Heterologous Expression and Characterization of Two 1-Hydroxy-2-Naphthoic Acid Dioxygenases from *Arthrobacter phenanthrenivorans*

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A protein fraction exhibiting 1-hydroxy-2-naphthoic acid (1-H2NA) dioxygenase activity was purified via ion exchange, hydrophobic interactions, and gel filtration chromatography from *Arthrobacter phenanthrenivorans* sp. nov. strain Sphe3 isolated from a Greek creosote-oil-polluted site. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and tandem MS (MS-MS) analysis revealed that the amino acid sequences of oligopeptides of the major 45-kDa protein species, as analyzed by SDS-PAGE and silver staining, comprising 29% of the whole sequence, exhibited strong homology with 1-H2NA dioxygenase of *Nocardioideis* sp. strain KP7. A BLAST search of the recently sequenced Sphe3 genome revealed two putative open reading frames, named *diox1* and *diox2*, showing 90% nucleotide identity to each other and 85% identity at the amino acid level with the *Nocardia* sp. homologue. *diox1* was found on an indigenous Sphe3 plasmid, whereas *diox2* was located on the chromosome. Both genes were induced by the presence of phenanthrene used as a sole carbon and energy source, and as expected, both were subject to carbon catabolite repression. The relative RNA transcription level of the chromosomal (*diox2*) gene was significantly higher than that of its plasmid (*diox1*) homologue. Both *diox1* and *diox2* putative genes were PCR amplified, cloned, and overexpressed in *Escherichia coli*. Recombinant *E. coli* cells expressed 1-H2NA dioxygenase activity. Recombinant enzymes exhibited Michaelis-Menten kinetics with an apparent K_m of 35 μM for *Diox1* and 29 μM for *Diox2*, whereas they showed similar kinetic turnover characteristics with K_{cat}/K_m values of $11 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $12 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, respectively. Occurrence of two *diox1* and *diox2* homologues in the Sphe3 genome implies that a replicative transposition event has contributed to the evolution of 1-H2NA dioxygenase in *A. phenanthrenivorans*.

Polycyclic aromatic hydrocarbons (PAHs) are widespread pollutants found in many soils contaminated by natural or industrial activities and threatening human and animal life. Their removal from polluted environmental niches largely depends on microbial degradation (5, 23, 42, 46). Apart from its environmental impact, microbial transformation of PAHs is also important for various technological applications, such as wastewater treatment, biodegradation, bioremediation, and biocatalysis. Among PAHs, phenanthrene, a three-benzene ring compound, has often been used as a model for experimental biodegradation. Two metabolic pathways are currently known for the metabolism of phenanthrene (3, 10, 17, 27)—one leading to the formation of protocatechuic acid via *o*-phthalic acid and the other leading to catechol via salicylic acid—which follow a further catabolic pattern through *ortho*- and *meta*-cleavage (Fig. 1). Both pathways involve the formation of 1-hydroxy-2-naphthoic acid (1-H2NA) at early steps. In bacterial pathways for the degradation of phenanthrene via *o*-phthalate, the 1-H2NA is ring cleaved by a 1-H2NA dioxygenase, producing 2-carboxybenzalpyruvate. Ring cleavage dioxygenases play a central role in the decomposition of doubly hydroxylated aromatic compounds. 1-H2NA dioxygenase is unique among such dioxygenases because it can cleave a singly hydroxylated aromatic ring. However, although degradative pathways via *o*-phthalate are described in several microorganisms (24, 27, 47), 1-H2NA dioxygenase has been purified only from *Nocardioideis* sp. strain KP7 and *Pseudomonas* sp. strain PPD (8, 18). The deduced amino acid sequence of the enzyme was different from those of other dioxygenases cleaving doubly hydroxylated aromatic rings (18). No significant similarity to any other dioxygenases, except for a moderate similarity (33%) to the gentisate 1,2-dioxygenase from *Xanthobacter polyaromaticivorans* 127W, has

been reported (15). Comparative studies of the properties of different 1-H2NA dioxygenases from various bacterial species will illuminate the identification of the structural determinants of particular functions, such as substrate specificity and catalysis, and are expected to facilitate the engineering of microorganisms with improved degrading abilities.

We have previously reported the isolation of *Arthrobacter phenanthrenivorans* strain Sphe3, which can grow on phenanthrene as the sole source of carbon and energy, efficiently catabolizing phenanthrene up to 400 $\text{mg} \cdot \text{liter}^{-1}$ at high rates (22). In the present work, we describe the purification, catalytic properties, cloning, and characterization of two 1-H2NA dioxygenases isolated from *A. phenanthrenivorans* sp. nov. (20).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *A. phenanthrenivorans* strain Sphe3 was isolated from a creosote-polluted area in Epirus, Greece (12 km north of the city of Ioannina), as described previously (20). M9 minimal

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This article is in memory of Constantin Drinas, who so unexpectedly lost his life in a car accident.

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FIG 1 Proposed pathways for bacterial degradation of phenanthrene. 1, 1-hydroxy-2-naphthoate dioxygenase; 2, 2-carboxybenzylpyruvate hydratase/aldolase; 3, 2-carboxybenzaldehyde dehydrogenase; 4, 1-hydroxy-2-naphthoate hydroxylase; 5, salicylic hydroxylase. The single arrows indicate one-step reactions, the double arrows transformations with two or more steps. TCA, tricarboxylic acid.

medium (MM M9) was supplemented with 0.02% (wt/vol) phenanthrene or 0.04% (wt/vol) glucose as described previously (22). Cultures were incubated at 30°C on a rotary shaker agitated at 180 rpm.

Escherichia coli strains DH5 α and BL21(DE3) were cultured in lysogeny broth (LB) medium in the presence of the appropriate antibiotics when necessary for selection.

Preparation of cell extracts. Cells were harvested by centrifugation (6,000 $\times$ g; 20 min) at 4°C; washed with 10 mM Tris-H₂SO₄ buffer, pH 7.5, containing 1 mM dithiothreitol (DTT); resuspended in 2 ml of the same buffer; and disrupted with a mini Bead Beater (Biospec Product, Oklahoma) (10 1-min periods using zirconium beads 0.1 mm in diameter). The homogenate was centrifuged (12,000 rpm; 30 min; 4°C), the supernatant was centrifuged again (40,000 rpm; 60 min; 4°C), and the final supernatant was used as the crude cell extract. Protein concentrations were determined by the Bradford method, using an assay kit (Bio-Rad) with bovine serum albumin (BSA) as a standard (4).

Enzyme assays. All enzyme assays were performed at 25°C using a Shimadzu UV-1201 spectrophotometer. The activity of 1-H2NA dioxygenase under standard conditions was estimated spectrophotometrically by measuring the product formation as the increase in absorbance at 300

nm (extinction coefficient [ϵ_{300}] = 11.5 mM⁻¹ cm⁻¹) in 10 mM Tris-H₂SO₄ (pH 7.5) containing 0.1 mM 1-hydroxy-2-naphthoate as described by Iwabuchi and Harayama (18). 2-Carboxybenzaldehyde dehydrogenase, 4,5-protocatechuic dioxygenase, 3,4-protocatechuic dioxygenase, 1-hydroxy-2-naphthoate hydroxylase, catechol 2,3-dioxygenase, and salicylate hydroxylase were assayed as described previously (11, 17, 26, 34, 49, 50). Kinetic data were calculated with Origin Microcal software, ORIGIN (32), by nonlinear curve fitting of the Michaelis-Menten equation.

Purification of native 1-H2NA dioxygenases. 1-H2NA dioxygenases were purified from crude cell extract of Sphe3 cells grown for 34 h at 30°C in 20 liters of MM M9 containing 0.02% (wt/vol) phenanthrene. Crude cell extract was prepared as described above and loaded onto an anion-exchange column (DEAE-Sepharose CL-6B; 12 cm by 1.6 cm; Pharmacia). Proteins were eluted from the column by a linear gradient of 0.0 to 0.5 M Na₂SO₄ in 120 ml of 10 mM Tris-H₂SO₄ buffer (pH 7.5) containing 1 mM DTT at a flow rate of 2 ml $\cdot$ min⁻¹. Active fractions were pooled and dialyzed against 10 mM Tris-H₂SO₄ buffer (pH 7.5) containing 1 mM DTT. The dialyzed fractions were adjusted to 0.5 M (NH₄)₂SO₄ at 4°C. The precipitated proteins were removed by centrifugation at 12,000 rpm for 30 min at 4°C. 1-H2NA dioxygenase activity was recovered in the supernatant fluid. This supernatant was loaded onto a hydrophobic interaction column (Phenyl-Sepharose CL-4B; 6 cm by 1.6 cm; Pharmacia) preequilibrated with 10 mM Tris-H₂SO₄ buffer (pH 7.5) containing 1 mM DTT and 0.5 M Na₂SO₄. Proteins were eluted from the column by a linear gradient of 0.5 to 0.0 M Na₂SO₄ in 120 ml of 10 mM Tris-H₂SO₄ buffer (pH 7.5) containing 1 mM DTT at a flow rate of 1 ml $\cdot$ min⁻¹. Active fractions were pooled, condensed to 2 ml, and loaded onto a gel filtration column (Sephacryl S-200 High Resolution; 75 cm by 1.6 cm; Pharmacia). The proteins were eluted from the column with a mobile phase of 20 mM Tris-H₂SO₄ buffer (pH 7.5) containing 1 mM DTT and 100 mM Na₂SO₄ at a flow rate of 0.5 ml $\cdot$ min⁻¹. The molecular mass of the enzyme was determined from its mobility through the gel filtration column relative to those of standard proteins (Blue Dextran, 2,000 kDa; β -amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa; carbonic anhydrase, 29 kDa; and cytochrome c, 12.4 kDa). The absorbance of the protein effluent was monitored at 280 nm (Shimadzu UV-1201).

SDS-PAGE of native 1-H2NA dioxygenases. The purity of protein preparations was evaluated by Coomassie- or silver-stained 12% SDS-PAGE in the presence of molecular mass markers (non-stained marker; Fermentas) by standard methodology (28, 33).

Amino acid sequencing. Protein bands of 55, 45, and 35 kDa were excised from SDS-PAGE of the fraction samples collected from gel filtration chromatography on the basis of 1-H2NA dioxygenase activity and analyzed by mass spectrometry (matrix-assisted laser desorption ionization-time of flight mass spectrometry [MALDI-TOF MS] and tandem MS [MS-MS]).

Mass spectrometry analysis was done at the Proteomics Research Unit, Biomedical Research Foundation, Academy of Athens, under the supervision of G. T. Tsangaris as previously described (48).

Gene cloning. The complete genome sequence of *A. phenanthrenivorans* Sphe3 was analyzed by the Department of Energy (DOE)-Joint Genome Institute (JGI) and published recently (Genomes OnLine Database accession number Gi01675) (21, 30). The identification of the Sphe3 1-H2NA dioxygenase genes was based on BLAST searches within the IMG-ER platform (31), using the sequences of the oligopeptide fragments deduced from the mass spectrometry analysis described above. This search revealed two putative ORFs (open reading frames), each one coding for a putative protein of 387 amino acids with 90% sequence similarity to each other at the nucleotide level. One of the ORFs was found on an indigenous plasmid (pASPHE301) and named *diox1*. The second ORF was located on the chromosome and named *diox2*. Because the two ORFs share strong sequence similarity, primers Diox1F/Diox1R and Diox2F/Diox2R (Table 1) were designed according to sequences flanking *diox1*

TABLE 1 Oligonucleotides used in this study

Designation	Oligonucleotide sequence	Purpose
Diox.1F	5'-AAAGTCCGGGTGCATCGCG-3'	Cloning of flanked genes
Diox.1R	5'-GGTCTCGTCGAGATTGATCG-3'	
Diox.2F	5'-ACGATGAGCTCGACCATGCG-3'	
Diox.2R	5'-CGTTCGCCGGACCTTTACG-3'	
pETdiox.1F	5'-CGAAGGCAACATATGGATTCAATC-3'	
pETdiox.1R	5'-GAGGATCCCATAGTTCATCC-3'	pET29c cloning
pETdiox.2F	5'-CGAAGGAGACCACATATGGATTCA GTC-3'	
pETdiox.2R	5'-CGTGC GG GATCCACAGTTCG-3'	
1mRNA _{for}	5'-GACGCGGGCAACCCCTTA-3'	Quantitative real-time PCR
1mRNA _{rev}	5'-TGATCGGTGACGAACGA-3'	
2mRNA _{for}	5'-TCAGCCGGGAATGAGTT-3'	
2mRNA _{rev}	5'-GTGTCTAGTCTGCATCGT-3'	
gyrβ _{for}	5'-GGCTAACGACAATACAGATA-3'	
gyrβ _{rev}	5'-ACCACTTCATAAACAAGGT-3'	

and *diox2* at either side. Using these primers in a PCR with total DNA from Sphe3 as a template, we obtained 1,373- and 1,408-nt amplification products for the two ORFs, respectively. These fragments were cloned in the vector pCR-blunt (Invitrogen), verified by nucleotide sequence analysis, and used as templates in a PCR for a second amplification of *diox1* and *diox2* in an effort to overexpress their putative products in *E. coli*. For this purpose, a second pair of primers was designed (pETdiox1F/pETdiox1R and pETdiox2F/pETdiox2R [Table 1]) carrying NdeI-BamHI restriction sites, appropriate for subcloning in the expression vector pET29c(+). The amplified fragments were once again cloned in vector pCR-blunt (Invitrogen) and verified by nucleotide sequence analysis. Finally, the *diox1* and *diox2* ORFs were cloned in the pET29c(+) expression vector (Novagen) following restriction with NdeI-BamHI. These constructs were transformed in *E. coli* BL21(DE3) for expression analysis.

The PCR cycles applied here were as follows: an initial step at 95°C for 5 min, followed by 30 cycles consisting of three steps each (denaturation at 95°C for 1 min, annealing at 60°C for 2 min, and extension for 3 min) and a final elongation step at 72°C for 10 min.

Amplification reactions were carried out in a PTC-100 version 7.0 thermocycler (MJ Research Inc.) using the Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) and 1.5 mM MgCl₂ (final concentration). The amplicons produced were purified using NucleoSpin Extract 2 in 1 (Macherey-Nagel, Germany). Molecular cloning was performed using a Zero Blunt Kit (Invitrogen) according to the manufacturer's recommendations. *E. coli* strain DH5α was used as the recombinant plasmid host (13). When *E. coli* strain BL21(DE3) was used as a recipient, transformation was carried out according to the method of Chung and Miller (6). Cloned fragments were sequenced by Macrogen (South Korea). Sequence alignments were performed using the program BLAST (1) at the NCBI website.

Sphe3 genomic DNA was isolated from cells grown on MM M9 containing 0.02% (wt/vol) phenanthrene according to the standard JGI (California) protocol for bacterial genomic DNA isolation using cetyl trimethylammonium bromide (CTAB). Restriction enzyme digestions, ligations, and agarose gel electrophoresis were carried out using standard methodology (40).

DNA sequences were aligned with Lalign software (16). Amino acid sequences exhibiting similarity were retrieved from protein databases and aligned using CLUSTALW (45). Phylogenetic tree and molecular evolutionary analyses were conducted using MEGA version 4 (44).

Purification of recombinant 1-H2NA dioxygenases. An *E. coli* BL21(DE3)-pET29c(+) host-vector overexpression system was used to overproduce the Diox1 and Diox2 polypeptides. Cells containing the pET29c::diox1 or pET29c::diox2 plasmid were incubated in 2 liters LB containing kanamycin sulfate (Kn) (50 μg ml⁻¹). The culture was grown

at 37°C with aeration in an orbital shaker (Forma Scientific) fermentation system. Cultures were induced at an A₆₀₀ of 0.6 by adding 1 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside), and incubation was continued for four additional hours. The cells were harvested by centrifugation, washed with 100 mM Tris-HCl, pH 8.0, and stored at -20°C.

Frozen cells were thawed in the appropriate volume (5 ml · g of cells⁻¹) of 100 mM Tris-HCl, pH 8.0, containing 0.5 M sodium chloride, 20 mM imidazole hydrochloride, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was subjected to ultrasonic treatment (8 pulses/10 s/300 W with interval pauses of 10 s for cooling of the samples on ice) and centrifuged. The supernatant was applied to an Ni²⁺-chelating column (Qiagen) previously equilibrated with 100 mM Tris-HCl, pH 8.0, containing 0.5 M sodium chloride and 20 mM imidazole (flow rate, 2 ml · min⁻¹). The column was washed with 50 ml of 100 mM Tris-HCl, pH 8.0, containing 0.5 M sodium chloride and 20 mM imidazole and run with a gradient of 20 to 100 mM imidazole in 20 ml of 100 mM Tris-HCl, pH 8.0, followed by a gradient of 100 to 500 mM imidazole in 100 mM Tris-HCl, pH 8.0, containing 0.5 M sodium chloride (total volume, 120 ml) (38). Fractions exhibiting 1-H2NA dioxygenase activity were collected, analyzed by SDS-PAGE, pooled, and used for further kinetic analysis. The K_m values were determined in a broad concentration range of substrates varying from 0.005 to 0.15 mM in the presence of 0.1 mM FeSO₄ and 0.1 mM L-ascorbic acid.

RNA isolation and quantitative real-time PCR (qPCR). Total RNA was isolated from 5 ml fresh Sphe3 cultures grown on phenanthrene, glucose, or glucose plus phenanthrene (as the sole carbon source) at the mid-exponential phase by using the NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. Residual DNA was removed by additional treatment with RNase-free DNase I (TaKaRa). RNA integrity was observed by electrophoresis in a 1.2% agarose gel and quantified by measuring the absorbance at 260 nm (Shimadzu UV-1201). Reverse transcription (RT) was performed by using 0.5 μg of hot denatured DNA-free RNA with the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa), using 100 pmol of random hexamer primers and 200 U of PrimeScript reverse transcriptase at a final volume of 20 μl, according to the manufacturer's instructions.

qPCR was performed in 96-well plates on the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) using the Kapa SYBR Fast qPCR Kit Master Mix (2×) Universal. The 20-μl reaction mixtures contained 19 μl of SYBR green master mix (containing the Kapa SYBR DNA polymerase), 200 nM each oligonucleotide primer, and 1 μl of different dilutions of the reverse transcriptase product. The sequences of the primers are shown in Table 1. To determine the specificities of the primer pairs, experiments were initially performed under the optimum conditions determined by conventional PCR. The reaction conditions were as follows: 3 min at 95°C, followed by 40 cycles of 10 s at 95°C, 20 s at 58°C, and 30 s at 72°C. To confirm the accuracy and reproducibility of the qPCR, each sample was analyzed in triplicate. A nontemplate control for each primer pair was included in all real-time plates to detect any possible contamination. Fluorescence measurements were taken during the final temperature ramp. The observation of a unique fluorescence fall at the fusion temperature specific for the PCR product certified its purity.

The exact amount and quality of the total RNA were determined in each sample by quantifying transcripts of the gyrase β (*gyrβ*) gene, a housekeeping gene used as a reference. Each unknown sample was normalized to the *gyrβ* mRNA content. The relative standard curves were plotted using the *diox1*, *diox2*, and *gyrβ* primers amplified with 10, 5, 1, 0.5, 0.2, and 0.1 ng of total RNA from glucose-grown cells (19). qPCR efficiencies were calculated from the given slopes. The corresponding qPCR efficiency (*E*) of one cycle within the exponential phase was calculated according to the following equation: $E = 10^{(-1/\text{slope})}$. The transcripts under investigation exhibited high qPCR efficiency rates, such as 1.76, 1.86, and 1.77 for *diox1*, *diox2*, and *gyrβ*, respectively, within the cDNA input range of 0.1 to 10 ng and with high linearity (correlation

TABLE 2 Purification of 1-H2NA dioxygenase from *A. phenanthrenivorans* strain Sphe3

Purification step	Total activity ($\mu\text{mol}/\text{min}$)	Sp act ($\mu\text{mol}/\text{min}/\text{mg}$)	Yield (%)	Purification (fold)
Crude extract	9.7	1.0	100	1
DEAE-Sepharose	9	2.0	93	2
Phenyl-Sepharose	3.6	5.8	37	5.8
Sephacryl S-200	0.1	15.5	1	15.5

factors, $0.9924 < r^2 < 0.9968$) (7, 35). The qPCR results were analyzed by the relative-quantification method, which calculates the amount of a target transcript in relation to an internal standard (a suitable housekeeping gene, in our case, *gyrB*) within the same sample. Moreover, samples were normalized through a calibrator introduced in each run. The results were expressed as the target/internal-standard concentration ratio of the sample divided by the target/internal-standard concentration ratio of the calibrator (2).

Nucleotide sequence accession numbers. The *diox1* and *diox2* sequences were deposited at EMBL under accession no. [AM931437](#) and [AM931438](#), respectively.

RESULTS

Phenanthrene metabolism. Evidence for the phenanthrene degradation pathway utilized by strain Sphe3 was obtained by assaying the activities of several key enzymes in crude cell extracts after growth on phenanthrene. The enzyme activities of 1-H2NA dioxygenase, 2-carboxybenzaldehyde dehydrogenase, and 4,5-protocatechuate dioxygenase were estimated as 1, 0.2, and $0.1 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, respectively, in cells grown on phenanthrene. However, no 1-H2NA hydroxylase, 3,4-protocatechuate dioxygenase, 2,3-catechol dioxygenase, or salicylate hydroxylase activity was found in these samples. In addition, Sphe3 could grow on 2-carboxybenzaldehyde, protocatechuate, and phthalate as sole carbon sources, all intermediates of phenanthrene degradation via the phthalate pathway (3), whereas no growth was observed on salicylic acid.

Purification of 1-H2NA dioxygenases. 1-H2NA dioxygenases were purified from crude cell extract of strain Sphe3 grown on MM M9 in the presence of phenanthrene as a sole carbon and energy source (Table 2). 1-H2NA dioxygenase activity was not detectable in crude cell extracts from cells grown in the presence of glucose. The active fraction was eluted from a DEAE column (anion exchange) at an Na_2SO_4 concentration of 0.3 M, followed by phenyl-Sepharose hydrophobic interaction chromatography by a declining concentration gradient of Na_2SO_4 . Finally, the active fraction exhibiting 1-H2NA dioxygenase activity was eluted as a single peak equivalent to a molecular mass of 180 kDa by gel filtration chromatography as described in Materials and Methods (Table 2). SDS-PAGE analysis of this fraction showed one major protein band corresponding to a molecular mass of ~ 45 kDa and two minor bands at 55 and 35 kDa, respectively (Fig. 2). The protein bands were excised from the gel and analyzed by mass spectrometry (MALDI-TOF MS and MS-MS). The amino acid sequences of the oligopeptides from the individual 55- and 45-kDa bands, covering up to 18 and 29% of the total sequence, respectively, were quite revealing. BLAST analysis of the 55-kDa band fragments showed 100% sequence identity with parts of (PhnF) dehydrogenase Q9ZHHF from *Burkholderia* sp. strain RP007 (29). Most interestingly, peptides deduced from the 45-kDa band showed 100% sequence identity with parts of 1-H2NA

dioxygenase from *Nocardioide* sp. strain KP7. The peptides from the third band (35 kDa) did not perfectly match any known protein sequence.

Catalytic properties of native 1-H2NA dioxygenases. The fraction obtained from the gel filtration chromatography exhibiting 1-H2NA dioxygenase activity was used for further kinetic studies. The temperature and pH optima of 1-H2NA dioxygenase catalytic activity were 40°C and 8.5, respectively (see Fig. S1 in the supplemental material). The purified enzyme was stable when stored at 4°C for 48 h in the presence of DTT (1 mM). No activity was detected when 3-hydroxy-2-naphthoic acid was used as a substrate at a concentration of 0.1 mM in a 10 mM Tris- H_2SO_4 buffer (pH 7.5) at 25°C .

The effects of chelators on the activity of 1-H2NA dioxygenase were examined in a 10 mM Tris- H_2SO_4 buffer (pH 7.5) containing 0.1 mM 1-H2NA at 25°C . EDTA (at 1 or 10 mM) had no influence on the activity of the enzyme, but inhibition was observed in the presence of *o*-phenanthroline (10 μM)—a chelator specific for ferrous iron. Addition of 0.1 mM FeSO_4 to the *o*-phenanthroline-treated enzyme restored the activity, implying that Sphe3 1-H2NA dioxygenase is iron dependent.

Cloning and heterologous expression of *diox1* and *diox2*. The amino acid sequences deduced from the 45-kDa band were further used in a BLAST search analysis of the strain Sphe3 genome (21). This analysis led to the identification of two putative ORFs in the Sphe3 genome coding for 1-H2NA dioxygenase, thereafter named *diox1*, located on an indigenous plasmid, pASPHE301, and *diox2*, located on the chromosome (Fig. 3).

The predicted protein products of the two putative 1-H2NA dioxygenase genes, *diox1* and *diox2*, shared 93% sequence identity to each other at the amino acid level (90% identity at the nucleotide level) and 85% identity at the amino acid level with 1-H2NA dioxygenase from *Nocardioide* sp. strain KP7. Multiple alignment of Diox1 and Diox2 with other ring cleavage dioxygenases revealed the presence of conserved cupin domains (see Fig. S2 in the supplemental material). Figure 4 shows the phylogenetic analysis of Diox1 and Diox2 and related ring cleavage dioxygenases.

Heterologous expression of Diox1 and Diox2 in *E. coli* BL21 resulted in intense protein bands of ~ 42 kDa, as determined by SDS-PAGE analysis (Fig. 2), corresponding well to the native subunit molecular mass of ~ 45 kDa. Purification of Diox1 and Diox2

FIG 2 SDS-PAGE protein analysis. (A) Purification of native 1-H2NA dioxygenases. Lane M, protein molecular mass markers in kDa; lane 1, cell extract DEAE-Sepharose; lane 2, CL-6B column eluate; lane 3, phenyl Sepharose CL-4B column eluate; lane 4, Sephacryl S-200 column eluate. (B) Purification of overexpressed Diox1 (i) and Diox2 (ii) from *A. phenanthrenivorans*. Lanes M, protein molecular mass markers in kDa; lanes 5 and 7, cell-free crude extract from *E. coli* BL21/pET29c::diox1 (lane 5) and *E. coli* BL21/pET29c::diox2 (lane 7); lanes 6 and 8, samples of the Ni^{2+} -NTA chromatography-purified Diox1 (lane 6) and Diox2 (lane 8) proteins.

FIG 3 Graphical representation of the gene neighborhood of the *diox1* (A) and *diox2* (B) genes in the genome of *A. phenanthrenivorans* sp. nov. strain Sphe3. (A) 1, *diox1*; 2, *trans*-2-carboxybenzalpyruvate hydratase-aldolase; 3, ribulose-5-phosphate-4-epimerase; 4, NAD-dependent aldehyde dehydrogenase; 5, hypothetical proteins; 6, DNA replication protein; 7, transposases. (B) 1, *diox2*; 2, *trans*-2-carboxybenzalpyruvate hydratase-aldolase; 3, arabinose efflux permease; 4, hypothetical proteins; 5, integrase core domain; 6, transposases and hypothetical proteins; 7, transposase IS4 family.

was achieved by a single-step Ni^{2+} -nitrilotriacetic acid (NTA) column, using a step gradient as described in Materials and Methods. Overexpressed protein fractions were eluted at 110 mM imidazole, pooled, analyzed by SDS-PAGE (Fig. 2), and assayed for activity. Both recombinant Diox1 and Diox2 exhibited 1-H2NA dioxygenase activity estimated at $2.44 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $3.17 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, respectively. Their activity increased more than 50% when Fe(II) and ascorbic acid were added to the reaction buffer. The purified Diox1 and Diox2 lost 95% and 60% of their initial activity when they were stored for 1 week at 4°C or -80°C , respectively. Ethanol (10% [vol/vol]), glycerol (20% [vol/vol]), DTT (1 mM), and Fe(II) (0.1 mM) did not stabilize the enzyme during storage.

Catalytic properties of recombinant Diox1 and Diox2. The apparent K_m of Diox1 and Diox2 for 1-H2NA estimated at 25°C were $35 \pm 5.7 \mu\text{M}$ and $29 \pm 5.8 \mu\text{M}$, respectively. In addition, the turnover numbers were similar, exhibiting K_{cat} values of 385 s^{-1} and 358 s^{-1} for Diox1 and Diox2, respectively, whereas the K_{cat}/K_m values were $11 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $12 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, respectively. The presence of $10 \mu\text{M}$ *o*-phenanthroline completely inactivated both Diox1 and Diox2 polypeptides, whereas the presence of Fe(II) restored the activities to their initial levels. Concentrations of ferrous ion exceeding 0.1 mM caused a decrease in the activity of both Diox1 and Diox2 homologues.

Transcription of *diox1* and *diox2*. Using qPCR, we were able to distinguish quantitative differences in the relative transcripts of the two genes encoding 1-H2NA dioxygenase in strain Sphe3. Expression of the *diox1* and *diox2* genes was measured in cultures of Sphe3 on M9 with glucose, phenanthrene, or glucose plus phenanthrene as the sole carbon source. The gyrase β (*gyrB*) housekeeping gene was used as a reference gene. Both genes were induced in cells grown on phenanthrene, whereas only minimal transcription was detected in cultures grown in the presence of glucose or glucose plus phenanthrene. Interestingly, *diox2* up-regulation was about twice as much as *diox1* (69-fold versus 34-fold, respectively).

FIG 4 Phylogenetic tree of Diox1, Diox2, and other related ring cleavage dioxygenases. The bootstrap values are indicated at the nodes; accession numbers are in parentheses. Diox1, *A. phenanthrenivorans* 1-hydroxy-2-naphthoic acid dioxygenase (YP_004243241.1); Diox2, *A. phenanthrenivorans* 1-hydroxy-2-naphthoic acid dioxygenase (YP_004241483.1); Noc_HNDO, *Nocardia* sp. KP7 1-hydroxy-2-naphthoic acid dioxygenase (BAA31235.2); Myc_HNDO, *Mycobacterium* sp. CH1 1-hydroxy-2-naphthoic acid dioxygenase (ACN38281.1); B.hal_GDO, *Bacillus halodulcerans* C-125 gentisate 1,2-dioxygenase (NP242868.1); P.sal_SDO, *Pseudomonas salicylatoxidans* salicylate 1,2-dioxygenase (AAQ91293.1); X.pol_GDO: *Xanthobacter polyaromaticivorans* gentisate 1,2-dioxygenase (BAC98955.1); E.col_GDO, *E. coli* O157:H7 strain EDL933 gentisate 1,2-dioxygenase (Q8X655); B.cen_GDO, *Burkholderia cenocepacia* MCO-3 gentisate 1,2-dioxygenase (YP_001777018.1); A.aven_GDO, *Acidovorax avenae* ATCC 19860 gentisate 1,2-dioxygenase (YP_004232675.1).

DISCUSSION

Two 1-H2NA dioxygenase homologous genes were identified in *A. phenanthrenivorans* strain Sphe3 involved in the degradation of phenanthrene via the *o*-phthalate pathway. Strain Sphe3 catabolizes phenanthrene at concentrations up to $400 \text{ mg} \cdot \text{liter}^{-1}$ as a sole source of carbon and energy at higher rates than those reported for any other member of the genus (22).

Phenanthrene degradation by two distinct routes, via either phthalate or salicylate, has been well documented (17, 39). Both pathways involve the formation of 1-hydroxy-2-naphthoic acid, which is further metabolized via either dioxygenation and subsequent ring opening to *trans*-2-carboxybenzalpyruvate as previously reported for *Nocardioide*s and *Mycobacterium* (18, 43) or decarboxylation and hydroxylation to produce naphthalene-1,2-diol (36, 37). Little is known about PAH degradation within the genus *Arthrobacter* (39). Recently, Seo et al. (41) reported that phenanthrene degradation by *Arthrobacter* sp. strain P1-1 occurred via a dominant phthalic acid or a minor salicylic acid pathway (41). Our results suggest that Sphe3 metabolizes 1-H2NA via dioxygenation and subsequent ring opening to *trans*-2-carboxybenzalpyruvate. This pathway is evident (i) from the detection of 1-H2NA dioxygenase and 2-carboxybenzaldehyde dehydrogenase activities in phenanthrene-grown cells and (ii) from the absence of 1-H2NA hydroxylase or salicylate hydroxylase activity, enzymes involved in the decarboxylation and hydroxylation of 1-H2NA via the salicylate pathway. In addition, Sphe3 cells could grow on 1-H2NA, 2-carboxybenzaldehyde, protocatechuic acid, and phthalic acid as sole C sources, whereas no growth was observed on salicylic acid. Phenanthrene catabolism by strain Sphe3 via the phthalate pathway is also confirmed, since putative operons containing genes that encode enzymes involved in the degradation of phthalic acid and protocatechuic acid have been

found in its total genome (21). It is likely that the enzymes involved in phenanthrene degradation are expressed only in the presence of phenanthrene, as no activity was detected in glucose-grown cells. These results are consistent with a previous report demonstrating that phenanthrene can cross the membranes of Sphe3 cells by two mechanisms: (i) a low-activity passive-diffusion system also detected in glucose-grown cells and (ii) a phenanthrene-inducible active transport dependent on proton motive force detected in phenanthrene-grown cells (22).

Our data demonstrate that Sphe3 cells, when grown in the presence of phenanthrene as a sole carbon source, produce a 180-kDa 1-H2NA dioxygenase, as evaluated by gel filtration chromatography. The subunit molecular mass of the enzyme was determined to be 45 kDa, indicating that the enzyme apparently should be a homotetramer (Fig. 2), which is similar in subunit molecular mass (as evaluated by SDS-PAGE) to a previously reported 1-H2NA dioxygenase from *Nocardioides* sp. strain KP7 that also degrades phenanthrene via the *o*-phthalate pathway (18). However, the *Nocardioides* enzyme was found to be a homohexamer with a native molecular mass of 270 kDa (18). Very recently, Deveryshetty and Phale (8) reported the purification of 1-H2NA dioxygenase from *Pseudomonas* sp. strain PPD. The mass of the denatured enzyme was 39 kDa, and the molecular mass of the native proteins was 160 kDa, indicating that the enzyme was also a homotetramer. Apparently, the native enzyme isolated from column chromatography is a mixture of the products of two homologous genes, one plasmid and one chromosomal. The catalytic properties of the polypeptides of the two genes, both overexpressed in *E. coli* and purified, were found to be similar, with the chromosomally encoded protein exhibiting higher substrate affinity. Moreover, no substrate inhibition was found for concentrations up to 150 μ M. Generally, this result is in agreement with those reported for *Nocardioides* sp. KP7 (18), whereas Deveryshetty and Phale (8) have shown that the enzyme from strain PPD exhibited substrate inhibition at a concentration over 25 μ M, with a K_i value of 116 μ M. The Sphe3 enzyme was specific for 1-H2NA, since no activity was determined using 3-H2NA as a substrate. The specificity of Sphe3 1-H2NA dioxygenase for 1-H2NA is in agreement with what was found previously in *Nocardioides* sp. KP7 and *Pseudomonas* sp. strain PPD (8, 18). The K_m values of Diox1 and Diox2 were 2-fold and 3-fold higher, respectively, than previously reported for those of *Nocardioides* sp. KP7 and *Pseudomonas* sp. strain PPD, whereas the specificity constants (K_{cat}/K_m) of both recombinant enzymes were about 2-fold higher (8, 18). In addition, 1-H2NA dioxygenase from strain Sphe3 required Fe(II), as previously reported for its *Nocardioides* sp. KP7 and *Pseudomonas* sp. strain PPD homologues (8, 18). 1-H2NA dioxygenase seems to be a member of the cupin family of proteins, which contain a set of motifs, a metal-binding motif, G(X)₅HXH(X)_{3,4}E(X)₆G, and a second motif, G(X)₅PXG(X)₂H(X)₃N. Two histidines (HXH) in the first motif and a histidine in the second motif, essential for dioxygenase activity, were completely conserved in both Diox1 and Diox2 (see Fig. S2 in the supplemental material) (15).

diox1 (plasmid) and *diox2* (chromosomal), although differentially expressed, are both induced in the presence of phenanthrene and are both subject to carbon catabolite repression. The individual dioxygenase activities of both recombinant enzymes were verified but were found to be lower than those of their native equivalents, even after the addition of Fe(II). Protein misfolding, in combination with the presence of a His tag on the carboxyl end of

the recombinant enzymes, could be a plausible explanation of the lower activities of the individually overexpressed enzymes. Furthermore, the native enzymes are partially purified, and their higher specific activities may be due to coeluting unidentified cofactors. The difference in the -10 promoter sequences predicted for the two genes may account for the higher expression of the chromosomal homologue *diox2* (see Fig. S3 in the supplemental material), but this remains to be investigated. Nevertheless, induction of PAH-degrading enzymes in the presence of their substrate apparently is a common occurrence in nature, as confirmed recently with the increase in the pool of dioxygenase genes in environments spiked with phenanthrene (9). This observation, in combination with the significant sequence conservation between the two 1-H2NA dioxygenase genes identified in the Sphe3 genome at the nucleotide level, strongly indicates that induction, along with the collateral existence of multiple copies of PAH degradation genes as a result of replicative transposition events, facilitates the versatile adaptation of microorganisms to more efficient degradation of organic pollutants. The syntenic occurrence of the 1-H2NA genes, regardless of whether they are found on the chromosome or on a plasmid, with a proximal *trans*-2'-carboxybenzalpyruvate hydratase-aldolase, an enzyme involved in the transformation of *trans*-2'-carboxybenzalpyruvate (the product of 1-H2NA oxidation), and transposase/integrase genes in close proximity in both locations (Fig. 3) are rather supportive of a replicative transposition event. Additionally, in strain Sphe3, both the *diox1* and *diox2* genes do not cluster with all the structural genes required for phenanthrene degradation, as reported for aromatic degradation by Gram-negative bacteria (14). Our results are similar to those published for *Mycobacterium vanbaaleenii* PYR-1, where genes involved in particular degradation processes are dispersed throughout several different gene clusters (25). In any case, the occurrence of two copies of 1-H2NA dioxygenase in Sphe3 renders the strain more advantageous for PAH biodegradation and hence more adjustable to polluted environments. It is well known that gene duplications provide an advantage for microbial adaptation to environmental changes (12). Our present results demonstrate that *A. phenanthrenivorans* isolated from creosote-polluted soil provides an evolutionary demonstration that such duplications facilitate survival in contaminated environments.

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