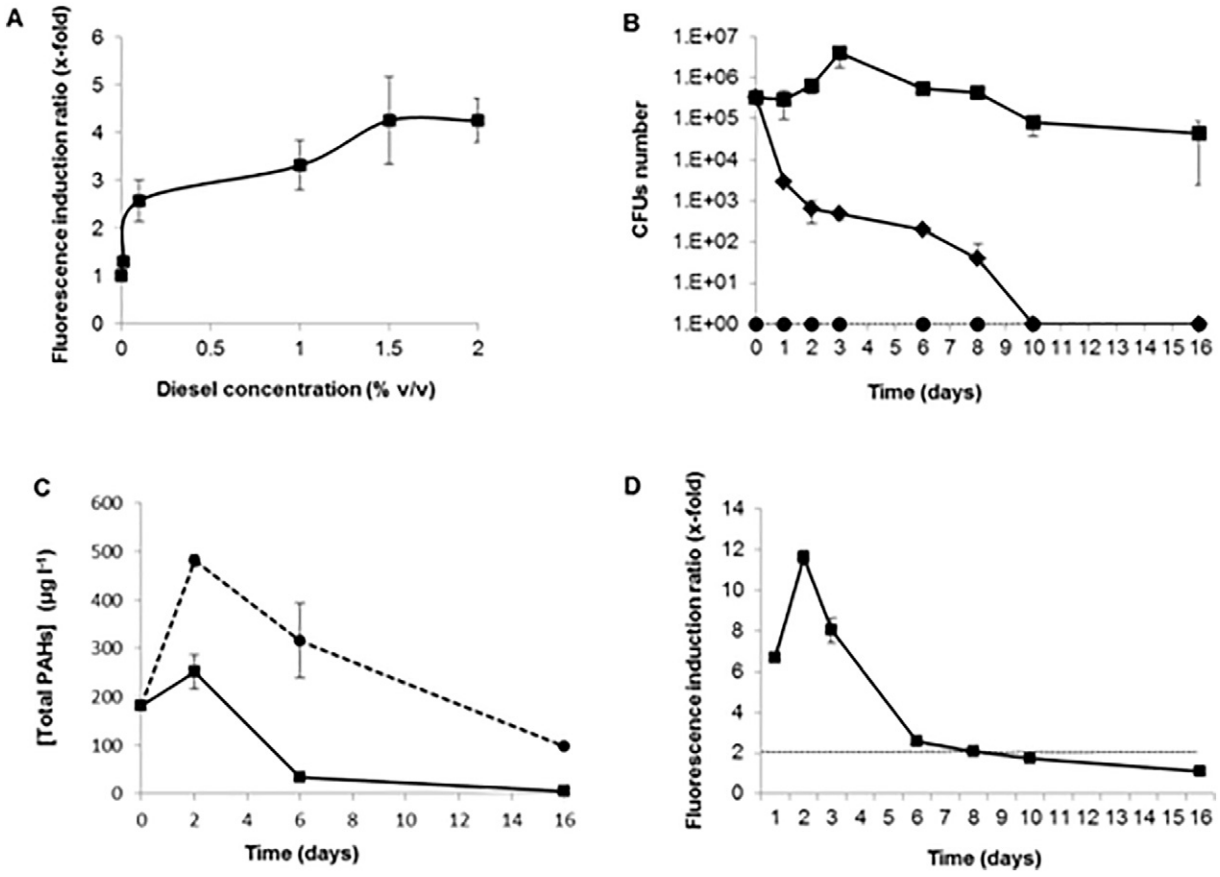
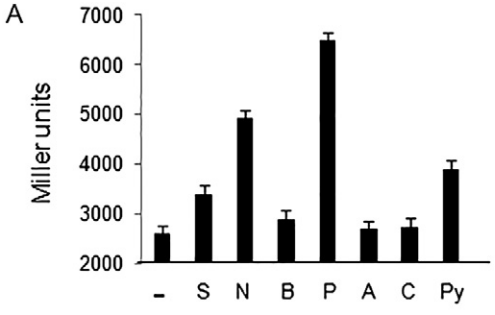


Fig. 7. Performance of the biosensor *Novosphingobium* sp. HR1a (pKSPA-1) in an ONR7 marine medium contaminated with diesel A) Detection of diesel in ONR7a marine medium using biosensor *Novosphingobium* sp. HR1a (pKSPA-1). B) Growth of *Novosphingobium* sp. HR1a (pKSPA-1) in an ONR7a minimal medium with 0.5% (v/v) of diesel (squares) and without diesel (diamonds). CFUs in the mixture without inoculum are shown in dotted line (circles). C) Total concentration of PAHs in the samples in the presence (squares) and in the absence (circles) of *Novosphingobium* sp. HR1a (pKSPA-1). D) Detection of diesel compounds in the samples by the biosensor *Novosphingobium* sp. HR1a (pKSPA-1).

faster response than *Novosphingobium* sp. HR1a (pKSPA-1); the signal was maximum at 5 h instead of 7.5 h in M9-glucose and 5 instead of 24 h in marine medium ONR7a plus glucose; *Novosphingobium* sp. HR1a (pKSPA-R) responded to salicylate, phenanthrene, 2-methylnaphthalene, naphthalene and biphenyl. Interestingly it also responded to monoaromatic compounds such as 3-methyl benzoate and *o*- and *p*-



B

tcgataactgagagctgggcttgcccgatcgacttcggtctggctctgcgcacttgcttg

gccatgaatggcgtaaaatggaa*gtc*gcggcatcccgaaaagggatgcggcctagacta

agttgcaaggggggtaatcagagagaaaaatccccattcgggtgca*actgt*cggttgggggg

tcgcgcggtctgaccaacgcgatctttgccaGcgcgcccgatcacgagcgccagaATG

-24 -12 +1

Fig. 8. Analysis of the promoter region of *pahR* A) Expression from the P_{pahR} promoter in presence of different PAHs. S (salicylate), N (naphthalene), B (biphenyl), P (phenanthrene), A (anthracene), C (chrysene), Py (pyrene). B) P_{pahR} promoter sequence: The tss is indicated by +1 (bold) and in capital letter; −12/−24 positions are in bold and indicated by numbers. Inverted repeats constituting the putative UASs are in bold and underlined.

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xylene (Fig. 9A). This biosensor was more sensitive to PAHs than the biosensor based on *pahA* promoter (Table 2).

When we tested this biosensor in the presence of environmental samples, we observed that the detection limit was lower for the biosensor that incorporated the regulatory protein (Fig. 9B). Similarly, this detection limit was lower when tested with water contaminated with diesel or petrol (Fig. 9C).

4. Discussion

Because of the great ecological impact that PAHs have on the environment and health, it is important to understand the molecular mechanisms that rule PAH degradation in bacteria. *Novosphingobium* sp. HR1a has the capacity to degrade/transform a wide range of PAHs and, as in other sphingomonads, this capacity is granted by the existence of a nonspecific PAH hydroxylating dioxygenase (Demanèche et al., 2004: Jouanneau and Meyer, 2006; Schuler et al., 2009; Lyu et al., 2014). In most cases, the functionality of this protein was assigned because the encoding gene was induced in the presence of PAHs or because heterologous expression conferred the host strain with the capacity to oxidize the compounds (Khan et al., 2001; Krivobok et al., 2003; Kim et al., 2006; Singleton et al., 2009; Yun et al., 2014; Lyu et al., 2014). In this study we have obtained direct evidence of the crucial role of the PahA enzyme in the degradation of PAHs by *Novosphingobium* sp. HR1a; a mutant in the *pahA* gene (encoding the α-subunit of the dioxygenase) was unable to use and transform a wide range of PAHs. The *pahA* gene was inducible by different PAHs and intermediate metabolites of the degradative pathway and we have demonstrated that induction requires the regulatory protein *PahR*, which belongs to the NtrC family of σ^{54}-dependent transcriptional regulators. However, there was no consensus sequence for σ^{54}-dependent promoters in the P_{pahA} promoter, suggesting that *PahR* indirectly regulates the expression of *pahA*. Whatever the mechanism by which *PahR* regulates the expression of the dioxygenase gene, this regulatory protein is essential for PAH degradation as a knock-out mutant in the *pahR* gene is totally unable to grow with PAHs as the sole carbon source. Given that the basal expression of the *pahA* gene in both the wild-type and *pahR* mutant strains is relatively high, it is possible that *PahR* is also regulating other genes involved in PAH utilization.

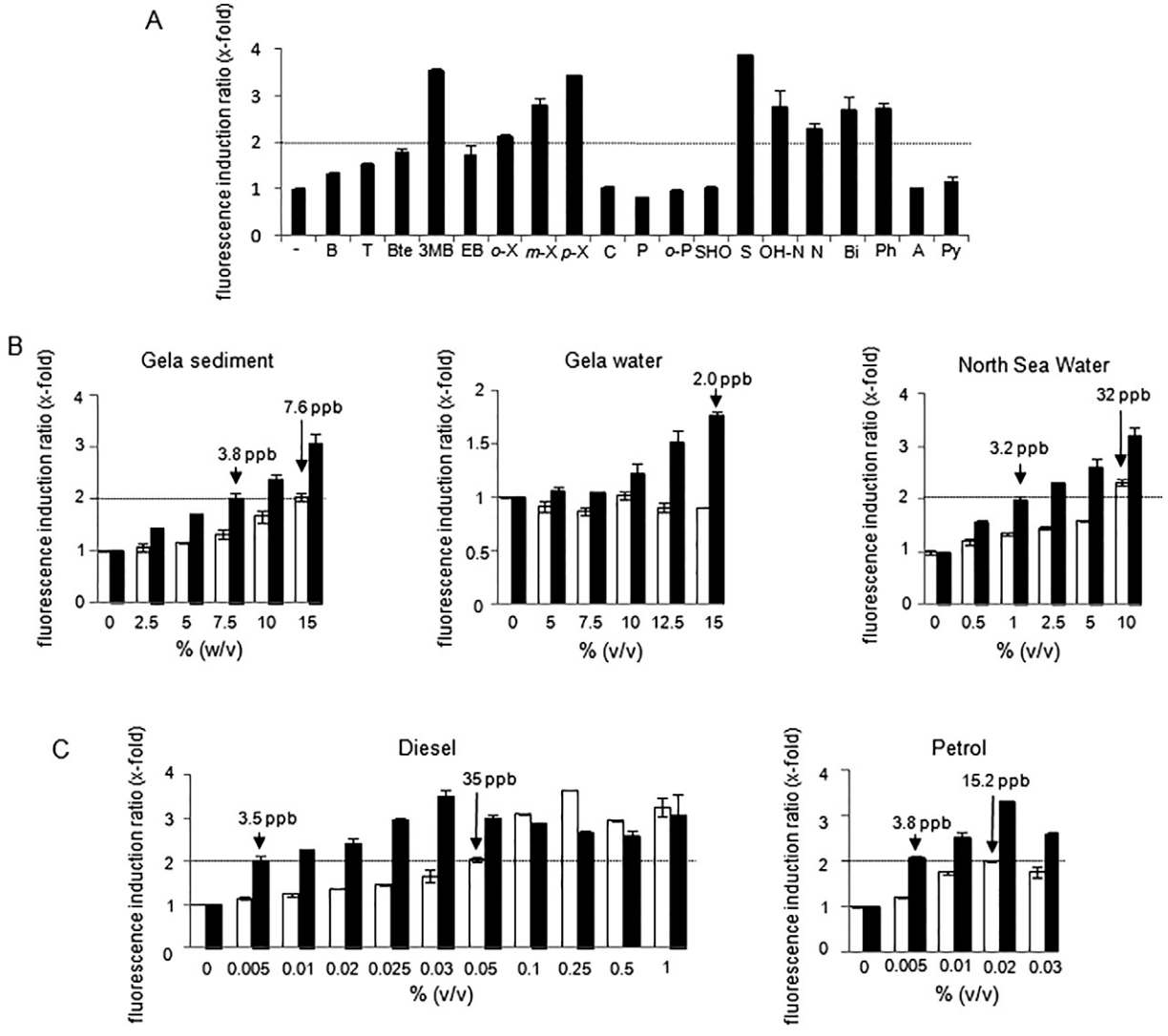

Fig. 9. Performance of *Novosphingobium* sp. HR1a (pKSP-R) with pure compounds and contaminated samples. A) Fluorescence induction ratio in the presence of B: benzene (0.025% v/v); T: toluene (0.05% v/v); Bte: benzoate (1 mM); 3 MB: 3 methylbenzoate (1 mM); EB: ethylbenzene (0.01% v/v); *o*-X: *o*-xylene (0.025%); *m*-X: *m*-xylene (0.025% v/v); *p*-X: *p*-xylene (0.025% v/v); C: catechol (1 mM), P: protocatechuate (1 mM); *o*-P: *o*-phthalate (1 mM); SHO: salicyladehyde (0.001% v/v); S: salycilate (1 mM); and saturated solutions of OHN: 1-hydroxy-2 naphthoic acid; N: naphthalene; Bi: biphenyl; P: Phenanthrene; A: Anthracene; Py: pyrene. B) Comparison between fluorescence induction ratio of *Novosphingobium* sp. HR1a (pKSPA-R) (black bars) and *Novosphingobium* sp. HR1a (pKSPA-1) (white bars) in the present of different concentrations of contaminated samples. C) Comparison between fluorescence induction ratios of the two biosensors in water samples contaminated with diesel or petrol.

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Table 2
Saturation and detection limits of pKSPA-1 and pKSPA-R for pure compounds and environmental samples.

	Novosphingobium HR1a (pKSPA-1)				*Novosphingobium* HR1a (pKSPA-R)			
	M9-glucose		ONR7a		M9-glucose		ONR7a	
	DL	SL	DL	SL	DL	SL	DL	SL
Pure compounds								
Naphthalene	0.79	31.60	5.20	70.0	0.32	31.6	3.16	70.0
2-Methyl-naphthalene	0.20	1.50	1.0	3.0	0.08	1.50	0.50	3.0
Phenanthrene	0.65	1.60	1.60	60.0	0.34	1.60	0.80	60.0
Salicylate	0.69	6.90	1.60	40.0	0.35	6.90	0.80	60.0
Oil and derivatives (total PAHs)								
Diesel	0.035	1.05	0.04	1.05	0.0035	0.035	0.007	1.05
Petrol	0.015	0.02 (toxic)	(High toxicity)		0.0038	0.02 (toxic)	toxic	
North sea oil	0.032	n.d	0.032	n.d	0.0032	n.d	0.006	n.d
Crude oil	0.060	n.d	0.12	n.d	0.0060	n.d	0.006	n.d
Prestige spill	u.d.l		u.d.l		u.d.l		u.d.l	
Sediments								
Gela	0.0038	n.d	0.0038	n.d	0.0038	n.d	0.0038	n.d
Messina	u.d.l		u.d.l		u.l.d		u.d.l	
Water								
Salobreña harbor	u.d.l				u.d.l		u.d.l	
Motril harbor	u.d.l				u.d.l		u.d.l	
Messina harbor	u.d.l				u.d.l		u.d.l	
Gela	0.0038	n.d	0.0038	n.d	0.0038	n.d	0.0038	n.d

Concentration data is given in mg l^{-1}.
DL: detection limit. Concentration when the fluorescence induction ratio is 2.
SL: saturation limit. Concentration when the fluorescence induction ratio is maximum.
n.d.: not determined.
u.d.l: under our detection limits.

The identification of the genetic determinants for PAH degradation allowed us to utilize these elements in the construction of biosensors based on the promoters of the two genes (*pahA* and *pahR*) and on the regulatory protein *PahR*. Both biosensors *Novosphingobium* sp. (pKSPA-1) and *Novosphigobium* sp. (pKSPA-R) were able to sense similar compounds when present at saturation concentrations. However, *Novosphigobium* sp. (pKSPA-R) was able to detect lower amounts of the contaminants in polluted samples. Interestingly, the construction with *lacZ* gene as reporter (pHR1a) was more sensitive than the one that carried *gfp* as reporter gene (pKSPA-1); this is probably due to the high basal expression levels of the promoter and the sensitivity of the fluorescent assay. However, because of the manipulations required to measure β-galactosidase assays, bioreporters with *gfp* have been described as more appropriate to monitor environmental samples than those using β-galactosidase or luciferase (Van der Meer and Belkin, 2010).

In most cases, the concentrations of PAHs quantified by GC-MS in the environmental samples that gave positive results with both biosensors were lower than the detection limit for a single PAH. This has been observed previously using other biosensors (Tecon et al., 2010; Hernández-Sánchez et al., 2016) and it is probably related to the presence in the samples of different mixtures of contaminants that may exert synergic effects on the biosensor, or to a higher solubility of the PAHs due to the presence of possible organic solvents in the sample. Interestingly, expression of *pahA* in the presence of crude oil and *Prestige* samples was not as high as expected on the basis of the PAH content determined by GC-MS (Fig. 6; Table 1), probably reflecting the low bioavailability of the PAHs in these particular samples.

There are some other biosensors for the detection of PAHs described in the literature (Heitzer et al., 1998; Dorn et al., 2003; Werlen et al., 2004; Bastiaens et al., 2001). Particularly relevant in this context is the PAH biosensor based on the PhnR protein of *Burkholderia* sp. RP007 (Tecon et al., 2006) that is able to detect naphthalene and phenanthrene. This biosensor, as those described here, was able to detect fluxes of phenanthrene, as the host strain was able to degrade the PAHs. The detection limit of this biosensor was very similar to that determined for *Novosphingobium* sp. HR1a (pKSPA-1) but higher than that of *Novosphingobium* sp. HR1a (pKSPAR). *Novosphingobium* sp. HR1a (pKSPA-R) was able to detect quantities as low as 2–4 ppb of total PAHs depending on the sample, whilst *Novosphingobium* sp. HR1a (pKSPA-1) was able to detect 8–35 ppb of total PAHs. This means two or three orders of magnitude lower than the detection limit for a single PAH.

These bioreporters have been introduced into a host strain, *Novosphingobium* sp. HR1a that is able to degrade the contaminants sensed by the bioreporter and therefore it is possible, as shown in Fig. 7, to use our biosensors to proceed with the bioremediation concomitantly with the monitoring of the outcome of the remediation. It is worth mentioning that the detection limit for the PAHs of *Novosphingobium* sp. HR1a (pKSPA-1) when grown with diesel as the sole carbon source was similar to the detection limit observed in assays with glucose plus diesel (40 ppb), suggesting that biosensor's nutritional conditions did not significantly interfere with the its performance.

5. Conclusions

Novosphingobium sp. HR1a is able to degrade a broad range of PAHs and is a robust strain for utilization in the bioremediation of soil and water. We have identified and characterized the genetic elements that are involved in the degradation of PAHs in this strain and we have used this information for the construction of biosensors that allow us to determine the presence of contaminants in water. *Novosphingobium* sp. HR1a (pKSPA-R) is able to detect concentrations as low as 4 ppb of total PAHs in contaminated samples, being one of the best PAH biosensors described so far.

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Conflict of interests

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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