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Characterization of a ring-hydroxylating dioxygenase from phenanthrene-degrading *Sphingomonas* sp. strain LH128 able to oxidize benz[*a*]anthracene.

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ABSTRACT

Sphingomonas sp. strain LH128 was isolated from a polycyclic aromatic hydrocarbon (PAH) contaminated soil using phenanthrene as the sole source of carbon and energy. A dioxygenase complex, *phnA1fA2f* encoding the α and β subunit of a terminal dioxygenase responsible for the initial attack on PAHs, was identified and isolated from this strain. PhnA1f showed 98%, 78% and 78% identity to the α subunit of *Novosphingobium aromaticivorans* strain F199, *Sphingomonas* sp. strain CHY-1 and *Sphingobium yanoikuyae* strain B1 respectively. When overexpressed in *E. coli*, PhnA1fA2f was able to oxidize low molecular weight PAHs, chlorinated biphenyls, dibenzo-*p*-dioxin and the high molecular weight PAHs benz[*a*]anthracene, chrysene and pyrene. The action of PhnA1fA2f on benz[*a*]anthracene produced two benz[*a*]anthracene dihydrodiols.

INTRODUCTION

Polycyclic aromatic hydrocarbons are found ubiquitously in nature (natural oil seeps, bushfires, volcanoes etc.) but anthropogenic activities have led to an increased incidence of these recalcitrant pollutants due to, amongst others, the burning, handling or disposal of organic matter including coal tars, crude oil and petroleum products. For the purpose of bioremediation, microorganisms able to use these pollutants as the sole source of carbon and energy are extensively studied (Cerniglia 1992; Johnsen et al. 2005). Amongst these, sphingomonads have received much attention due to their ability to degrade a wide range of aromatic hydrocarbons. *Sphingomonas* species able to degrade mono- and polycyclic aromatic hydrocarbons (Pinyakong et al. 2000; Schuler et al. 2008; Story et al. 2001), phenols (Cai and Xun 2002), carbofuran (Feng et al. 1997; Kim et al. 2004), estradiol (Fujii et al. 2003), dibenzofurans (Bunz and Cook 1993; Fortnagel et al. 1990), biphenyls (Happe et al. 1993; Kim and Zylstra 1999; Peng et al 2002; Zylstra and Kim 1997), dibenzo-*p*-dioxin (Bunz and Cook 1993; Hong et al. 2002) and herbicides (Johannesen et al. 2003; Sorensen et al. 2001) have been isolated. In the last few years, attention has been turned towards identifying and characterizing the genes involved in PAH degradation, allowing a closer look at pathways potentially useful in bioremediation (Pinyakong et al. 2003a).

PAH degradation by aerobic bacteria is generally initiated by the introduction of both atoms of O_2 to the aromatic ring of the substrate (Butler and Mason 1997; Wackett 2002). This initial reaction, which is catalysed by aromatic ring hydroxylating dioxygenases, involves the dihydroxylation of the carbon-carbon double bond of adjacent carbon atoms. The enzymes responsible for the initial attack on PAHs from *Sphingomonas* sp. strain CHY-1, which was isolated for its ability to degrade chrysene (Demaneche et al. 2004; Jounneau et al. 2006) and *Sphingobium yanoikuyae* strain B1, which was isolated for its ability to degrade biphenyl (Ni Chadhain et al. 2007), are known and their respective crystal structures were determined (Jakoncic et al. 2007a; 2007b, Yu et al. 2007). In a recent study we have successfully

identified the genes governing the angular attack on fluorene by the gram-negative *Sphingomonas* sp. strain LB126 which uses fluorene as the sole source of carbon and energy (Schuler et al. 2008).

Although the complete sequence of plasmid pNL1 which harbours a catabolic gene cluster of 40 kb as well as the putative initial dioxygenase of *Novosphingobium aromaticivorans* F199 has been sequenced, the activity of the initial dioxygenase has not yet been investigated (Romine et al. 1999). Sphingomonads harbour multiple copies of genes predicted to encode the terminal component of Rieske-type oxygenases (Pinyakong et al. 2000; Romine et al. 1999). They constitute a large family of two- or three-component metalloenzymes whose catalytic activity component is generally a heteromeric α3β3 hexamer containing one Rieske-type [2Fe-2S] cluster and one nonheme iron atom per α subunit. The fact that all phenanthrene-degrading sphingomonads carry a similar pathway organization as found in *Sphingomonas* sp. strain CHY-1, *Sphingobium yanoikuyae* strain B1, *Novosphingobium aromaticivorans* strain F199 and *Sphingobium* sp. strain P2, indicates that this organization has been conserved for a long time and is quite stable despite the apparent complex organization compared to the more 'logical' organization of PAH-degradation genes in members of the genus *Pseudomonas*. These data could help to explain that *Sphingomonas* spp. started as phenanthrene degraders and their respective initial dioxygenases became substrate-relaxed in order to oxidize a large variety of PAHs.

Sphingomonas sp. strain LH128 was isolated from a heavily polluted soil (Bastiaens et al. 2000) and is capable of growing on phenanthrene as the sole source of carbon and energy. Strain LH128 is also able to transform indole to indigo in the presence of phenanthrene (data not shown). No indigo formation was observed when the strain was grown in the presence of glucose suggesting that the dioxygenase oxidizing indole must be induced by phenanthrene. Moreover strain LH128 is able to degrade anthracene, dibenzothiophene, fluorene (Bastiaens et al. 2000) and the N-heterocyclic PAHs acridine, phenanthridine, benzo[*f*]quinoline and

benzo[*h*]quinoline (van Herwijnen et al. 2004). In this study the multicomponent ring hydroxylating dioxygenase from *Sphingomonas* sp. strain LH128 was cloned and its function towards a variety of substrates was investigated. This newly characterized dioxygenase is shown to be closely related to BphA1fA2f from *Novosphingobium aromaticivorans* strain F199 (98 % identities) but to display significant differences in catalytic behaviour as reflected by a broad substrate range notably including the capacity to oxidize benz[a]anthracene.

MATERIALS AND METHODS

Reagents. PAHs and antibiotics were obtained from Sigma-Aldrich (St. Louis, MO). Primers were purchased from Sigma-Genosys. Silicone oil (Rhodorsil 47V20) was purchased from VWR International (France). Restriction enzymes were from New England Biolabs (Ipswich, MA).

Bacterial strains, plasmids, and media.

Sphingomonas sp. strain LH128 was kindly provided by VITO (Vlaamse Instelling voor Technologisch Onderzoek, Belgium). *Escherichia coli* Top10 (Invitrogen, Carlsbad, CA) was used as the recipient strain in all cloning experiments. *E. coli* BL21(DE3) was used for gene expression analysis. PCR amplicons were either cloned into pDrive (Qiagen, Valencia, CA) while pET30f (Novagen, San Diego, CA) and pVLT31 (de Lorenzo et al. 1993) were used as expression vector. MM284 minimal medium (Mergeay et al. 1985) was used for growing *Sphingomonas* sp. strain LH128 and was supplemented with phosphate buffer (50 mM; KH_2PO_4, K_2HPO_4, pH 7.2) instead of Tris buffer. Phenanthrene was provided as crystals in both solid and liquid media. LB broth (Sambrook et al. 1990) was used as complete medium for growing *E. coli* strains. Solid media contained 2% agar. When needed, ampicillin, streptomycin, tetracycline or kanamycin was added to the medium at 100, 200, 10 and 20 µg/ml, respectively. *Sphingomonas* sp. strain LH128 was grown at 30°C, and *E. coli* strains

were grown at 37°C. Bacterial growth was determined by optical density readings at 600 nm (OD_{600}).

DNA manipulations and molecular techniques. Total DNA from pure cultures of *Sphingomonas* sp. strain LH128 was extracted using the Ultra Clean DNA Isolation Kit (MoBio, Carlsbad, CA) following the manufacturer's recommendations or using standard methods (Sambrook et al. 1990) when a higher DNA concentration was needed. Plasmid DNA extractions, restriction enzyme digestions, ligations, transformations, sequencing and agarose gel electrophoresis were carried out using standard methods (Sambrook et al. 1990).

Polymerase chain reaction (PCR) and primer design. PCR primers RHDA1f-F (5'-CACCGCGGCAACCAGAT –3') and RHDA2f-R (5'- ACCATGGTATAGGTCCA-3') were constructed based upon conserved nucleic acid alignments of the initial dioxygenase from *Sphingomonas yanoikuyae* strain B1 (EF152282) *Novosphingobium aromaticivorans* strain F199 (AF079317) and *Sphingomonas* sp. strain CHY-1 (AJ633551) using Clustal X software (Thompson et al. 1997). All PCR reactions were carried out using PCR Master Mix (Abgene, Surrey, UK) and were performed in a programmable T-Gradient Thermocycler (Biometra, Göttingen, Germany). PCR products were purified and cloned into either the pDrive or pGEMT-easy plasmids.

Construction of plasmids for protein overexpression. Construction of the plasmids used in this study involved multiple PCR amplifications and cloning steps. The *phnA1fA2f* fragment (2048 bp) was amplified by PCR with the primers pairs: 5'- *CATATG*AATGGATCGTCGG -3' and 5'- *AAGCTT*GATCGAATTTGCTTATGCG -3', introducing NdeI and HindIII sites (italics) at the ends of the amplicon. The PCR amplicon was cloned into pDrive, sequenced, then subcloned into the NdeI and HindIII site of expression vector pET30f (Novagen, San

Diego, CA). The *phnA1fA2f* pair of genes was also transferred into pVLT31 (de Lorenzo et al. 1993) as a XbaI - HindIII fragment from pET30f*phnA1fA2f*. These constructs were transformed into *E. coli* BL21(DE3) for expression analysis.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Bacterial cells were pelleted by centrifugation and washed with 10 ml ice-cold phosphate buffer (140 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM NaH_2PO_4, pH 7.4). 1 ml of ice-cold phosphate buffer was added to the pellet and 550 µl of the suspension was subjected to sonication on ice for 20 s (5 s pulse interval; 40% of maximum amplitude). After centrifugation the supernatant and the pellet were mixed with an equal volume of loading solution. SDS-PAGE was performed on 13.3 % polyacrylamide mini gels. After electrophoresis, protein staining was performed with Coomassie brilliant blue R-250.

Dioxygenase overexpression and in vivo assays. Strains BL21(DE3)(pET30f*phnA1fA2f*) or BL21(DE3)(pVLT31*phnA1fA2f*) complemented with pEB431, carrying ferredoxin (*phnA3*) and ferredoxin reductase (*phnA4*) genes from *Sphingomonas* sp. strain CHY-1 (Demaneche et al. 2004), were grown overnight in 5 ml LB medium with the suitable antibiotics. This culture was used to inoculate 25 ml LB medium (0.1% vol/vol), which was incubated at 37°C until an OD_{600} of 0.5. IPTG was added to a final concentration of 0.5 mM. The cells were further incubated overnight at 25°C. For in vivo assays, cells were centrifuged, washed and resuspended to an OD_{600} of approximately 2 in M9 medium (Sambrook et al. 1990) containing 0.2% glucose. Cells (12 ml) overexpressing PhnA1fA2f, PhnA3 and PhnA4 were incubated overnight at 25 °C with 2 ml silicone oil containing 400 µM of each tested substrate.

GC-MS analysis of PAH oxidation products. Water-soluble products resulting from PAH oxidation were extracted from the aqueous phase of bacterial suspension by using columns filled with reverse phase-bonded silica (Upti-clean C18U, 0.5 g, Interchim, Montluçon, France). Columns were washed with 10 ml water then eluted with 1 ml ethyl acetate. The solvent was dried over sodium sulphate and evaporated under nitrogen gas. The dried extracts were then dissolved in 100 or 200 µl acetonitrile, before being derivatized with *N,O-bis*(trimethylsilyl)trifluoroacetamide containing trimethylchlorosilane (BSTFA) or *n*-butylboronate (NBB). In order to quantify the dihydrodiols formed upon incubation of BL21(DE3)(pET30f*phnA1fA2f*) recombinant cells with PAHs, 2,3-dihydrobiphenyl (Sigma-Aldrich, St. Louis, MO) was added to 0.1 µM final concentration in the aqueous phase prior to solid phase extraction, and was used as an internal standard. After derivatization and GC-MS analysis, NBB dihydrodiol derivates were quantified on the basis of peak area using a calibration curve generated by analysing known amounts of 3,4-phenanthrenedihydrodiol. GC-MS analysis of trimethylsilyl derivatives was carried out as previously described (Jouanneau et al. 2006). NBB derivatives were separated on MDN-12 capillary column (30 m, 0.25 mm internal diameter; Supelco) using helium as carrier gas at 1 ml/min. The oven temperature was held at 75°C for 1 min, then increased to 300°C at a rate of 14°C min^{-1}, and held at 300°C for 8 min. The mass spectrometer was operated in the selected ion-monitoring mode, selecting *m/z* values corresponding to the expected masses (M^+) of the dihydrodiol derivatives.

DNA and protein sequence analysis. Sequence analysis was performed using the DNASTAR software package (Lasergene Inc., Madison, WI*)*. The BLAST search tool was used for homology searches (Altschul et al. 1997). Multiple alignments were produced using the DNASTAR software.

Nucleotide sequence accession numbers. The nucleotide sequences described in this report have been deposited in the Genbank database under accession number EU024111 and EU024112 for the salicylate 1-hydroxylase and lower pathway enzymes and the terminal dioxygenase, respectively.

RESULTS

Cloning and sequence analysis of genes encoding a terminal dioxygenase.

Sphingomonas sp. strain LH128 has been studied for its ability to degrade three-ring azaarenes in cometabolism with phenanthrene but no genetic analysis was undertaken (van Herwijnen et al. 2004). In order to detect genes potentially involved in the initial attack of PAHs, a PCR strategy was chosen. The genes involved in phenanthrene oxidation by strain LH128 were expected to display some similarity with counterparts already described in other phenanthrene-degrading *Sphingomonas* species. Based on sequence similarities between a conserved catabolic gene cluster encoding genes of central metabolism from *Novosphingobium aromaticivorans* strain F199, *Sphingomonas* sp. strain CHY-1, *Sphingobium yanoikuyae* strain B1 and *Sphingomonas* sp. strain LH128 (GenBank accession number EU024111), we hypothesized that the genes encoding the terminal component of the initial dioxygenase from strain LH128 showed conserved sequences and could be amplified by PCR using primers RHDA1f-F and RHDA2f-R. A fragment of 2048 bp was obtained with genomic DNA from *Sphingomonas* sp. strain LH128 as template. The encoded proteins (PhnA1fA2f) shared 99%, 78%, 78% identity (α subunit) and 98%, 70% and 63% (β subunit) with counterparts from *Novosphingobium aromaticivorans* F199, *Sphingobium yanoikuyae* B1, and *Sphingomonas* sp. strain CHY-1 respectively. Since the counterparts of the *Sphingomonas* sp. strain LH128 isolated genes have been shown to be involved in the initial attack of their respective substrate, the genes were called *phnA1fA2f* (substrate phenanthrene, see below). Here we present functional data regarding a ring hydroxylating dioxygenase closely related to BphA1fA2f from strain F199 for which no functional data are available.

Functional expression of PhnA1fA2f in *E. coli*.

In order to investigate the substrate range of PhnA1fA2f, the corresponding genes were PCR-amplified and cloned into the expression vector pET30f. The resulting construction was

introduced into *E. coli* BL21(DE3) for SDS-PAGE analysis of IPTG-induced proteins. The cells overproduced two polypeptides with the expected size of 50,000 Da and 20,000 Da (Fig. 1). However, the proteins were mainly insoluble (inclusion bodies) and recombinant cells did not show detectable oxygenase activity. The *phnA1fA2f* sequence was therefore subcloned behind the P*tac* promoter into the broad host-range vector pVLT31 (de Lorenzo et al. 1993) and introduced into *E. coli* BL21(DE3). When induced with IPTG, the recipient cells produced appreciable levels of 50-kDa and 20-kDa polypeptides, which appeared to form a soluble recombinant protein (Fig. 1). In order to provide the terminal oxygenase component with an appropriate electron transport chain, plasmid pEB431, expressing *phnA3* and *phnA4* (Demaneche et al. 2004) was co-transformed into *E. coli* BL21(DE3). PhnA3 and PhnA4 formed with PhnA1fA2f a competent enzymatic complex in the *E. coli* host as proved by indigo formation compared to cells lacking pEB431.

Substrate range of PhnA1fA2f.

The recombinant *E. coli* strain producing PhnA1f, PhnA2f, PhnA3 and PhnA4 was incubated overnight separately with several representative PAHs, dibenzo-*p*-dioxin and PCBs. The water-soluble products released into the culture medium were extracted and analysed by GC-MS (Table 1) as described elsewhere (Krivobok et al. 2003). Since *Sphingomonas* sp. strain LH128 is able to use fluorene, dibenzothiophene, and anthracene in cometabolic degradation (Bastiaens et al. 2000) we tested whether PhnA1fA2f was responsible for the initial attack on these compounds. The relative activity toward each PAH was calculated from the GC-MS selected ion monitoring peak areas of the NBB derivatives compared to an internal standard (2,3-dihydroxybiphenyl). Naphthalene was the preferred substrate (100 %), then phenanthrene (43.3 %), biphenyl (31.8 %) and anthracene (28.7 %) were converted at significant but lower rates to the corresponding dihydrodiols. Since naphthalene cannot support growth of strain LH128, the genes were called *phnA1fA2f.* Interestingly, PhnA1fA2f was also able to oxidize

the heteroatomic analogues of fluorene i.e. dibenzofuran, dibenzothiophene and carbazole. Strain LH128 is able to degrade fluorene in cometabolism with phenanthrene as the main carbon source (Bastiaens et al. 2000). However, only traces of fluorenedihydrodiol were detected after *n*-butylboronate (NBB) derivatization, a result that did not account for the substantial cometabolic activity of strain LH128 towards fluorene. GC-MS analysis of TMS derivatives of fluorene oxidation products allowed identification of a large peak of monohydroxyfluorene (RT 16.262 min) with significant fragment ions at m/z 254 (100), 239 (95), 165 (80), 152 (19), 73 (31). Moreover dihydroxyfluorene (RT: 17.577 min; 342 (36), 327 (4), 253 (33), 223 (7), 73 (100) was detected, which most likely resulted from hydroxylation of fluorene on two non-adjacent carbon atoms because it could not be detected by NBB derivatization. Detection of monohydroxycarbazole (RT: 17.092 min; *m/z* 255 (100), 239 (57), 224 (47), 166 (11)) after BSTFA derivatization suggests that PhnA1fA2f transforms carbazole to an unstable dihydrodiol by lateral dioxygenation. Fluoranthene was also probably oxidized to an unstable dihydrodiol, which was further converted to 8-hydroxyfluoranthene, since the TMS derivative had the same GC-MS characteristics as those reported for the oxidation product of fluoranthene by the PhnI dioxygenase from strain CHY-1 (RT: 20.365 min; *m/z* 290 (100), 275 (55), 215 (15), 201 (19), 200 (18), 189 (30)) (Jouanneau et al. 2006). Since PhnA1fA2f displayed a relatively high activity towards biphenyl (31.8%), we tested whether PhnA1fA2f could oxidize halogenated biphenyls. Monochlorinated biphenyls such as 2-chlorobiphenyl (relative activity 6.6 %) and 4-chlorobiphenyl (6.1 %) were oxidized to corresponding dihydrodiols, but 2,3-dichlorobiphenyl was not. Moreover PhnA1fA2f was able to perform lateral oxygenation of dibenzo-*p*-dioxin. Interestingly, the four-ring PAH benz[*a*]anthracene was transformed into two compounds with masses and retention times consistent with those of two dihydrodiol isomers. These products most likely bear hydroxyls in positions 1,2 and 10,11 since the homologous enzyme from strain CHY-1 preferentially hydroxylated benz[*a*]anthracene on these carbons (Jouanneau et al, 2006). Chrysene and

pyrene were oxidized to *cis*-3,4-dihydroxy-3,4-dihydrochrysene and *cis*-4,5-dihydroxy-4,5-dihydropyrene based on the retention times of the purified dihydrodiols obtained with Phn1 (Jouanneau et al. 2006) and Pdo1 (Krivobok et al. 2003), respectively. The 5-ring PAH benz[*a*]pyrene did not produce any detectable dihydrodiol under identical conditions. These data demonstrate that the PhnA1fA2f terminal oxygenase from strain LH128 displays exceptionally broad substrate specificity towards a wide range of aromatic hydrocarbons.

DISCUSSION

Sphingomonads are known to degrade a large spectrum of pollutants, ranging from mono- and polycyclic hydrocarbons (Pinyakong et al. 2000; Story et al. 2001) to naphthalene sulfonate (Stolz 1999), dibenzo-*p*-dioxin (Armengaud et al. 1998; Hong et al. 2002), and methylated PAHs (Dimitriou-Christidis et al. 2007; Zylstra and Kim 1997). Most known degradation pathways of homocyclic PAHs start with the formation of a dihydroxy PAH by hydroxylation of two adjacent carbon atoms. This step is catalysed by dioxygenase enzymes with relaxed substrate specificity, which determines the substrate range of the organism. The compounds are further degraded to a limited number of intermediates such as *o*-phthalic acid or salicylic acid, and then via *ortho* or *meta* cleavage to tricarboxylic acid cycle intermediates. The genes for aromatic hydrocarbon degradation by sphingomonads are quite different from those found in other genera both in terms of nucleotide sequence and of gene order (Pinyakong et al. 2003a). This unique gene arrangement, which is remarkably conserved among strains of various origins, contrasts with that found in other degraders, such as pseudomonads.

To date only a few sphingomonads' initial dioxygenases have been well characterized: BphA1fA2f from strain B1 (Ni Chadhain et al. 2007) and PhnI (Jouanneau et al. 2006) from strain CHY-1. BphA1fA2f from strain F199 has been identified but further investigation to assess its catalytic abilities is missing. While the initial dioxygenases from strains LH128 and CHY-1 are related (78 % identity), strain CHY-1 is able to grow on chrysene as the sole source of carbon (Willison 2004) while strain LH128 cannot use chrysene as a substrate.

Likewise, the dioxygenases from strains CHY-1 and B1 show apparent differences of substrate specificity despite sharing an almost identical structure (Demaneche et al. 2004; Jouanneau et al. 2006; Ni Chadhain et al. 2007). These observations suggest that there exists a pool of highly conserved multicomponent dioxygenases in sphingomonads, with subtle structural variations that would appear to be responsible for differences in selectivity toward PAHs (Fig. 2). Six homologues to both large and small substrate binding components of ring hydroxylating dioxygenases were identified (*bphA1*$_{[a-f]}$-*bphA2*$_{[a-f]}$) in *Sphingomonas yanoikuyae* strain B1 (Zylstra and Kim 1997), *Sphingomonas* sp. strain P2 (Pinyakong et al. 2003b) and *Novosphingobium aromaticivorans* strain F199 (Romine et al. 1999). Since the genes isolated from strain LH128 display high homologies to catabolic genes from these species, one can expect to find the missing dioxygenase encoding genes in strain LH128 (*bphA1*$_{[a,b.e]}$-*bphA2*$_{[a,b,e]}$). Moreover, studies of *Sphingomonas* population structures of several PAH-contaminated soils by PCR-DGGE revealed that soils with the highest phenanthrene concentrations showed the lowest *Sphingomonas* diversity (Leys et al. 2004). This indicates that *Sphingomonas* species share a set of dioxygenases that probably originated as phenanthrene catabolic genes and then, by duplication, evolved to degrade different substrates. For instance, the enzymes involved in the initial step of PAH degradation exhibit a greater variety than those involved in the catabolism of central metabolites such as salicylate (Table 2). The overall identities between salicylate 1-hydroxylases are higher than the identities between the respective ring-hydroxylating dioxygenases of the different strains. This clearly indicates that the enzymes involved in the upper PAH catabolic pathways have a more relaxed substrate specificity then the enzymes involved in the lower pathway.

When overexpressed in *E. coli* BL21(DE3), PhnA1fA2f was found to be responsible for the oxidation of low and high molecular weight PAHs, dibenzo-*p*-dioxin and monochlorinated biphenyls but not 2,3-dichlorobiphenyl. Traces of carbazole dihydrodiol were detected after NBB derivatization, but monohydroxycarbazole was abundant. Resnick et al. (1993) reported

the formation of monohydroxycarbazole, possibly as a result of dehydration of unstable dihydrodiols. Phenanthrene (43.3 %), biphenyl (31.8 %) and anthracene (28.7 %) were transformed into high levels of the corresponding *cis*-dihydrodiols. Oxidation products of benz[a]anthracene, chrysene and pyrene (Table 1) were also identified in contrast with naphthalene dioxygenases whose selectivity is limited to only two and three ring PAHs (Ferraro et al. 2004; Gakhar et al. 2005; Kauppi et al. 1998). The five ring PAH benz[a]pyrene did not give any detectable products. This suggests that benz[a]pyrene probably does not fit into the catalytic pocket of PhnA1fA2f.

The catalytic pocket of the ring-hydroxylating dioxygenase from *Sphingomonas* sp. strain CHY-1 has been recently described on the basis of its crystal structure, and the amino acids lining the catalytic pocket were identified (Jakoncic et al. 2007a; 2007b). These residues are conserved in the enzymes from *Sphingomonas* sp. strain LH128, *Novosphingobium aromaticivorans* strain F199 and, with only two substitutions, in *Sphingobium yanoikuyae* strain B1 (Jakoncic et al. 2007a) (data not shown), suggesting that the topology of the substrate binding pocket is almost identical. However, these structural resemblances do not explain the differences in substrate specificity of the dioxygenases. The crystal structure of the ring hydroxylating dioxygenase from strain CHY-1 showed that the entrance of the catalytic pocket is covered by two flexible loops L1 and L2, exposed to the solvent. These loops are predicted to control the substrate's access to the catalytic pocket (Jakoncic et al. 2007b). Since the sequence of these loops is only partly conserved in the LH128 enzyme (83 % and 63 % identities for L1 and L2, respectively), it seems plausible that these structural differences may be responsible for the lower activity of the LH128 dioxygenase towards high molecular weight PAHs and its inability to oxidize benz[a]pyrene. The effects on the catalytic activity of residue substitutions in the active site have been well investigated in the case of naphthalene dioxygenase and biphenyl dioxygenases (Parales 2003; Parales et al. 1999; 2000a; 2000b), but the effect of substitutions outside the catalytic pocket is less well

documented (Furukawa et al. 2004; Zielinski et al. 2003; 2006). Our results indicate that residues in the loops at the entrance of the catalytic pocket are potentially interesting targets for mutagenesis as a means to better understand the structural determinants of selectivity.

In summary, we identified the genes encoding the dioxygenase responsible for the initial attack on various PAHs by *Sphingomonas* sp. strain LH128 and expressed them in *E. coli*. The dioxygenase PhnA1fA2f was closely related to BphA1fA2f from *Novosphingobium aromaticivorans* strain F199 and, to a lower extent, to PhnI from *Sphingomonas* sp. strain CHY-1 and BphA1fA2f *Sphingobium yanoikuyae* strain B1. Characterization of the activity of the dioxygenase cloned in *E. coli* showed significant differences in catalytic activity compared to the proteins PhnI from strain CHY-1 and BphA1fA2f from strain B1. This indicates that small variations in amino acid sequence outside the catalytic pocket can have substantial impact on dioxygenase selectivity. Significantly, PhnA1fA2f was able to oxidize the four ring PAH benz[a]anthracene and yielded two dihydrodiols.

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Table 1. PAH selectivity of PhnA1A2f from *Sphingomonas* sp. LH128 as expressed in *E. coli*.

Substrate[a]	Products	Molecular mass of NBB derivative	Retention Time (min)	Relative activity (%)[b]	µM Diol/ h mg Prot[c]
Biphenyl	*cis*-2,3-Dihydroxy-2,3-dihydrobiphenyl	254	16.199	31.8	0.097
Naphthalene	*cis*-1,2-Dihydroxy-1,2-dihydronaphthalene	228	14.479	100	0.306
Phenanthrene	*cis*-3,4-Dihydroxy-3,4-dihydrophenanthrene	278	19.239	43.3	0.133
Fluorene[d]	Fluorenedihydrodiol	266	16.043	0.9	0.003
	Monohydroxyfluorene		15.836	N.D.	N.D.
	Monohydroxyfluorene		16.163	N.D.	N.D.
	Dihydroxyfluorene		17.577	N.D.	N.D.
Anthracene	*cis*-1,2-Dihydroxy-1,2-dihydroanthracene	278	19.668	28.7	0.088
Fluoranthene[d]	Fluoranthene-diol	302	21.973	0.1	2.686E-04
	Monohydroxyfluoranthene		20.365	N.D.	N.D.
Benz[*a*]anthracene	*cis*-1,2-Benz[*a*]anthracenedihydrodiol	328	23.563	5.5	0.017
	cis-10,11-Benz[*a*]anthracenedihydrodiol	328	24.513	4.4	0.014
Pyrene	*cis*-4,5-Dihydroxy-4,5-dihydropyrene[e]	302	21.721	Traces	Traces
Chrysene	*cis*-3,4-Dihydroxy-3,4-dihydrochrysene[f]	328	24.801	0.3	9.452E-04
Benzo-*p*-dioxin	Benzo-*p*-dioxindihydrodiol	284	17.936	2.4	0.007
Dibenzothiophene	Dibenzothiophenedihydrodiol	284	18.949	12.6	0.039
Dibenzofuran	*cis*-1,2-Dihydroxy-1,2-dihydrodibenzofuran[g]	268	17.181	17.2	0.053
	Dibenzofurandihydrodiol	268	17.611	5.3	0.016

[a] Acenaphthene, benz[*a*]pyrene, benzo[*k*]fluoranthene, benzo[*e*]fluoranthene and 2,3'-dichlorobiphenyl did not give any detectable products.

[b] Calculated from the GC-MS-selected ion monitoring peak areas of the NBB derivatives of the products formed after 24 h of incubation and expressed as percentages of relative activity (with respect to the maximum obtained with naphthalene as substrate). The values are averages of two separate determinations.

[c] Calculated from the GC-MS-selected ion monitoring peak areas of the NBB derivatives of the products formed after 24 h of incubation per mg of total proteins. The values are averages of two separate determinations.

[d] Dihydrodiols appear to be unstable and are spontaneously transformed to the corresponding monohydroxylated compounds by dehydration as detected after BSTFA derivatization. Therefore no relative activity is determined for these substrates (N.D.).

[e] Same retention time and mass spectrum as *cis*-4,5-dihydroxy-4,5-dihydropyrene produced by Pdo1 (Krivobok et al. 2003).

[f] Same retention time and mass spectrum as *cis*-3,4-dihydroxy-3,4-dihydrochrysene produced by Phn1 (Demaneche et al. 2004).

[g] Same retention time and mass spectrum as oxidation products of dibenzofuran from Phn1 (Jouanneau et al. unpublished data).

Table 2. Comparisons amongst salicylate 1-hydroxylase and the initial ring-hydroxylating dioxygenase from *Sphingomonas* sp. strain P2, *Novosphingobium aromaticivorans* strain F199, *Sphingomonas* sp. strain LH128, *Sphingobium yanoikuyae* strain B1 and *Sphingomonas* sp. strain CHY1.

Ring-hydroxylating dioxygenase	BphA1 P2[a]	BphA1f F199[a]	PhnA1f LH128[a]	BphA1f B1[a]	PhnA1a CHY-1[a]
BphA1f F199 (YP_001165670)		100	99	78	78
PhnA1f LH128 (EU024112)			100	77	77
BphA1f B1 (2GBW_A)				100	99
PhnA1a CHY-1 (2CKF_A)					100
Salicylate 1-hydroxylase	**BphA1c P2**	**BphA1c F199**	**PhnA1c LH128**	**BphA2c B1**	**PhnA1b CHY-1**
BphA1c P2 (BAC65426)	100	79	79	96	79
BphA1c F199 (NP_049213)		100	97	76	97
PhnA1c LH128 (EU024111)			100	76	76
BphA2c B1 (ABM79781)				100	76
PhnA1b CHY-1 (CAG17582)					100

[a] Amino acid identity to their respective counterparts is shown.

FIGURE LEGENDS

Figure 1. Detection of PhnA1fA2f overproduced in *E. coli* BL21(DE3). A: *E. coli* BL21(DE3)(pET30f*phnA1fA2f*) overproduced high amounts of 50-kDa and 20-kDa that were mainly insoluble. B: *E. coli* BL21(DE3) harbouring pVLT31*phnA1fA2f* produced soluble proteins. However the β subunit could not be detected by SDS-PAGE. *E. coli* BL21(DE3) harbouring pET30f (A) or pVLT31 (B) lacking the *phnA1fA2f* insert were used as controls (C). Protein extracts from 4 clones induced by IPTG are shown (lanes 1-4). Molecular mass (kDa): M1: Prestained PAGE Ruler (Fermentas, St. Leon Rot, Germany), M2: Prestained Protein Marker, Broad Range (New England Biolabs, Ipswich, MA). The arrows show the PhnA1fA2f subunits.

Figure 2. [Modified and updated after Pinyakong et al. (2003a)]. Comparison of the conserved catabolic operon from *Novosphingobium aromaticivorans* strain F199 (Romine et al. 1999), *Sphingobium* sp. strain P2 (Pinyakong et al. 2003b), *Sphingobium yanoikuyae* strain B1 (Zylstra and Kim 1997; Ni Chadhain et al. 2007), *Sphingomonas* sp. strain CHY-1 (Demaneche et al. 2004), *Sphingomonas* sp. strain HV3 (Yrjala et al. 1994) and *Sphingomonas chungbukensis* strain DJ77 (Kim et al. 2000). The protein sequence identities to the counterparts from strain F199 are indicated.

562 Fig. 1

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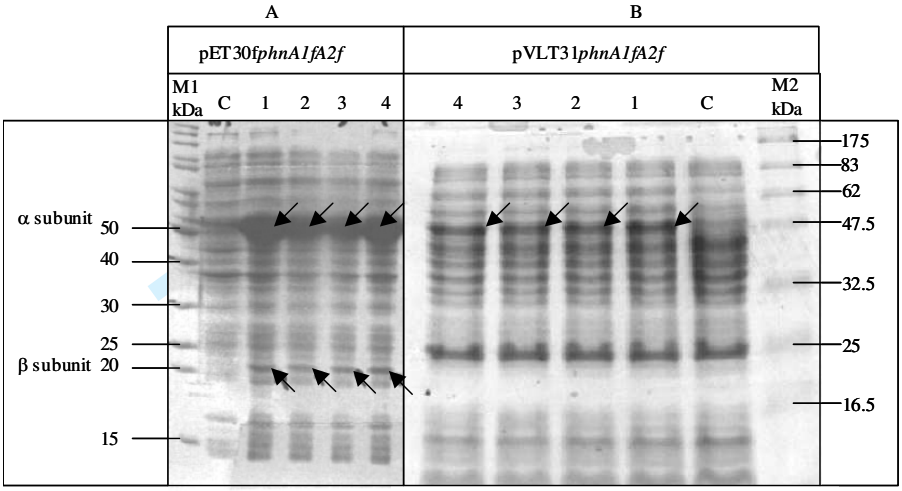

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Fig. 2