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Manuscript for review

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

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Keyword:	polycyclic aromatic hydrocarbons, dioxygenase, ring hydroxylation, substrate specificity, overexpression, catabolic genes

1
2 1 **Characterization of a ring-hydroxylating dioxygenase from phenanthrene-degrading**
3
4 2 ***Sphingomonas* sp. strain LH128 able to oxidize benz[*a*]anthracene.**
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31
32
33 14 **Running Title:** Dioxygenase from *Sphingomonas* sp. strain LH128

34
35 15 **Keywords:** Bioremediation, *meta*-cleavage operon genes, indigo formation, Rieske non-heme
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37 16 iron oxygenase

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ABSTRACT

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2 25
3
4 26 *Sphingomonas* sp. strain LH128 was isolated from a polycyclic aromatic hydrocarbon (PAH)
5
6 27 contaminated soil using phenanthrene as the sole source of carbon and energy. A dioxygenase
7
8 28 complex, *phnA1fA2f* encoding the α and β subunit of a terminal dioxygenase responsible for
9
10 29 the initial attack on PAHs, was identified and isolated from this strain. PhnA1f showed 98%,
11
12 30 78% and 78% identity to the α subunit of *Novosphingobium aromaticivorans* strain F199,
13
14 31 *Sphingomonas* sp. strain CHY-1 and *Sphingobium yanoikuyae* strain B1 respectively. When
15
16 32 overexpressed in *E. coli*, PhnA1fA2f was able to oxidize low molecular weight PAHs,
17
18 33 chlorinated biphenyls, dibenzo-*p*-dioxin and the high molecular weight PAHs
19
20 34 benz[*a*]anthracene, chrysene and pyrene. The action of PhnA1fA2f on benz[*a*]anthracene
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22 35 produced two benz[*a*]anthracene dihydrodiols.
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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 O₂ 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

1
2 62 identified the genes governing the angular attack on fluorene by the gram-negative
3
4 63 *Sphingomonas* sp. strain LB126 which uses fluorene as the sole source of carbon and energy
5
6 64 (Schuler et al. 2008).
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8
9 65 Although the complete sequence of plasmid pNL1 which harbours a catabolic gene cluster of
10
11 66 40 kb as well as the putative initial dioxygenase of *Novosphingobium aromaticivorans* F199
12
13 67 has been sequenced, the activity of the initial dioxygenase has not yet been investigated
14
15 68 (Romine et al. 1999). Sphingomonads harbour multiple copies of genes predicted to encode
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17 69 the terminal component of Rieske-type oxygenases (Pinyakong et al. 2000; Romine et al.
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19 70 1999). They constitute a large family of two- or three-component metalloenzymes whose
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21 71 catalytic activity component is generally a heteromeric $\alpha_3\beta_3$ hexamer containing one Rieske-
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23 72 type [2Fe-2S] cluster and one nonheme iron atom per α subunit. The fact that all
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25 73 phenanthrene-degrading sphingomonads carry a similar pathway organization as found in
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27 74 *Sphingomonas* sp. strain CHY-1, *Sphingobium yanoikuyae* strain B1, *Novosphingobium*
28
29 75 *aromaticivorans* strain F199 and *Sphingobium* sp. strain P2, indicates that this organization
30
31 76 has been conserved for a long time and is quite stable despite the apparent complex
32
33 77 organization compared to the more 'logical' organization of PAH-degradation genes in
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35 78 members of the genus *Pseudomonas*. These data could help to explain that *Sphingomonas*
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37 79 spp. started as phenanthrene degraders and their respective initial dioxygenases became
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39 80 substrate-relaxed in order to oxidize a large variety of PAHs.
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47 81 *Sphingomonas* sp. strain LH128 was isolated from a heavily polluted soil (Bastiaens et
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49 82 al. 2000) and is capable of growing on phenanthrene as the sole source of carbon and energy.
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51 83 Strain LH128 is also able to transform indole to indigo in the presence of phenanthrene (data
52
53 84 not shown). No indigo formation was observed when the strain was grown in the presence of
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55 85 glucose suggesting that the dioxygenase oxidizing indole must be induced by phenanthrene.
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57 86 Moreover strain LH128 is able to degrade anthracene, dibenzothiophene, fluorene (Bastiaens
58
59 87 et al. 2000) and the N-heterocyclic PAHs acridine, phenanthridine, benzo[*f*]quinoline and
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1
2 88 benzo[*h*]quinoline (van Herwijnen et al. 2004). In this study the multicomponent ring
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4 89 hydroxylating dioxygenase from *Sphingomonas* sp. strain LH128 was cloned and its function
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6 90 towards a variety of substrates was investigated. This newly characterized dioxygenase is
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8 91 shown to be closely related to BphA1fA2f from *Novosphingobium aromaticivorans* strain
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10 92 F199 (98 % identities) but to display significant differences in catalytic behaviour as reflected
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12 93 by a broad substrate range notably including the capacity to oxidize benz[*a*]anthracene.
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20 MATERIALS AND METHODS

21 96 **Reagents.** PAHs and antibiotics were obtained from Sigma-Aldrich (St. Louis, MO). Primers
22
23 97 were purchased from Sigma-Genosys. Silicone oil (Rhodorsil 47V20) was purchased from
24
25 98 VWR International (France). Restriction enzymes were from New England Biolabs (Ipswich,
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27 99 MA).
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31 101 **Bacterial strains, plasmids, and media.**

32
33 102 *Sphingomonas* sp. strain LH128 was kindly provided by VITO (Vlaamse Instelling voor
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35 103 Technologisch Onderzoek, Belgium). *Escherichia coli* Top10 (Invitrogen, Carlsbad, CA) was
36
37 104 used as the recipient strain in all cloning experiments. *E. coli* BL21(DE3) was used for gene
38
39 105 expression analysis. PCR amplicons were either cloned into pDrive (Qiagen, Valencia, CA)
40
41 106 while pET30f (Novagen, San Diego, CA) and pVLT31 (de Lorenzo et al. 1993) were used as
42
43 107 expression vector. MM284 minimal medium (Mergeay et al. 1985) was used for growing
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45 108 *Sphingomonas* sp. strain LH128 and was supplemented with phosphate buffer (50 mM;
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47 109 KH₂PO₄, K₂HPO₄, pH 7.2) instead of Tris buffer. Phenanthrene was provided as crystals in
48
49 110 both solid and liquid media. LB broth (Sambrook et al. 1990) was used as complete medium
50
51 111 for growing *E. coli* strains. Solid media contained 2% agar. When needed, ampicillin,
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53 112 streptomycin, tetracycline or kanamycin was added to the medium at 100, 200, 10 and 20
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55 113 µg/ml, respectively. *Sphingomonas* sp. strain LH128 was grown at 30°C, and *E. coli* strains
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2 114 were grown at 37°C. Bacterial growth was determined by optical density readings at 600 nm
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4 115 (OD₆₀₀).

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9 117 **DNA manipulations and molecular techniques.** Total DNA from pure cultures of
10
11 118 *Sphingomonas* sp. strain LH128 was extracted using the Ultra Clean DNA Isolation Kit
12
13 119 (MoBio, Carlsbad, CA) following the manufacturer's recommendations or using standard
14
15 120 methods (Sambrook et al. 1990) when a higher DNA concentration was needed. Plasmid
16
17 121 DNA extractions, restriction enzyme digestions, ligations, transformations, sequencing and
18
19 122 agarose gel electrophoresis were carried out using standard methods (Sambrook et al. 1990).
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21 123

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25 124 **Polymerase chain reaction (PCR) and primer design.** PCR primers RHDA1f-F (5'-
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27 125 CACCGCGGCAACCAGAT -3') and RHDA2f-R (5'- ACCATGGTATAGGTCCA-3') were
28
29 126 constructed based upon conserved nucleic acid alignments of the initial dioxygenase from
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31 127 *Sphingomonas yanoikuyae* strain B1 (EF152282) *Novosphingobium aromaticivorans* strain
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33 128 F199 (AF079317) and *Sphingomonas* sp. strain CHY-1 (AJ633551) using Clustal X software
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35 129 (Thompson et al. 1997). All PCR reactions were carried out using PCR Master Mix (Abgene,
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37 130 Surrey, UK) and were performed in a programmable T-Gradient Thermocycler (Biometra,
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39 131 Göttingen, Germany). PCR products were purified and cloned into either the pDrive or
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41 132 pGEMT-easy plasmids.
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49 134 **Construction of plasmids for protein overexpression.** Construction of the plasmids used in
50
51 135 this study involved multiple PCR amplifications and cloning steps. The *phnA1fA2f* fragment
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53 136 (2048 bp) was amplified by PCR with the primers pairs: 5'- *CATATGAATGGATCGTCCG* -
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55 137 3' and 5'- *AAGCTTGATCGAATTTGCTTATGCG* -3', introducing NdeI and HindIII sites
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57 138 (*italics*) at the ends of the amplicon. The PCR amplicon was cloned into pDrive, sequenced,
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59 139 then subcloned into the NdeI and HindIII site of expression vector pET30f (Novagen, San
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1
2 140 Diego, CA). The *phnA1fA2f* pair of genes was also transferred into pVLT31 (de Lorenzo et al.
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4 141 1993) as a XbaI - HindIII fragment from pET30f*phnA1fA2f*. These constructs were
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6 142 transformed into *E. coli* BL21(DE3) for expression analysis.
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11 144 **Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).** Bacterial
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14 145 cells were pelleted by centrifugation and washed with 10 ml ice-cold phosphate buffer (140
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16 146 mM NaCl, 10 mM Na₂HPO₄, 2.7 mM KCl, 1.8 mM NaH₂PO₄, pH 7.4). 1 ml of ice-cold
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18 147 phosphate buffer was added to the pellet and 550 µl of the suspension was subjected to
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21 148 sonication on ice for 20 s (5 s pulse interval; 40% of maximum amplitude). After
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23 149 centrifugation the supernatant and the pellet were mixed with an equal volume of loading
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26 150 solution. SDS-PAGE was performed on 13.3 % polyacrylamide mini gels. After
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28 151 electrophoresis, protein staining was performed with Coomassie brilliant blue R-250.
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33 153 **Dioxygenase overexpression and in vivo assays.** Strains BL21(DE3)(pET30f*phnA1fA2f*) or
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35 154 BL21(DE3)(pVLT31*phnA1fA2f*) complemented with pEB431, carrying ferredoxin (*phnA3*)
36
37 155 and ferredoxin reductase (*phnA4*) genes from *Sphingomonas* sp. strain CHY-1 (Demaneche et
38
39 156 al. 2004), were grown overnight in 5 ml LB medium with the suitable antibiotics. This culture
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42 157 was used to inoculate 25 ml LB medium (0.1% vol/vol), which was incubated at 37°C until an
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44 158 OD₆₀₀ of 0.5. IPTG was added to a final concentration of 0.5 mM. The cells were further
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47 159 incubated overnight at 25°C. For in vivo assays, cells were centrifuged, washed and
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50 160 resuspended to an OD₆₀₀ of approximately 2 in M9 medium (Sambrook et al. 1990)
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52 161 containing 0.2% glucose. Cells (12 ml) overexpressing PhnA1fA2f, PhnA3 and PhnA4 were
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54 162 incubated overnight at 25 °C with 2 ml silicone oil containing 400 µM of each tested
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56 163 substrate.
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2 165 **GC-MS analysis of PAH oxidation products.** Water-soluble products resulting from PAH
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4 166 oxidation were extracted from the aqueous phase of bacterial suspension by using columns
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6 167 filled with reverse phase-bonded silica (Upti-clean C18U, 0.5 g, Interchim, Montluçon,
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8 168 France). Columns were washed with 10 ml water then eluted with 1 ml ethyl acetate. The
9
10 169 solvent was dried over sodium sulphate and evaporated under nitrogen gas. The dried extracts
11
12 170 were then dissolved in 100 or 200 µl acetonitrile, before being derivatized with *N,O*-
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14 171 *bis*(trimethylsilyl)trifluoroacetamide containing trimethylchlorosilane (BSTFA) or *n*-
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16 172 butylboronate (NBB). In order to quantify the dihydrodiols formed upon incubation of
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18 173 BL21(DE3)(pET30f*phnA1fA2f*) recombinant cells with PAHs, 2,3-dihydrobiphenyl (Sigma-
19
20 174 Aldrich, St. Louis, MO) was added to 0.1 µM final concentration in the aqueous phase prior
21
22 175 to solid phase extraction, and was used as an internal standard. After derivatization and GC-
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24 176 MS analysis, NBB dihydrodiol derivates were quantified on the basis of peak area using a
25
26 177 calibration curve generated by analysing known amounts of 3,4-phenanthrenedihydrodiol.
27
28 178 GC-MS analysis of trimethylsilyl derivatives was carried out as previously described
29
30 179 (Jouanneau et al. 2006). NBB derivatives were separated on MDN-12 capillary column (30 m,
31
32 180 0.25 mm internal diameter; Supelco) using helium as carrier gas at 1 ml/min. The oven
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34 181 temperature was held at 75°C for 1 min, then increased to 300°C at a rate of 14°C min⁻¹, and
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36 182 held at 300°C for 8 min. The mass spectrometer was operated in the selected ion-monitoring
37
38 183 mode, selecting *m/z* values corresponding to the expected masses (M^+) of the dihydrodiol
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40 184 derivatives.
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52 186 **DNA and protein sequence analysis.** Sequence analysis was performed using the
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54 187 DNASTAR software package (Lasergene Inc., Madison, WI). The BLAST search tool was
55
56 188 used for homology searches (Altschul et al. 1997). Multiple alignments were produced using
57
58 189 the DNASTAR software.
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1
2 191 **Nucleotide sequence accession numbers.** The nucleotide sequences described in this report
3
4 192 have been deposited in the Genbank database under accession number EU024111 and
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6 193 EU024112 for the salicylate 1-hydroxylase and lower pathway enzymes and the terminal
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9 194 dioxygenase, respectively.
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For Peer Review

RESULTS

196 **Cloning and sequence analysis of genes encoding a terminal dioxygenase.**

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

217

218 **Functional expression of PhnA1fA2f in *E. coli*.**

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

1
2 221 introduced into *E. coli* BL21(DE3) for SDS-PAGE analysis of IPTG-induced proteins. The
3
4 222 cells overproduced two polypeptides with the expected size of 50,000 Da and 20,000 Da (Fig.
5
6
7 223 1). However, the proteins were mainly insoluble (inclusion bodies) and recombinant cells did
8
9 224 not show detectable oxygenase activity. The *phnA1fA2f* sequence was therefore subcloned
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11 225 behind the *Ptac* promoter into the broad host-range vector pVLT31 (de Lorenzo et al. 1993)
12
13 226 and introduced into *E. coli* BL21(DE3). When induced with IPTG, the recipient cells
14
15 227 produced appreciable levels of 50-kDa and 20-kDa polypeptides, which appeared to form a
16
17 228 soluble recombinant protein (Fig. 1). In order to provide the terminal oxygenase component
18
19 229 with an appropriate electron transport chain, plasmid pEB431, expressing *phnA3* and *phnA4*
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21 230 (Demaneche et al. 2004) was co-transformed into *E. coli* BL21(DE3). PhnA3 and PhnA4
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23 231 formed with PhnA1fA2f a competent enzymatic complex in the *E. coli* host as proved by
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25 232 indigo formation compared to cells lacking pEB431.
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234 **Substrate range of PhnA1fA2f.**

34
35 235 The recombinant *E. coli* strain producing PhnA1f, PhnA2f, PhnA3 and PhnA4 was incubated
36
37 236 overnight separately with several representative PAHs, dibenzo-*p*-dioxin and PCBs. The
38
39 237 water-soluble products released into the culture medium were extracted and analysed by GC-
40
41 238 MS (Table 1) as described elsewhere (Krivobok et al. 2003). Since *Sphingomonas* sp. strain
42
43 239 LH128 is able to use fluorene, dibenzothiophene, and anthracene in cometabolic degradation
44
45 240 (Bastiaens et al. 2000) we tested whether PhnA1fA2f was responsible for the initial attack on
46
47 241 these compounds. The relative activity toward each PAH was calculated from the GC-MS
48
49 242 selected ion monitoring peak areas of the NBB derivatives compared to an internal standard
50
51 243 (2,3-dihydroxybiphenyl). Naphthalene was the preferred substrate (100 %), then phenanthrene
52
53 244 (43.3 %), biphenyl (31.8 %) and anthracene (28.7 %) were converted at significant but lower
54
55 245 rates to the corresponding dihydrodiols. Since naphthalene cannot support growth of strain
56
57 246 LH128, the genes were called *phnA1fA2f*. Interestingly, PhnA1fA2f was also able to oxidize
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1
2 247 the heteroatomic analogues of fluorene i.e. dibenzofuran, dibenzothiophene and carbazole.
3
4 248 Strain LH128 is able to degrade fluorene in cometabolism with phenanthrene as the main
5
6 249 carbon source (Bastiaens et al. 2000). However, only traces of fluorenedihydrodiol were
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8
9 250 detected after *n*-butylboronate (NBB) derivatization, a result that did not account for the
10
11 251 substantial cometabolic activity of strain LH128 towards fluorene. GC-MS analysis of TMS
12
13 252 derivatives of fluorene oxidation products allowed identification of a large peak of
14
15 253 monohydroxyfluorene (RT 16.262 min) with significant fragment ions at *m/z* 254 (100), 239
16
17 254 (95), 165 (80), 152 (19), 73 (31). Moreover dihydroxyfluorene (RT: 17.577 min; 342 (36),
18
19 255 327 (4), 253 (33), 223 (7), 73 (100) was detected, which most likely resulted from
20
21 256 hydroxylation of fluorene on two non-adjacent carbon atoms because it could not be detected
22
23 257 by NBB derivatization. Detection of monohydroxycarbazole (RT: 17.092 min; *m/z* 255 (100),
24
25 258 239 (57), 224 (47), 166 (11)) after BSTFA derivatization suggests that PhnA1fA2f transforms
26
27 259 carbazole to an unstable dihydrodiol by lateral dioxygenation. Fluoranthene was also probably
28
29 260 oxidized to an unstable dihydrodiol, which was further converted to 8-hydroxyfluoranthene,
30
31 261 since the TMS derivative had the same GC-MS characteristics as those reported for the
32
33 262 oxidation product of fluoranthene by the PhnI dioxygenase from strain CHY-1 (RT: 20.365
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35 263 min; *m/z* 290 (100), 275 (55), 215 (15), 201 (19), 200 (18), 189 (30)) (Jouanneau et al. 2006).
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37 264 Since PhnA1fA2f displayed a relatively high activity towards biphenyl (31.8%), we tested
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39 265 whether PhnA1fA2f could oxidize halogenated biphenyls. Monochlorinated biphenyls such as
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41 266 2-chlorobiphenyl (relative activity 6.6 %) and 4-chlorobiphenyl (6.1 %) were oxidized to
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43 267 corresponding dihydrodiols, but 2,3-dichlorobiphenyl was not. Moreover PhnA1fA2f was
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45 268 able to perform lateral oxygenation of dibenzo-*p*-dioxin. Interestingly, the four-ring PAH
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47 269 benz[*a*]anthracene was transformed into two compounds with masses and retention times
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49 270 consistent with those of two dihydrodiol isomers. These products most likely bear hydroxyls
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51 271 in positions 1,2 and 10,11 since the homologous enzyme from strain CHY-1 preferentially
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53 272 hydroxylated benz[*a*]anthracene on these carbons (Jouanneau et al, 2006). Chrysene and
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2 273 pyrene were oxidized to *cis*-3,4-dihydroxy-3,4-dihydrochrysene and *cis*-4,5-dihydroxy-4,5-
3
4 274 dihydropyrene based on the retention times of the purified dihydrodiols obtained with PhnI
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7 275 (Jouanneau et al. 2006) and Pdo1 (Krivobok et al. 2003), respectively. The 5-ring PAH
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9 276 benz[*a*]pyrene did not produce any detectable dihydrodiol under identical conditions. These
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11 277 data demonstrate that the PhnA1fA2f terminal oxygenase from strain LH128 displays
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13
14 278 exceptionally broad substrate specificity towards a wide range of aromatic hydrocarbons.

16 279 DISCUSSION

18 280 Sphingomonads are known to degrade a large spectrum of pollutants, ranging from mono- and
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20
21 281 polycyclic hydrocarbons (Pinyakong et al. 2000; Story et al. 2001) to naphthalene sulfonate
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23 282 (Stolz 1999), dibenzo-*p*-dioxin (Armengaud et al. 1998; Hong et al. 2002), and methylated
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26 283 PAHs (Dimitriou-Christidis et al. 2007; Zylstra and Kim 1997). Most known degradation
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28 284 pathways of homocyclic PAHs start with the formation of a dihydroxy PAH by hydroxylation
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30 285 of two adjacent carbon atoms. This step is catalysed by dioxygenase enzymes with relaxed
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33 286 substrate specificity, which determines the substrate range of the organism. The compounds
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35 287 are further degraded to a limited number of intermediates such as *o*-phthalic acid or salicylic
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37 288 acid, and then via *ortho* or *meta* cleavage to tricarboxylic acid cycle intermediates. The genes
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40 289 for aromatic hydrocarbon degradation by sphingomonads are quite different from those found
41
42 290 in other genera both in terms of nucleotide sequence and of gene order (Pinyakong et al.
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44
45 291 2003a). This unique gene arrangement, which is remarkably conserved among strains of
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47 292 various origins, contrasts with that found in other degraders, such as pseudomonads.
48
49 293 To date only a few sphingomonads' initial dioxygenases have been well characterized:
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51 294 BphA1fA2f from strain B1 (Ni Chadhain et al. 2007) and PhnI (Jouanneau et al. 2006) from
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53
54 295 strain CHY-1. BphA1fA2f from strain F199 has been identified but further investigation to
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56 296 assess its catalytic abilities is missing. While the initial dioxygenases from strains LH128 and
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59 297 CHY-1 are related (78 % identity), strain CHY-1 is able to grow on chrysene as the sole
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298 source of carbon (Willison 2004) while strain LH128 cannot use chrysene as a substrate.

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2 299 Likewise, the dioxygenases from strains CHY-1 and B1 show apparent differences of
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4 300 substrate specificity despite sharing an almost identical structure (Demaneche et al. 2004;
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6 301 Jouanneau et al. 2006; Ni Chadhain et al. 2007). These observations suggest that there exists a
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8 302 pool of highly conserved multicomponent dioxygenases in sphingomonads, with subtle
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10 303 structural variations that would appear to be responsible for differences in selectivity toward
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12 304 PAHs (Fig. 2). Six homologues to both large and small substrate binding components of ring
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14 305 hydroxylating dioxygenases were identified (*bphA1*_[a-f]-*bphA2*_[a-f]) in *Sphingomonas*
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16 306 *yanoikuyae* strain B1 (Zylstra and Kim 1997), *Sphingomonas* sp. strain P2 (Pinyakong et al.
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18 307 2003b) and *Novosphingobium aromaticivorans* strain F199 (Romine et al. 1999). Since the
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20 308 genes isolated from strain LH128 display high homologies to catabolic genes from these
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22 309 species, one can expect to find the missing dioxygenase encoding genes in strain LH128
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24 310 (*bphA1*_[a,b,e]-*bphA2*_[a,b,e]). Moreover, studies of *Sphingomonas* population structures of several
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26 311 PAH-contaminated soils by PCR-DGGE revealed that soils with the highest phenanthrene
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28 312 concentrations showed the lowest *Sphingomonas* diversity (Leys et al. 2004). This indicates
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30 313 that *Sphingomonas* species share a set of dioxygenases that probably originated as
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32 314 phenanthrene catabolic genes and then, by duplication, evolved to degrade different
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34 315 substrates. For instance, the enzymes involved in the initial step of PAH degradation exhibit a
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36 316 greater variety than those involved in the catabolism of central metabolites such as salicylate
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38 317 (Table 2). The overall identities between salicylate 1-hydroxylases are higher than the
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40 318 identities between the respective ring-hydroxylating dioxygenases of the different strains.
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42 319 This clearly indicates that the enzymes involved in the upper PAH catabolic pathways have a
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44 320 more relaxed substrate specificity than the enzymes involved in the lower pathway.
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46 321 When overexpressed in *E. coli* BL21(DE3), PhnA1fA2f was found to be responsible for the
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48 322 oxidation of low and high molecular weight PAHs, dibenzo-*p*-dioxin and monochlorinated
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50 323 biphenyls but not 2,3-dichlorobiphenyl. Traces of carbazole dihydrodiol were detected after
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52 324 NBB derivatization, but monohydroxycarbazole was abundant. Resnick et al. (1993) reported

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2 325 the formation of monohydroxycarbazole, possibly as a result of dehydration of unstable
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4 326 dihydrodiols. Phenanthrene (43.3 %), biphenyl (31.8 %) and anthracene (28.7 %) were
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7 327 transformed into high levels of the corresponding *cis*-dihydrodiols. Oxidation products of
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9 328 benz[a]anthracene, chrysene and pyrene (Table 1) were also identified in contrast with
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11 329 naphthalene dioxygenases whose selectivity is limited to only two and three ring PAHs
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13
14 330 (Ferraro et al. 2004; Gakhar et al. 2005; Kauppi et al. 1998). The five ring PAH
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16 331 benz[a]pyrene did not give any detectable products. This suggests that benz[a]pyrene
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18 332 probably does not fit into the catalytic pocket of PhnA1fA2f.

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21 333 The catalytic pocket of the ring-hydroxylating dioxygenase from *Sphingomonas* sp. strain
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23 334 CHY-1 has been recently described on the basis of its crystal structure, and the amino acids
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25 335 lining the catalytic pocket were identified (Jakoncic et al. 2007a; 2007b). These residues are
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28 336 conserved in the enzymes from *Sphingomonas* sp. strain LH128, *Novosphingobium*
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30 337 *aromaticivorans* strain F199 and, with only two substitutions, in *Sphingobium yanoikuyae*
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32 338 strain B1 (Jakoncic et al. 2007a) (data not shown), suggesting that the topology of the
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34 339 substrate binding pocket is almost identical. However, these structural resemblances do not
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37 340 explain the differences in substrate specificity of the dioxygenases. The crystal structure of
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40 341 the ring hydroxylating dioxygenase from strain CHY-1 showed that the entrance of the
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42 342 catalytic pocket is covered by two flexible loops L1 and L2, exposed to the solvent. These
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45 343 loops are predicted to control the substrate's access to the catalytic pocket (Jakoncic et al.
46
47 344 2007b). Since the sequence of these loops is only partly conserved in the LH128 enzyme (83
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49 345 % and 63 % identities for L1 and L2, respectively), it seems plausible that these structural
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51 346 differences may be responsible for the lower activity of the LH128 dioxygenase towards high
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54 347 molecular weight PAHs and its inability to oxidize benz[a]pyrene. The effects on the catalytic
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56 348 activity of residue substitutions in the active site have been well investigated in the case of
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59 349 naphthalene dioxygenase and biphenyl dioxygenases (Parales 2003; Parales et al. 1999;
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350 2000a; 2000b), but the effect of substitutions outside the catalytic pocket is less well

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2 351 documented (Furukawa et al. 2004; Zielinski et al. 2003; 2006). Our results indicate that
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4 352 residues in the loops at the entrance of the catalytic pocket are potentially interesting targets
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7 353 for mutagenesis as a means to better understand the structural determinants of selectivity.
8
9 354 In summary, we identified the genes encoding the dioxygenase responsible for the initial
10
11 355 attack on various PAHs by *Sphingomonas* sp. strain LH128 and expressed them in *E. coli*.
12
13 356 The dioxygenase PhnA1fA2f was closely related to BphA1fA2f from *Novosphingobium*
14
15 357 *aromaticivorans* strain F199 and, to a lower extent, to PhnI from *Sphingomonas* sp. strain
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17 358 CHY-1 and BphA1fA2f *Sphingobium yanoikuyae* strain B1. Characterization of the activity
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19 359 of the dioxygenase cloned in *E. coli* showed significant differences in catalytic activity
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21 360 compared to the proteins PhnI from strain CHY-1 and BphA1fA2f from strain B1. This
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23 361 indicates that small variations in amino acid sequence outside the catalytic pocket can have
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25 362 substantial impact on dioxygenase selectivity. Significantly, PhnA1fA2f was able to oxidize
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30 363 the four ring PAH benz[a]anthracene and yielded two dihydrodiols.
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For Peer Review

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525 **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	<i>cis</i> -2,3-Dihydroxy-2,3-dihydrobiphenyl	254	16.199	31.8	0.097
Naphthalene	<i>cis</i> -1,2-Dihydroxy-1,2-dihydronaphthalene	228	14.479	100	0.306
Phenanthrene	<i>cis</i> -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	<i>cis</i> -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[<i>a</i>]anthracene	<i>cis</i> -1,2-Benz[<i>a</i>]anthracenedihydrodiol	328	23.563	5.5	0.017
	<i>cis</i> -10,11-Benz[<i>a</i>]anthracenedihydrodiol	328	24.513	4.4	0.014
Pyrene	<i>cis</i> -4,5-Dihydroxy-4,5-dihydropyrene ^e	302	21.721	Traces	Traces
Chrysene	<i>cis</i> -3,4-Dihydroxy-3,4-dihydrochrysene ^f	328	24.801	0.3	9.452E-04
Benzo- <i>p</i> -dioxin	Benzo- <i>p</i> -dioxindihydrodiol	284	17.936	2.4	0.007
Dibenzothiophene	Dibenzothiophenedihydrodiol	284	18.949	12.6	0.039
Dibenzofuran	<i>cis</i> -1,2-Dihydroxy-1,2-dihydrodibenzofuran ^g	268	17.181	17.2	0.053
	Dibenzofurandihydrodiol	268	17.611	5.3	0.016

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

528 ^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
529 as percentages of relative activity (with respect to the maximum obtained with naphthalene as substrate). The values are averages of two separate
530 determinations.

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^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-dihdropyrene 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).

538 **Table 2.** Comparisons amongst salicylate 1-hydroxylase and the initial ring-hydroxylating
 539 dioxygenase from *Sphingomonas* sp. strain P2, *Novosphingobium aromaticivorans* strain
 540 F199, *Sphingomonas* sp. strain LH128, *Sphingobium yanoikuyae* strain B1 and *Sphingomonas*
 541 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

542 ^a Amino acid identity to their respective counterparts is shown.

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2 543 **FIGURE LEGENDS**

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6 545 Figure 1. Detection of PhnA1fA2f overproduced in *E. coli* BL21(DE3). A: *E. coli*
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8 546 BL21(DE3)(pET30f*phnA1fA2f*) overproduced high amounts of 50-kDa and 20-kDa that were
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10 547 mainly insoluble. B: *E. coli* BL21(DE3) harbouring pVLT31*phnA1fA2f* produced soluble
11 548 proteins. However the β subunit could not be detected by SDS-PAGE. *E. coli* BL21(DE3)
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13 549 harbouring pET30f (A) or pVLT31 (B) lacking the *phnA1fA2f* insert were used as controls
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15 550 (C). Protein extracts from 4 clones induced by IPTG are shown (lanes 1-4). Molecular mass
16 551 (kDa): M1: Prestained PAGE Ruler (Fermentas, St. Leon Rot, Germany), M2: Prestained
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18 552 Protein Marker, Broad Range (New England Biolabs, Ipswich, MA). The arrows show the
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20 553 PhnA1fA2f subunits.
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30 555 Figure 2. [Modified and updated after Pinyakong et al. (2003a)]. Comparison of the conserved
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32 556 catabolic operon from *Novosphingobium aromaticivorans* strain F199 (Romine et al. 1999),
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34 557 *Sphingobium* sp. strain P2 (Pinyakong et al. 2003b), *Sphingobium yanoikuyae* strain B1
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36 558 (Zylstra and Kim 1997; Ni Chadhain et al. 2007), *Sphingomonas* sp. strain CHY-1
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38 559 (Demaneche et al. 2004), *Sphingomonas* sp. strain HV3 (Yrjala et al. 1994) and
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40 560 *Sphingomonas chungbukensis* strain DJ77 (Kim et al. 2000). The protein sequence identities
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44 561 to the counterparts from strain F199 are indicated.
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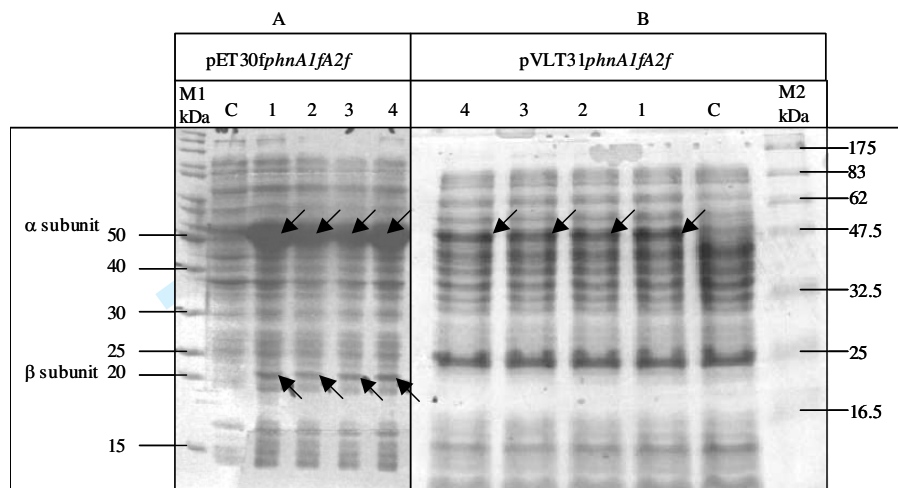
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Fig. 2

