Appl Microbiol Biotechnol (2009) 83:465–475
DOI 10.1007/s00253-009-1858-2

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

Luc Schuler · Yves Jouanneau · Sinéad M. Ní Chadhain · Christine Meyer · Maria Pouli · Gerben J. Zylstra · Pascal Hols · Spiros N. Agathos

Received: 4 October 2008 / Revised: 3 January 2009 / Accepted: 5 January 2009 / Published online: 27 January 2009

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 PAH dioxygenase from *Novosphingobium aromaticivorans* strain F199, *Sphingomonas* sp. strain CHY-1, and *Sphingobium yanoikuyae* strain B1, respectively. When overexpressed in *Escherichia 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.

Keywords Bioremediation · *Meta*-cleavage operon genes · Indigo formation · Rieske nonheme iron oxygenase

L. Schuler · M. Pouli · S. N. Agathos (✉)
Unité de Génie Biologique, Institut des Sciences de la Vie,
Université Catholique de Louvain,
Place Croix du Sud, 2/19,
1348 Louvain-la-Neuve, Belgium
e-mail: spiros.agathos@uclouvain.be

Y. Jouanneau · C. Meyer
CEA, iRTSV, Laboratoire de Chimie et Biologie
des Métaux and UMR 5249 CNRS, Université J. Fourier,
38054 Grenoble Cedex 9, France

S. M. Ní Chadhain · G. J. Zylstra
Biotechnology Center for Agriculture and the Environment,
Cook College, Rutgers University,
New Brunswick, NJ 08901-8520, USA

P. Hols
Unité de Génétique, Institut des Sciences de la Vie,
Université Catholique de Louvain,
Place Croix du Sud 5,
1348 Louvain-la-Neuve, Belgium

Introduction

Polycyclic aromatic hydrocarbons (PAHs) 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 monocyclic aromatic hydrocarbons and PAHs (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 toward 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 catalyzed 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; Jouanneau 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, b; 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 harbors 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 harbor 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 component is generally a heterohexamer $\alpha_3\beta_3$ 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, *N. 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 genes encoding the oxygenase component of the ring-hydroxylating dioxygenase from *Sphingomonas* sp. strain LH128 were cloned and its function toward a variety of substrates was investigated. This newly characterized dioxygenase is shown to be closely related to BphA1fA2f from *N. aromaticivorans* strain F199 (98% identities) but to display significant differences in catalytic behavior 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, USA). 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, USA).

Bacterial strains, plasmids, and media

Sphingomonas sp. strain LH128 was kindly provided by Vlaamse Instelling voor Technologisch Onderzoek (VITO, Belgium). *Escherichia coli* Top10 (Invitrogen, Carlsbad, CA, USA) was used as the recipient strain in all cloning experiments. *E. coli* BL21(DE3) was used for gene expression analysis. Polymerase chain reaction (PCR) amplicons were cloned into pDrive (Qiagen, Valencia, CA, USA) while pET30f (Novagen, San Diego, CA, USA) and pVLT31 (de Lorenzo et al. 1993) were used as expression vectors. 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. Luria–Bertani (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, USA) 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 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) *N. 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 pGEM-T 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 (2,048 bp) was amplified by PCR with the primers pairs: 5′-*CATATG*AATGGATCGTCGG-3′ and 5′-AAGCTTGATCGAATTTGCTTATGCG-3′, introducing the *Nde*I and *Hin*dIII sites (in italics) at the ends of the amplicon. The PCR amplicon was cloned into pDrive, sequenced, and then subcloned into the *Nde*I and *Hin*dIII sites of the expression vector pET30f (Novagen, San Diego, CA, USA). The *phnA1fA2f* pair of genes was also transferred into pVLT31 (de Lorenzo et al. 1993) as a *Xba*I–*Hin*dIII fragment from pET30f*phnA1fA2f*. These constructs were transformed into *E. coli* BL21(DE3) for expression analysis.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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). One milliliter 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. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 13.3% polyacrylamide minigels. 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. Isopropyl β-D-1-thiogalactopyranoside (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 sulfate 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-dihydroxybiphenyl (Sigma-Aldrich, St. Louis, MO, USA) 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 gas chromatography–mass spectrometry (GC-MS) analysis, NBB dihydrodiol derivates were quantified on the basis of peak area using a calibration curve generated by analyzing known amounts of 3,4-phenanthrene dihydrodiol. GC-MS analysis of trimethylsilyl (TMS) 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, Madison, WI, USA). 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 numbers 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 *N. 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 2,048 bp was obtained with genomic DNA from *Sphingomonas* sp. strain LH128 as template. The encoded proteins (PhnA1fA2f) shared 99%, 78%, and 78% (α subunit) and 98%, 70%, and 63% (β subunit) identity with counterparts from *N. 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 BphA1-fA2f from strain F199 for which no functional data are available.

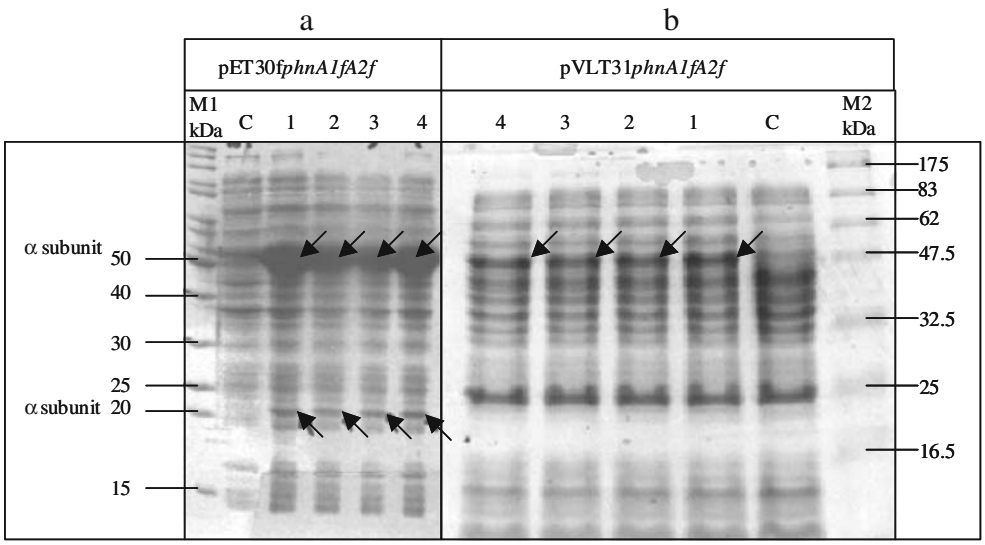

Fig. 1 Detection of PhnA1fA2f overproduced in *E. coli* BL21(DE3). **a** *E. coli* BL21(DE3)(pET30f*phnA1fA2f*) overproduced high amounts of 50 and 20 kDa that were mainly insoluble. **b** *E. coli* BL21(DE3) harboring pVLT31*phnA1fA2f* produced a soluble recombinant protein. However, the β subunit could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

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 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 polypeptide (Fig. 1). Although the 20-kDa subunit was barely detectable by SDS-PAGE, a significant amount of active enzyme appeared to be produced in a soluble form. 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 cotransformed 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 polychlorinated biphenyls. The water-soluble products released into the culture medium were extracted and analyzed 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).

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

Substrate[a]	Products	Molecular mass of NBB derivative	RT (min)	Relative activity (%)[b]	μM Diol/h mg Prot[c]
Biphenyl	*cis*-2,3-Dihydroxy-2,3-dihydrobiphenyl	254	16.20	31.8	0.097
Naphthalene	*cis*-1,2-Dihydroxy-1,2-dihydronaphthalene	228	14.48	100	0.306
Phenanthrene	*cis*-3,4-Dihydroxy-3,4-dihydrophenanthrene	278	19.24	43.3	0.133
Fluorene[d]	Fluorenedihydrodiol	266	16.04	0.9	0.0030
	Monohydroxyfluorene		15.84	N.D.	N.D.
	Monohydroxyfluorene		16.16	N.D.	N.D.
	Dihydroxyfluorene		17.58	N.D.	N.D.
Anthracene	*cis*-1,2-Dihydroxy-1,2-dihydroanthracene	278	19.67	28.7	0.088
Fluoranthene[d]	Fluoranthene-diol	302	21.97	0.1	0.00027
	Monohydroxyfluoranthene		20.37	N.D.	N.D.
Benz[*a*]	*cis*-1,2-Benz[*a*]anthracenedihydrodiol	328	23.56	5.5	0.017
anthracene	*cis*-10,11-Benz[*a*]anthracenedihydrodiol	328	24.51	4.4	0.014
Pyrene	*cis*-4,5-Dihydroxy-4,5-dihydropyrene[e]	302	21.72	Traces	Traces
Chrysene	*cis*-3,4-Dihydroxy-3,4-dihydrochrysene[f]	328	24.80	0.3	0.00095
Benzo-*p*-dioxin	Benzo-*p*-dioxindihydrodiol	284	17.94	2.4	0.007
Dibenzothiophene	Dibenzothiophenedihydrodiol	284	18.95	12.6	0.039
Dibenzofuran	*cis*-1,2-Dihydroxy-1,2-dihydrodibenzofuran[g]	268	17.18	17.2	0.053
	Dibenzofurandihydrodiol	268	17.61	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 RT and mass spectrum as *cis*-4,5-dihydroxy-4,5-dihydropyrene produced by Pdo1 (Krivobok et al. 2003)

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

[g] Same RT and mass spectrum as oxidation products of dibenzofuran from Phn1 (Jouanneau, unpublished data)

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 analogs 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 fluorene dihydrodiol were detected after NBB derivatization, a result that did not account for the substantial cometabolic activity of strain LH128 toward fluorene. GC-MS analysis of TMS derivatives of fluorene oxidation products allowed the identification of a large peak of monohydroxyfluorene (retention time [RT]= 16.26 min) with significant fragment ions at *m*/*z* 254 (100), 239 (95), 165 (80), 152 (19), 73 (31). Moreover, dihydroxyfluorene [RT=17.58 min; *m*/*z* 342 (36), 327 (4), 253 (33), 223 (7), 73 (100)] was detected, which most likely resulted from hydroxylation of fluorene on two nonadjacent carbon atoms because it could not be detected by NBB derivatization. Detection of monohydroxycarbazole [RT=17.09 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=20.37 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 toward 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 RTs 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 RTs of the purified dihydrodiols obtained with Phn1 (Jouanneau et al. 2006) and Pdo1 (Krivobok et al. 2003), respectively. The five-ring PAH benzo[*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 toward a wide range of aromatic hydrocarbons.

Discussion

Sphingomonads are known to degrade a large spectrum of pollutants, ranging from monocyclic 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 catalyzed 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 from strain CHY-1 (Jouanneau et al. 2006). 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 homologs to both large and small subunits of ring-hydroxylating dioxygenases were identified (*bphA1*$_{[a–f]}$–*bphA2*$_{[a–f]}$) in *Sphingomonas yanoikuyae* strain B1 (Zylstra and Kim 1997) and *N. 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

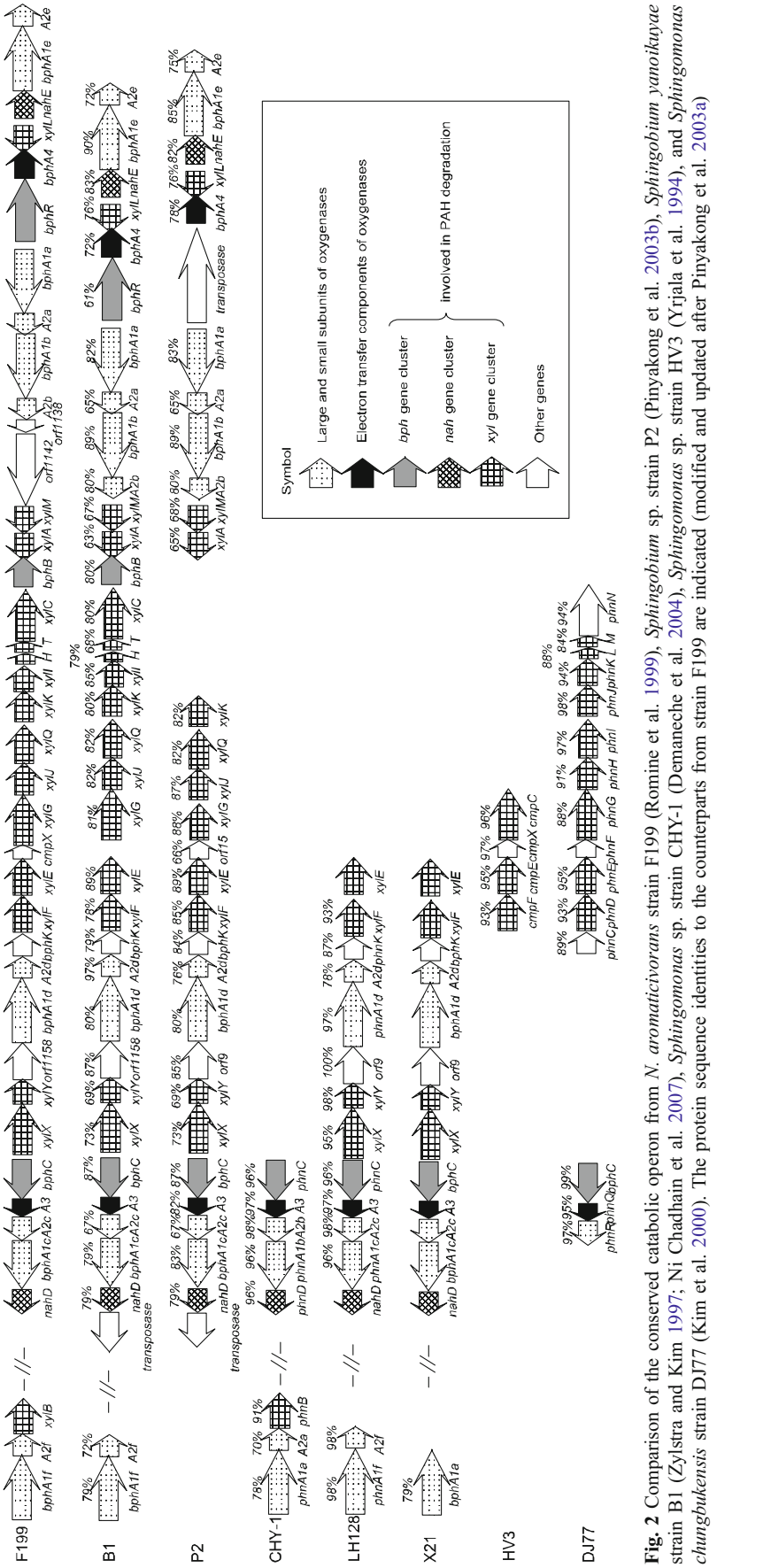

Fig. 2 Comparison of the conserved catabolic operon from *N. aromaticivorans* strain F199 (Romine et al. 1999), *Sphingobium* sp. strain P2 (Pinyakong et al. 2003b), *Sphingobium yanoikuyae* strain B1 (Zylstra and Kim 1997; Ni Chadhain et al. 2007), *Sphingomonas* sp. strain CHY-1 (Demaneche et al. 2004), *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 (modified and updated after Pinyakong et al. 2003a)

Table 2 Comparisons amongst salicylate 1-hydroxylase and the initial ring-hydroxylating dioxygenase from *Sphingomonas* sp. strain P2, *N. 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]	Salicylate 1-hydroxylase	BphA1c P2	BphA1c F199	PhnA1c LH128	BphA2c B1	PhnA1b CHY-1
BphA1f F199 (YP_001165670)		100	99	78	78	BphA1c P2 (BAC65426)	100	79	79	96	79
PhnA1f LH128 (EU024112)			100	77	77	BphA1c F199 (NP_049213)		100	97	76	97
BphA1f B1 (2GBW_A)				100	99	PhnA1c LH128 (EU024111)			100	76	76
PhnA1a CHY-1 (2CKF_A)					100	BphA2c B1 (ABM79781)				100	76
						PhnA1b CHY-1 (CAG17582)					100

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

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–denaturing gradient gel electrophoresis 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 (Table 2).

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. 2005; Gakhar et al. 2005; Kauppi et al. 1998). The five-ring PAH benzo[*a*]pyrene did not give any detectable products. This suggests that benzo[*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, b). These residues are conserved in the enzymes from *Sphingomonas* sp. strain LH128, *N. 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 toward high-molecular-weight PAHs and its inability to oxidize benzo[*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, b), 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 *N. aromaticivorans* strain F199 and, to a lower extent, to PhnI from *Sphingomonas* sp. strain CHY-1 and BphA1fA2f from *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.

Our results are important in view of the potential usefulness of such bacterial dioxygenases in biocatalysis and, more specifically, in the chemoenzymatic synthesis of chiral catechol derivatives (Boyd et al. 2001). Recently, for instance, a bacterial biphenyl dioxygenase has served as a catalyst for chiral dihydroxylation of 2-chloroquinoline, 2-chloro-3-methylquinoline, and 2-chloro-6-phenylpyridine into the corresponding enantiopure *cis*-dihydrodiols (Boyd et al. 2008). Until now, the only PAH dioxygenase that has shown industrially scaleable potential is naphthalene dioxygenase by means of whole-cell biocatalysis in biphasic (aqueous/nonaqueous) media (McIver et al. 2008). Still, the limited stability of heterologously overexpressed enzyme represents a major challenge; hence, whole cells of the host (*E. coli*) are proposed to meet the robustness required in such industrial applications (McIver et al. 2008).

Acknowledgements L.S. gratefully acknowledges the Fund for the Promotion of Research in Industry and Agriculture (F.R.I.A.), Belgium, for providing a doctoral fellowship. L.S. also wishes to thank the members of the Unit of Physiological Biochemistry (FYSA), Catholic University of Louvain, for their daily help and constructive remarks for many years. PH is a research associate at the Belgian National Fund for Scientific Research (FNRS).

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Armengaud J, Happe B, Timmis KN (1998) Genetic analysis of dioxin dioxygenase of *Sphingomonas* sp. strain RW1: catabolic genes dispersed on the genome. J Bacteriol 180:3954–3966

Bastiaens L, Springael D, Wattiau P, Harms H, deWachter R, Verachtert H, Diels L (2000) Isolation of adherent polycyclic aromatic hydrocarbon (PAH)-degrading bacteria using PAH-sorbing carriers. Appl Environ Microbiol 66:1834–1843

Boyd DR, Sharma ND, Allen CCR (2001) Aromatic dioxygenases: molecular biocatalysis and applications. Curr Opin Biotechnol 12:564–573

Boyd DR, Sharma ND, Sbircea L, Murphy D, Belhocine T, Malone JF, James SL, Allen CCR, Hamilton JTG (2008) Azaarene *cis*-dihydrodiol-derived 2,2′-bipyridine ligands for asymmetric allylic oxidation and cyclopropanation. Chem Commun 43:5535–5537

Bunz PV, Cook AM (1993) Dibenzofuran 4,4a-dioxygenase from *Sphingomonas* sp. strain RW1: angular dioxygenation by a three-component enzyme system. J Bacteriol 175:6467–6475

Butler CS, Mason JR (1997) Structure–function analysis of the bacterial aromatic ring-hydroxylating dioxygenases. Adv Microb Physiol 38:47–84

Cai M, Xun L (2002) Organization and regulation of pentachlorophenol-degrading genes in *Sphingobium chlorophenolicum* ATCC 39723. J Bacteriol 184:4672–4680

Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3:351–368

de Lorenzo V, Eltis L, Kessler B, Timmis KN (1993) Analysis of *Pseudomonas* gene products using *lacI*^{q}/*Ptrp-lac* plasmids and transposons that confer conditional phenotypes. Gene 123:17–24

Demaneche S, Meyer C, Micoud J, Louwagie M, Willison JC, Jouanneau Y (2004) Identification and functional analysis of two aromatic-ring-hydroxylating dioxygenases from a *Sphingomonas* strain that degrades various polycyclic aromatic hydrocarbons. Appl Environ Microbiol 70:6714–6725

Dimitriou-Christidis P, Autenrieth RL, McDonald TJ, Desai AM (2007) Measurement of biodegradability parameters for single unsubstituted and methylated polycyclic aromatic hydrocarbons in liquid bacterial suspensions. Biotechnol Bioeng 97:922–932

Feng X, Ou LT, Ogram A (1997) Plasmid-mediated mineralization of carbofuran by *Sphingomonas* sp. strain CF06. Appl Environ Microbiol 63:1332–1337

Ferraro DJ, Gakhar L, Ramaswamy S (2005) Rieske business: structure–function of rieske non-heme oxygenases. Biochem Biophys Res Commun 338:175–190

Fortnagel P, Harms H, Wittich RM, Krohn S, Meyer H, Sinnwell V, Wilkes H, Francke W (1990) Metabolism of dibenzofuran by *Pseudomonas* sp. strain HH69 and the mixed culture HH27. Appl Environ Microbiol 56:1148–1156

Fujii K, Satomi M, Morita N, Motomura T, Tanaka T, Kikuchi S (2003) *Novosphingobium tardaugens* sp. nov., an oestradiol-degrading bacterium isolated from activated sludge of a sewage treatment plant in Tokyo. Int J Syst Evol Microbiol 53:47–52

Furukawa K, Suenaga H, Goto M (2004) Biphenyl dioxygenases: functional versatilities and directed evolution. J Bacteriol 186:5189–5196

Gakhar L, Malik ZA, Allen CCR, Lipscomb DA, Larkin MJ, Ramaswamy S (2005) Structure and increased thermostability of *Rhodococcus* sp. naphthalene 1,2-dioxygenase. J Bacteriol 197:7222–7231

Happe B, Eltis LD, Poth H, Hedderich R, Timmis KN (1993) Characterization of 2,2′,3-trihydroxybiphenyl dioxygenase, an extradiol dioxygenase from the dibenzofuran- and dibenzo-p-dioxin-degrading bacterium *Sphingomonas* sp. strain RW1. J Bacteriol 175:7313–7320

Hong HB, Chang YS, Nam IH, Fortnagel P, Schmidt S (2002) Biotransformation of 2,7-dichloro- and 1,2,3,4-tetrachlorodibenzo-p-dioxin by *Sphingomonas wittichii* RW1. Appl Environ Microbiol 68:2584–2588

Jakoncic J, Jouanneau Y, Meyer C, Stojanoff V (2007a) The catalytic pocket of the ring-hydroxylating dioxygenase from *Sphingomonas* CHY-1. Biochem Biophys Res Commun 352:861–866

Jakoncic J, Jouanneau Y, Meyer C, Stojanoff V (2007b) The crystal structure of the ring-hydroxylating dioxygenase from *Sphingomonas* CHY-1. FEBS J 274:2470–2481

Johannesen H, Sorensen SR, Aamand J (2003) Mineralization of soil-aged isoproturon and isoproturon metabolites by *Sphingomonas* sp. strain SRS2. J Environ Qual 32:1250–1257

Johnsen AR, Wick LY, Harms H (2005) Principles of microbial PAH-degradation in soil. Environ Pollut 133:71–84

Jouanneau Y, Meyer C, Jakoncic J, Stojanoff V, Gaillard J (2006) Characterization of a naphthalene dioxygenase endowed with an exceptionally broad substrate specificity toward polycyclic aromatic hydrocarbons. Biochemistry 45:12380–12391

Kauppi B, Lee K, Carredano E, Parales RE, Gibson DT, Eklund H, Ramaswamy S (1998) Structure of an aromatic-ring-hydroxylating dioxygenase-naphthalene 1,2-dioxygenase. Structure 6:571–586

Kim E, Zylstra GJ (1999) Functional analysis of genes involved in biphenyl, naphthalene, phenanthrene, and m-xylene degradation by *Sphingomonas yanoikuyae* B1. J Ind Microbiol Biotechnol 23:294–302

Kim S, Chun J, Bae K, Kim Y (2000) Polyphasic assignment of an aromatic-degrading *Pseudomonas* sp., strain DJ77, in the genus *Sphingomonas* as *Sphingomonas chungbukensis* sp. nov. Int J Syst Evol Microbiol 50:1641–1647

Kim IS, Ryu JY, Hur HG, Gu MB, Kim SD, Shim JH (2004) *Sphingomonas* sp. strain SB5 degrades carbofuran to a new metabolite by hydrolysis at the furanyl ring. J Agric Food Chem 52:2309–2314

Krivobok S, Kuony S, Meyer C, Louwagie M, Willison JC, Jouanneau Y (2003) Identification of pyrene-induced proteins in *Mycobacterium* sp. strain 6PY1: evidence for two ring-hydroxylating dioxygenases. J Bacteriol 185:3828–3841

Leys NM, Ryngaert A, Bastiaens L, Verstraete W, Top EM, Springael D (2004) Occurrence and phylogenetic diversity of *Sphingomonas* strains in soils contaminated with polycyclic aromatic hydrocarbons. Appl Environ Microbiol 70:1944–1955

McIver AM, Janardhan Garikipati SVB, Bankole KS, Gyamerah M, Peeples TL (2008) Microbial oxidation of naphthalene to cis-1,2-naphthalene dihydrodiol using naphthalene dioxygenase in biphasic media. Biotechnol Prog 24:593–598

Mergeay M, Nies D, Schlegel HG, Gerits J, Charles P, Van Gijsegem F (1985) *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J Bacteriol 162:328–334

Ni Chadhain SM, Moritz EM, Kim E, Zylstra GJ (2007) Identification, cloning, and characterization of a multicomponent biphenyl dioxygenase from *Sphingobium yanoikuyae* strain B1. J Ind Microbiol Biotechnol 34:605–613

Parales RE (2003) The role of active-site residues in naphthalene dioxygenase. J Ind Microbiol Biotechnol 30:271–278

Parales RE, Parales JV, Gibson DT (1999) Aspartate 205 in the catalytic domain of naphthalene dioxygenase is essential for activity. J Bacteriol 181:1831–1837

Parales RE, Lee K, Resnick SM, Jiang H, Lessner DJ, Gibson DT (2000a) Substrate specificity of naphthalene dioxygenase: effect of specific amino acids at the active site of the enzyme. J Bacteriol 182:1641–1649

Parales RE, Resnick SM, Yu CL, Boyd DR, Sharma ND, Gibson DT (2000b) Regioselectivity and enantioselectivity of naphthalene dioxygenase during arene cis-dihydroxylation: control by phenylalanine 352 in the alpha subunit. J Bacteriol 182:5495–5504

Peng X, Masai E, Kitayama H, Harada K, Katayama Y, Fukuda M (2002) Characterization of the 5-carboxyvanillate decarboxylase gene and its role in lignin-related biphenyl catabolism in *Sphingomonas paucimobilis* SYK-6. Appl Environ Microbiol 68:4407–4415

Pinyakong O, Habe H, Supaka N, Pinpanichkarn P, Juntongjin K, Yoshida T, Furihata K, Nojiri H, Yamane H, Omori T (2000) Identification of novel metabolites in the degradation of phenanthrene by *Sphingomonas* sp. strain P2. FEMS Microbiol Lett 191:115–121

Pinyakong O, Habe H, Omori T (2003a) The unique aromatic catabolic genes in sphingomonads degrading polycyclic aromatic hydrocarbons (PAHs). J Gen Appl Microbiol 49:1–19

Pinyakong O, Habe H, Yoshida T, Nojiri H, Omori T (2003b) Identification of three novel salicylate 1-hydroxylases involved in the phenanthrene degradation of *Sphingobium* sp. strain P2. Biochem Biophys Res Commun 301:350–357

Resnick SM, Torok DS, Gibson DT (1993) Oxidation of carbazole to 3-hydroxycarbazole by naphthalene 1,2-dioxygenase and biphenyl 2,3-dioxygenase. FEMS Microbiol Lett 113:297–302

Romine MF, Stillwell LC, Wong KK, Thurston SJ, Sisk EC, Sensen C, Gaasterland T, Fredrickson JK, Saffer JD (1999) Complete sequence of a 184-kilobase catabolic plasmid from *Sphingomonas aromaticivorans* F199. J Bacteriol 181:1585–1602

Sambrook J, Fritsch EF, Maniatis T (1990) Molecular cloning: a laboratory manual. Cold Spring Laboratory Harbor Press, Cold Spring Harbor, NY

Schuler L, Ni Chadhain SM, Jouanneau Y, Meyer C, Zylstra GL, Hols P, Agathos SN (2008) Characterization of a novel angular dioxygenase from fluorene-degrading *Sphingomonas* sp. strain LB126. Appl Environ Microbiol 74:1050–1057

Sorensen SR, Ronen Z, Aamand J (2001) Isolation from agricultural soil and characterization of a *Sphingomonas* sp. able to mineralize the phenylurea herbicide isoproturon. Appl Environ Microbiol 67:5403–5409

Stolz A (1999) Degradation of substituted naphthalenesulfonic acids by *Sphingomonas xenophaga* BN6. J Ind Microbiol Biotechnol 23:391–399

Story SP, Parker SH, Hayasaka SS, Riley MB, Kline EL (2001) Convergent and divergent points in catabolic pathways involved in utilization of fluoranthene, naphthalene, anthracene, and phenanthrene by *Sphingomonas paucimobilis* var. EPA505. J Ind Microbiol Biotechnol 26:369–382

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

van Herwijnen R, de Graaf C, Govers HA, Parsons JR (2004) Estimation of kinetic parameter for the biotransformation of three-ring azaarenes by the phenanthrene-degrading strain *Sphingomonas* sp. LH128. Environ Toxicol Chem 23:331–338

Wackett LP (2002) Mechanism and application of Rieske non-heme iron dioxygenases. Enzyme Microb Technol 31:577–587

Willison JC (2004) Isolation and characterization of a novel sphingomonad capable of growth with chrysene as sole carbon and energy source. FEMS Microbiol Lett 241:143–150

Yrjala K, Paulin L, Kilpi S, Romantschuk M (1994) Cloning of cmpE, a plasmid-borne catechol 2,3-dioxygenase-encoding gene from the aromatic- and chloroaromatic-degrading *Pseudomonas* sp. HV3. Gene 138:119–121

Yu CL, Liu W, Ferraro DJ, Brown EN, Parales JV, Ramaswamy S, Zylstra GJ, Gibson DT, Parales RE (2007) Purification, characterization, and crystallization of the components of a biphenyl dioxygenase system from *Sphingobium yanoikuyae* B1. J Ind Microbiol Biotechnol 34:311–324

Zielinski M, Kahl S, Hecht HJ, Hofer B (2003) Pinpointing biphenyl dioxygenase residues that are crucial for substrate interaction. J Bacteriol 185:6976–6980

Zielinski M, Kahl S, Standfuss-Gabisch C, Camara B, Seeger M, Hofer B (2006) Generation of novel-substrate-accepting biphenyl dioxygenases through segmental random mutagenesis and identification of residues involved in enzyme specificity. Appl Environ Microbiol 72:2191–2199

Zylstra GJ, Kim E (1997) Aromatic hydrocarbon degradation by *Sphingomonas yanoikuyae* B1. J Ind Microbiol Biotechnol 19:408–414