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

Elpniiki Vandera,a Konstantinos Kavakiotis,a Aristeidis Kallimanis,a Nikos C. Kyripides,b Constantin Drainas,a† and Anna-Irini Koukkoua

Sector of Organic Chemistry and Biochemistry, University of Ioannina, Ioannina, Greece,a and Genome Biology Program, Department of Energy Joint Genome Institute, Walnut Creek, California, USAa

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 *Nocardioides* 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 *Kₘ* of 35 μM for *Diox1* and 29 μM for *Diox2*, whereas they showed similar kinetic turnover characteristics with *Kₗ/ₚ₃* values of 11 × 10⁻⁶ M⁻¹ s⁻¹ and 12 × 10⁻⁶ M⁻¹ 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 protocatechucic 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 *Nocardioides* 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 catalyzing phenanthrene up to 400 mg·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 medium (21) was used for culturing. The molar concentration of *L*-arginine was increased to 0.3 M for *E. coli* cells expressing 1-H2NA dioxygenase activity. *E. coli* strain DH5α was grown at 37°C for 24 h in LB broth (1 M NaCl, 10 g/L tryptone, 5 g/L yeast extract), and *E. coli* strain BL21 (DE3) was grown at 37°C for 24 h in LB broth (1 M NaCl, 10 g/L tryptone, 5 g/L yeast extract) supplemented with 25 μg/ml ampicillin. The bacteria were grown until the optical density at 600 nm reached 0.6. The cultures were induced with 1 mM isopropyl β-D-thiogalactopyranoside, and cells were harvested after 16 h.

**Cloning, expression, and purification of 1-H2NA dioxygenase.** *E. coli* strain BL21 cells transformed with pET22b-diox1 were grown in 2 L of LB broth (1 M NaCl, 10 g/L tryptone, 5 g/L yeast extract), and cells were harvested after 16 h. 

**Received 7 October 2011 Accepted 8 November 2011**

**Published ahead of print 18 November 2011**

Address correspondence to Anna-Irini Koukkou, akukku@cc.uoi.gr.

†Deceased 5 July 2011.

E. Vandera and K. Kavakiotis contributed equally to this article.

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

Supplemental material for this article may be found at http://aem.asm.org/

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

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.

Preparation of cell extracts. Cells were harvested by centrifugation (6,000 × g; 20 min) at 4°C; washed with 10 mM Tris-H$_2$SO$_4$ 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 $[\varepsilon_{300}] = 11.5$ mM$^{-1}$ cm$^{-1}$) in 10 mM Tris-H$_2$SO$_4$ (pH 7.5) containing 0.1 mM 1-hydroxy-2-naphthoate as described by Iwabuchi and Harayama (18). 2-Carboxybenzaldehyde dehydrogenase, 4,5-protocatechuate dioxygenase, 3,4-protocatechuate dioxygenase, 1-hydroxy-2-naphthoate hydroxylase, catechol 2,3-dioxygenase, and salicylylate 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 Na$_2$SO$_4$ in 120 ml of 10 mM Tris-H$_2$SO$_4$ buffer (pH 7.5) containing 1 mM DTT at a flow rate of 2 ml·min$^{-1}$. Active fractions were pooled and dialyzed against 10 mM Tris-H$_2$SO$_4$ buffer (pH 7.5) containing 1 mM DTT. The dialyzed fractions were adjusted to 0.5 M (NH$_4$)$_2$SO$_4$ 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-H$_2$SO$_4$ buffer (pH 7.5) containing 1 mM DTT and 0.5 M Na$_2$SO$_4$. Proteins were eluted from the column by a linear gradient of 0.5 to 0.0 M Na$_2$SO$_4$ in 120 ml of 10 mM Tris-H$_2$SO$_4$ buffer (pH 7.5) containing 1 mM DTT at a flow rate of 1 ml·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-H$_2$SO$_4$ buffer (pH 7.5) containing 1 mM DTT and 100 mM Na$_2$SO$_4$ at a flow rate of 0.5 ml·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 Gi016753) (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) excised from SDS-PAGE of the fraction samples collected from gel filtration chromatography on the basis of 1-H2NA dioxygenase activity and assigned 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).
**TABLE 1 Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Oligonucleotide sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diox.1F</td>
<td>5’-AAAGTCGGGCTGATCGG-3’</td>
<td>Cloning of flanked genes</td>
</tr>
<tr>
<td>Diox.1R</td>
<td>5’-GGCTGCGTGAGTTATGCG-3’</td>
<td></td>
</tr>
<tr>
<td>Diox.2F</td>
<td>5’-ACGATGACCTGCACTATTGG-3’</td>
<td></td>
</tr>
<tr>
<td>Diox.2R</td>
<td>5’-GGCTCCGCTGACCTTGG-3’</td>
<td></td>
</tr>
<tr>
<td>pETdiox.1F</td>
<td>5’-GAAGAGCCATATGATGCGATTC-3’</td>
<td>Cloning</td>
</tr>
<tr>
<td>pETdiox.1R</td>
<td>5’-GGAGATTCCATGCCTAGCGC-3’</td>
<td></td>
</tr>
<tr>
<td>pETdiox.2F</td>
<td>5’-GAAGAGCCATATGATGCGC-3’</td>
<td></td>
</tr>
<tr>
<td>pETdiox.2R</td>
<td>5’-CGAAGGAGACCACATATGGATTCA-3’</td>
<td></td>
</tr>
</tbody>
</table>

and diox2 at either side. Using these primers in a PCR with total DNA from Sphec as a template, we obtained 1,373- and 1,408-nl 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) 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 into 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 Reseurch Inc.) using the Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) and 1.5 mM MgCl2 (final concentration). The amplicons 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.

Sphec genomic DNA was isolated from cells grown on MM M9 containing 0.02% (wt/vol) phenanthrene according to the standard IG (California) protocol for bacterial genomic DNA isolation using cetyl trimethylammonium bromide (CTAB). Restriction enzyme digestions, ligation, 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 strain 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 (Km) (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 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 ultrasonication 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 Ni⁺-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 Kₘ 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 Sphec 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 (Machery-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 (2x) 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 reactions were analyzed in triplicate and normalized to the gyrB1 mRNA content. The relative standard curves were plotted using the diox1, diox2, and gyrB1 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/slope. The transcripts under investigation exhibited high qPCR efficiency rates, such as 1.76, 1.86, and 1.77 for diox1, diox2, and gyrB1, 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

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (μmol/min)</th>
<th>Sp act (μmol/min/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>9.7</td>
<td>1.0</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>9</td>
<td>2.0</td>
<td>93</td>
<td>2</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>3.6</td>
<td>5.8</td>
<td>37</td>
<td>5.8</td>
</tr>
<tr>
<td>Sephacryl S-200</td>
<td>0.1</td>
<td>15.5</td>
<td>1</td>
<td>15.5</td>
</tr>
</tbody>
</table>

Factors, 0.9924 < r² < 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-protocatechuic acid oxygenase were estimated as 1, 0.2, and 0.1 μmol·min⁻¹·mg⁻¹, respectively, in cells grown on phenanthrene. However, no 1-H2NA hydroxylase, 3,4-protocatechuic acid oxygenase, 2,3-catechol dioxygenase, or salicylate hydroxylase activity was found in these samples. In addition, Sphe3 could grow on 2-carboxybenzaldehyde, protocatechuic, 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 Na₂SO₄ concentration of 0.3 M, followed by phenyl-Sepharose hydrophobic interaction chromatography by a declining concentration gradient of Na₂SO₄. 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 1-H2NA dioxygenase from *Nocardoides* 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-H₂SO₄ 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-H₂SO₄ 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-phenanthrolene (10 μM)—a chelator specific for ferrous iron. Addition of 0.1 mM FeSO₄ to the o-phenanthrolene-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 *Nocardoides* 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** and *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*
was achieved by a single-step Ni\textsuperscript{2+}-nitrilotriacetate 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 μmol · min\textsuperscript{-1} · mg\textsuperscript{-1} and 3.17 μmol · min\textsuperscript{-1} · mg\textsuperscript{-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 ± 5.7 μM and 29 ± 5.8 μM, respectively. In addition, the turnover numbers were similar, exhibiting \( k_{cat} \) values of 385 s\textsuperscript{-1} and 358 s\textsuperscript{-1} for Diox1 and Diox2, respectively, whereas the \( K_{cat}/K_m \) values were \( 11 \times 10^6 \) M\textsuperscript{-1} s\textsuperscript{-1} and \( 12 \times 10^6 \) M\textsuperscript{-1} s\textsuperscript{-1}, respectively. The presence of 10 μM 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 \textit{diox1} and \textit{diox2} genes was measured in cultures of Sphe3 on M9 with glucose, phenanthrene, or glucose plus phenanthrene as the sole carbon source. The gyrase \( \beta \) (gyr\( \beta \)) 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, \textit{diox2} up-regulation was about twice as much as \textit{diox1} (69-fold versus 34-fold, respectively).

**DISCUSSION**

Two 1-H2NA dioxygenase homologous genes were identified in \textit{A. phenanthrenivorans} strain Sphe3 involved in the degradation of phenanthrene via the o-phthalate pathway. Strain Sphe3 catalyzes phenanthrene at concentrations up to 400 mg · liter\textsuperscript{-1} as a sole source of carbon and energy up 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 \textit{trans-2-carboxybenzalpyruvate} as previously reported for \textit{Nocardioides} and \textit{Mycobacterium} (18, 43) or decarboxylation and hydroxylation to produce naphthalene-1,2-diol (36, 37). Little is known about PAH degradation within the genus \textit{Arthrobacter} (39). Recently, Seo et al. (41) reported that phenanthrene degradation by \textit{Arthrobacter} sp. strain P1-1 occurred via a dominant phthlic acid or a minor salicylic acid pathway (41). Our results suggest that Sphe3 metabolizes 1-H2NA via dioxygenation and subsequent ring opening to \textit{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, protocatechue 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 protocatechue 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 p-phenylthylate 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 μM. 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 μM, with a K\text{m} value of 116 μM. 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\text{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\text{cat}/K\text{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)_4HXXH(X)_3E(X)_2G, and a second motif, G(X)_2PXXG(X)_2H(X)_2N. Two histidines (HXXH) 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 vanbaalenii 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.

ACKNOWLEDGMENTS

This research project was cofunded by the European Union-European Social Fund (ESF) and National Sources in the framework of the program “HRALKLEITOS II” of the “Operational Program Education and Life Long Learning” of the Hellenic Ministry of Education, Life Long Learning and Religious Affairs. N.C.K. is supported by the U.S. Department of Energy’s Office of Science, Biological and Environmental Research Program, and by the University of California, Lawrence Berkeley National Laboratory, under contract no. DE-AC02-05CH11231; by the Lawrence Livermore National Laboratory under contract no. DE-AC52-07NA27344; and by the Los Alamos National Laboratory under contract no. DE-AC02-06NA25396.

We thank J. Samelis of the National Agricultural Research Foundation, Dairy Research Institute, Katsikas, Ioannina, Greece, for providing the real-time PCR facilities and E. Papamichael, University of Ioannina, for his contribution to the determination of kinetics constants. Mass spectrometry analysis was performed at the Proteomics Research Unit, Biomedical Research Foundation, Academy of Athens, under the supervision of G. T. Tsangaris.
REFERENCES


Downloaded from http://aem.asm.org/ on August 15, 2017 by guest