J Ind Microbiol Biotechnol (2007) 34:311–324
DOI 10.1007/s10295-006-0199-8

ORIGINAL PAPER

Purification, characterization, and crystallization of the components of a biphenyl dioxygenase system from *Sphingobium yanoikuyae* B1

C. L. Yu · W. Liu · D. J. Ferraro · E. N. Brown · J. V. Parales · S. Ramaswamy · G. J. Zylstra · D. T. Gibson · R. E. Parales

Received: 20 September 2006 / Accepted: 5 December 2006 / Published online: 9 January 2007

Abstract *Sphingobium yanoikuyae* B1 initiates the catabolism of biphenyl by adding dioxygen to the aromatic nucleus to form (+)-*cis*-(2*R*, 3*S*)-dihydroxy-1-phenylcyclohexa-4,6-diene. The present study focuses on the biphenyl 2,3-dioxygenase system, which catalyzes the dioxygenation reaction. This enzyme has been shown to have a broad substrate range, catalyzing the dioxygenation of not only biphenyl, but also three- and four-ring polycyclic aromatic hydrocarbons. Extracts prepared from biphenyl-grown B1 cells contained three protein components that were required for the oxidation of biphenyl. The genes encoding the three components (*bphA4, bphA3* and *bphA1f,A2f*) were expressed in *Escherichia coli*. Biotransformations of biphenyl, naphthalene, phenanthrene, and benzo[a]pyrene as substrates using the recombinant *E. coli* strain resulted in the formation of the expected *cis*-dihydrodiol products previously shown to be produced by biphenyl-induced strain B1. The three protein components were purified to apparent homogeneity and characterized in detail. The reductase component (*bphA4*), designated reductase$_{BPH-B1}$, was a 43 kD monomer containing one mol FAD/mol reductase$_{BPH-B1}$. The ferredoxin component (*bphA3*), designated ferredoxin$_{BPH-B1}$, was a 12 kD monomer containing approximately 2 g-atoms each of iron and acid-labile sulfur. The oxygenase component (*bphA1f,A2f*), designated oxygenase$_{BPH-B1}$, was a 217 kD heterotrimer consisting of α and β subunits (approximately 51 and 21 kD, respectively). The iron and acid-labile sulfur contents of oxygenase$_{BPH-B1}$ per αβ were 2.4 and 1.8 g-atom per mol, respectively. Reduced ferredoxin$_{BPH-B1}$ and oxygenase$_{BPH-B1}$ each gave EPR signals typical of Rieske [2Fe-2S] proteins. Crystals of reductase$_{BPH-B1}$, ferredoxin$_{BPH-B1}$ and oxygenase$_{BPH-B1}$ diffracted to 2.5 Å, 2.0 Å and 1.75 Å, respectively. The structures of the three proteins are currently being determined.

Keywords Biphenyl · Dioxygenase · Biodegradation · Protein purification · Rieske non-heme iron oxygenase

C. L. Yu · W. Liu · D. T. Gibson
Department of Microbiology and Center for Biocatalysis and Bioprocessing, The University of Iowa, Iowa City, IA 52242, USA

D. J. Ferraro · E. N. Brown · S. Ramaswamy
Department of Biochemistry, The University of Iowa, Iowa City, IA 52242, USA

S. Ramaswamy
Department of Chemical and Biochemical Engineering, The University of Iowa, Iowa City, IA 52242, USA

G. J. Zylstra
Biotechnology Center for Agriculture and the Environment, Rutgers University, New Brunswick, NJ 08901, USA

J. V. Parales · R. E. Parales (✉)
Section of Microbiology, University of California, Davis, CA 95616, USA
e-mail: reparales@ucdavis.edu

Introduction

Sphingobium yanoikuyae B1 [22] (formerly *Beijerinckia* sp. strain B1 and *Sphingomonas yanoikuyae* B1[36, 60]) grows with biphenyl as its sole source of carbon and energy. The first step in the degradation of biphenyl by B1 is catalyzed by biphenyl 2,3-dioxygenase (BPDO), which catalyzes the stereospecific addition

of dioxygen to one of the aromatic rings to form (+)-*cis*-(2*R*,3*S*)-dihydroxy-1-phenylcyclohexa-4,6-diene (biphenyl *cis*-dihydrodiol). This reaction was established with *S. yanoikuyae* B8/36, a mutant lacking biphenyl *cis*-dihydrodiol dehydrogenase [22]. Strain B8/36 oxidizes several aromatic substrates to *cis*-dihydrodiols including the polycyclic aromatic hydrocarbons naphthalene [16], anthracene [1, 34], phenanthrene [34], chrysene [7], benz[a]anthracene [23, 35] and benzo[a]pyrene [23]. The major *cis*-dihydrodiols formed from these substrates are shown in Fig. 1. Interest in these products stems from the fact that *cis*-dihydrodiols formed from these aromatic hydrocarbons and related

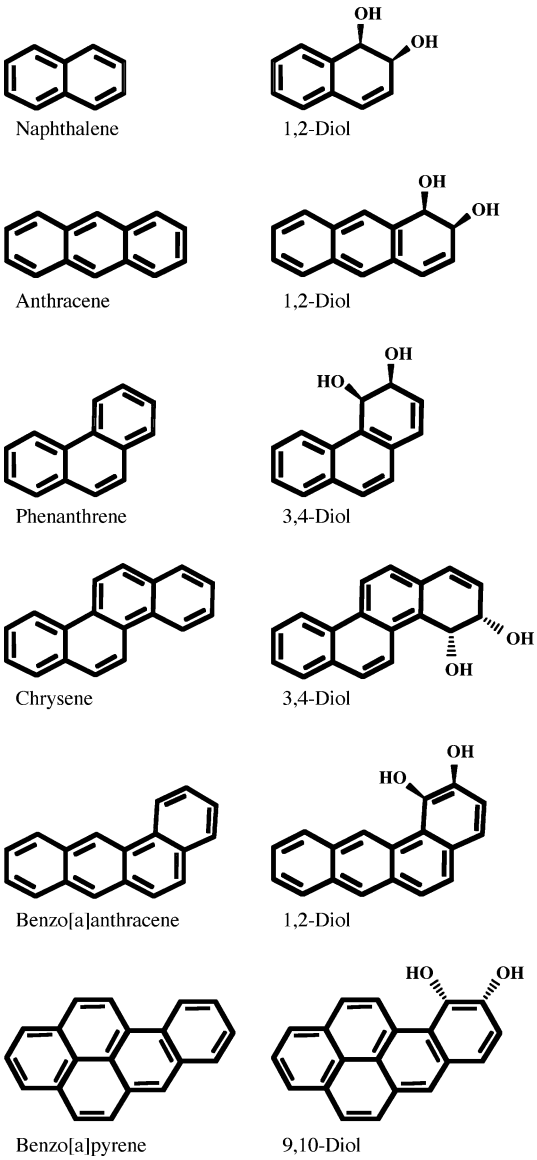

Fig. 1 Major *cis*-dihydrodiols formed from polycyclic aromatic hydrocarbons by *S. yanoikuyae* B1

substrates [5, 6, 8, 11, 16, 50, 53] are usually single enantiomers that are attractive intermediates for the asymmetric synthesis of biologically active molecules [29]. The above metabolic activities were observed in induced cells of strains B1 and B8/36 after growth in the presence of biphenyl, *m*-xylene, or salicylate [20], which could result from the presence of either a single inducible enzyme system with relaxed substrate specificity or multiple enzymes with overlapping substrate specificities. The potential utility of an enzyme capable of producing such a wide range of chiral *cis*-dihydrodiols prompted the present studies to identify and characterize the enzyme system responsible. However, the situation in strain B1 is complicated by the presence of multiple genes encoding different dioxygenase α and β subunits in its genome [64, 65]. The genes are located in a large complex chromosomal gene cluster that appears to encode enzymes for various steps in naphthalene, biphenyl, xylene, and toluate degradation. Such a complex gene arrangement seems to be characteristic of other related aromatic hydrocarbon degrading sphingomonads such as *Novosphingomoans aromaticivorans* F199 (formerly *Sphingomonas aromaticivorans* F199 [60]), and *Sphingobium* sp. strain P2 [48, 52]. One set of dioxygenase genes in B1 (*xylXY*) was shown to encode the oxygenase component of toluate dioxygenase [64], and one gene pair (*bphA2cA1c*) was recently shown to encode the oxygenase component of salicylate 1-hydroxylase [12]. However, individual inactivation of each of the dioxygenase gene pairs did not result in loss of the ability to oxidize biphenyl [64]. Interestingly, only single ferredoxin (*bphA3*), ferredoxin reductase (*bphA4*), and *cis*-dihydrodiol dehydrogenase (*bphB*) genes were found in the 40-kb region [37], a situation also seen for the plasmid-encoded cluster in *N. aromaticivorans* F199 [52]. Several studies have demonstrated that BphA3 and BphA4 are shared by multiple dioxygenase systems in this strain [2, 12, 37]. The genes encoding BPDO were finally identified following transposon mutagenesis of strain B1 and identification of a mutant unable to catalyze the oxidation of biphenyl or naphthalene. The BPDO genes (*bphA1fA2f*) were located in a cluster of putative aromatic compound degra-dation genes. When *bphA1fA2f* was co-expressed in *E. coli* with *bphA3A4*, the enzyme catalyzed the conversion of indole to indigo and the production of *cis*-dihydrodiols from naphthalene and biphenyl (G. J. Zylstra, unpublished data). These results suggest that this single oxygenase component (BphA1fA2f), together with shared reductase and ferredoxin components, catalyzes *cis*-dihydrodiol formation from polycyclic and heterocyclic aromatic hydrocarbons. In this study we

purified and characterized the three components of BPDO from recombinant *E. coli* strains expressing *bphA3A4* and *bphA1fA2f* and show that this oxygenase system oxidizes biphenyl, naphthalene, phenanthrene, and benzo[a]pyrene to the same *cis*-dihydrodiols formed by biphenyl-induced cells of *S. yanoikuyae* B1.

Materials and methods

Bacterial strains and growth conditions

Recombinant *E. coli* strains carrying cloned genes from strain B1 (G. J. Zylstra, unpublished data) were used for the expression and purification of oxygenase$_{BPH-B1}$, reductase$_{BPH-B1}$ and ferredoxin$_{BPH-B1}$. *E. coli* BL21star (Invitrogen, Inc., Carlsbad, CA, USA) carrying (pET101/D/*bphA1fA2f*), was used for the purification of oxygenase$_{BPH-B1}$. In this strain, the genes *bphA1fA2f* encoding the large and small subunits of oxygenase$_{BPH-B1}$ from strain B1 were cloned on the expression vector pET101/D (Invitrogen, Inc.). *E. coli* BL21(DE3)(pT7-7/*bphA3A4*) was used for the isolation of ferredoxin$_{BPH-B1}$ and reductase$_{BPH-B1}$. The plasmid pAlter-Ex2/*bphA3A4*, and vector pT7-7 [58] were digested with *Eco*RI and *Hin*dIII. Insertion of *bphA3A4* into pT7-7 gave plasmid pT7-7/*bphA3A4*, in which *bphA3A4* is under the control of the T7 promoter. *E. coli* BL21star(pET101D/*bphA1fA2f*) and *E. coli* BL21(DE3)(pT7-7/*bphA4A3*) were maintained on Luria-Bertani (LB) [15] plates containing 1.8% Bacto agar (Becton Dickinson, Sparks, MD, USA) and 100 μg/mL ampicillin.

For oxygenase$_{BPH-B1}$ purification, *E. coli* BL21star (pET101D/*bphA1fA2f*) was grown at 28°C in 9 l of 2× LB medium (Life Technologies, Rockville, MD, USA) containing 100 μg/ml ampicillin using a Biostat B-10 fermentor (B. Braun, Bethlehem, PA, USA). Air was supplied at a rate of 1-2 l/min and agitation at 400 rpm. Cells were grown to early exponential phase (OD_{660} = 0.5–0.7), at which time isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium (500 μM final concentration). Cells were incubated for an additional 4 h at 25°C and then harvested by centrifugation (14,000×g at 4°C for 15 min). For reductase$_{BPH-B1}$ and ferredoxin$_{BPH-B1}$ purification, *E. coli* BL21(DE3)(pT7–7/*bphA4A3*) was grown in LB medium containing 100 μg/ml ampicillin at 37°C with air supplied at a rate of 1–2 l/min and agitation at 400 rpm. When the culture reached mid-exponential phase (turbidity at 660 nm = 0.6–0.8), the temperature was reduced to 28°C and IPTG was added to a final concentration of 300 μM. After 4 h, cells were harvested by centrifugation. Cell pellets were resuspended in an equal volume of BTGD buffer (50 mM Bis-Tris [pH 6.8], 5% glycerol, 1 mM sodium dithio-threitol) and stored at −70°C.

Preparation of cell extracts and initial fractionation

Purification procedures were performed at 4°C using an automated FPLC system (Bio-Rad Laboratories, Hercules, CA, USA). Chromatography columns and column resins were from Amersham Biosciences, Piscataway, NJ, except for the ceramic hydroxyapatite (Bio-Rad Laboratories). DNase I (final concentration 0.01 mg/ml) was added to thawed cell suspensions of recombinant *E. coli* cultures. The cell suspensions were passed through a chilled French pressure cell, maintaining an internal cell pressure of approximately 20,000 psi. Cell debris and membranes were removed by centrifugation at 145,000×g for 60 min at 6°C. Cell extracts were applied to a XK50/30 column containing approximately 500 ml (bed volume) of a Q-Sepharose FF, pre-equilibrated with 1,500 ml of BTGD. Unbound proteins were eluted from the column with 500 ml of the same buffer at a rate of 2.0 ml/min. Bound proteins were eluted with a linear gradient from 0 to 0.6 M KCl in BTGD buffer at the same flow rate and 20 ml fractions were collected.

Purification of oxygenase$_{BPH-B1}$

Q-Sepharose fractions exhibiting oxygenase$_{BPH-B1}$ activity were pooled and concentrated under nitrogen by ultrafiltration with a 100 kDa-cut off membrane (Amico, Danvers, Mass). The concentrated fraction was adjusted to 0.8 M ammonium sulfate in BTGD buffer. After 1 h, the precipitate was removed by centrifugation at 14,000×g for 30 min. The supernatant was applied to a XK26/40 chromatography column containing 80 ml (bed volume) of butyl-sepharose that had been pre-equilibrated with 0.8 M ammonium sulfate in BTGD buffer. Unbound proteins were eluted at a flow rate of 1.0 ml/min with the same buffer. Bound proteins were eluted with a linear gradient from 0.8–0 M ammonium sulfate at the same flow rate. Fractions exhibiting oxygenase$_{BPH-B1}$ activity were combined and concentrated, and the buffer was exchanged to 5 mM potassium phosphate buffer, pH 6.8, by ultrafiltration under N_2 with a YM100 membrane. The concentrated protein solution was applied to a XK16/70 column containing 80 ml (bed volume) of hydroxyapatite that had been pre-equilibrated with 5 mM phosphate buffer (pH 6.8). The column was washed with 100 ml of the same buffer, and bound proteins were eluted from the

column with a linear gradient of 5–200 mM phosphate buffer, pH 6.8, at a flow rate of 1 ml/min. Fractions containing oxygenase$_{BPH-B1}$ were pooled and concentrated by ultrafiltration as described above. Purified oxygenase$_{BPH-B1}$ was frozen in liquid nitrogen and stored at −70°C.

Purification of reductase$_{BPH-B1}$

Q-Sepharose fractions exhibiting reductase$_{BPH-B1}$ activity were pooled and concentrated using an Amicon ultrafiltration system equipped with a YM30 membrane. Ammonium sulfate was added to the protein solution to a final concentration of 1.0 M, incubated for 1 h, and centrifuged for 30 min at 14,000×g. The supernatant was loaded (0.5 ml/min) onto a XK26/40 column containing 80 ml (bed volume) of butyl-sepharose pre-equilibrated with 400 ml BTGD buffer containing 0.85 M ammonium sulfate. Unbound protein was eluted at a flow rate of 0.7 ml/min with 80 ml BTGD buffer containing 0.85 M ammonium sulfate. Bound protein was eluted with a linear gradient from 0.85–0 M ammonium sulfate at the same flow rate. Fractions containing reductase$_{BPH-B1}$ were pooled and concentrated as described above. Ammonium sulfate was removed by buffer exchange with 5 mM potassium phosphate buffer, pH 6.8. The reductase preparation was then loaded onto a XK16/70 column containing 100 ml (bed volume) of ceramic hydroxyapatite that had been pre-equilibrated with 250 ml 5 mM potassium phosphate buffer, pH 6.8. Unbound protein was eluted with 100 ml of 5 mM potassium phosphate buffer, pH 6.8 at a flow rate of 1 ml/min. Bound proteins were eluted with a linear gradient from 5–100 mM potassium phosphate buffer, pH 6.8 at a flow rate of 1 ml/min. Fractions exhibiting reductase$_{BPH-B1}$ activity were pooled, and concentrated. Ammonium sulfate was added to a final concentration of 1.0 M, the protein solution was incubated for 1 h, and was centrifuged for 30 min at 14,000×g. The supernatant was loaded (0.5 ml/min) onto a Phenyl-sepharose High Performance column pre-equilibrated with 50 ml 5 mM potassium phosphate buffer containing 1.0 M ammonium sulfate. Unbound protein was eluted at a flow rate of 0.5 ml/min with 5 ml 5 mM potassium phosphate buffer containing 1.0 M ammonium sulfate. Bound protein was eluted with a linear gradient from 1.0–0 M ammonium sulfate at the same flow rate. Fractions containing reductase$_{BPH-B1}$ were pooled, concentrated and exchanged into 10 mM potassium phosphate buffer, pH 6.8. Purified reductase$_{BPH-B1}$ was frozen in liquid nitrogen and stored at −70°C.

Purification of ferredoxin$_{BPH-B1}$

Q-Sepharose fractions exhibiting ferredoxin$_{BPH-B1}$ activity were pooled and concentrated under N_2 by ultrafiltration with a YM10 membrane. Ammonium sulfate was added to the concentrated protein solution to give a final concentration of 1.2 M. After one hour, the solution was centrifuged for 30 min at 14,000×g. The supernatant solution was applied to a XK26/40 column containing 100 ml (bed volume) of octyl-sepharose that had been pre-equilibrated with 500 ml of BTGD buffer containing 1.2 M ammonium sulfate. Unbound protein was eluted at a flow rate of 0.5 ml/min with 250 ml BTGD buffer containing 1.2 M ammonium sulfate. Bound protein was eluted with a linear gradient from 1.2–0 M ammonium sulfate at the same flow rate. Fractions containing ferredoxin$_{BPH-B1}$ were pooled, concentrated and exchanged into 5 mM potassium phosphate buffer, pH 6.8, as described above. Ammonium sulfate was removed by buffer exchange with 5 mM potassium phosphate buffer, pH 6.8. The ferredoxin preparation was then loaded onto a XK16/70 column containing 80 ml (bed volume) of ceramic hydroxyapatite that had been pre-equilibrated with 250 ml 5 mM potassium phosphate buffer, pH 6.8. Unbound protein was eluted with 100 ml of 5 mM potassium phosphate buffer, pH 6.8 at a flow rate of 1 ml/min. Bound proteins were eluted with a linear gradient from 5 to 100 mM potassium phosphate buffer, pH 6.8 at a flow rate of 1 ml/min. Fractions containing ferredoxin$_{BPH-B1}$ were pooled, concentrated and exchanged into 10 mM potassium phosphate buffer, pH 6.8. Purified ferredoxin$_{BPH-B1}$ was frozen in liquid nitrogen and stored at −70°C.

Enzyme assays

BPDO activity was determined by measuring the formation of [^{14}C]biphenyl 2,3-dihydrodiol at room temperature as described previously [25]. The reaction mixture (0.25 ml) contained 50 mM MES buffer (pH 6.8), 400 μM NADH, 400 μM ferrous ammonium sulfate, 160 μM [U-^{14}C]biphenyl, 2 μg reductase$_{BPH-B1}$, 1 μg ferredoxin$_{BPH-B1}$ and appropriate amounts of crude cell extract, column fractions, or purified oxygenase$_{BPH-B1}$ (5–50 μg depend on the purity of enzyme). The reaction was initiated by the addition of [^{14}C]biphenyl, which was dissolved in 2 μl dimethylformamide. After incubation at room temperature for an appropriate period (typically 0.5–20 min), 25 μl of 37% formaldehyde was added to terminate the reaction. A 5 μl sample of each mixture was applied to the

origin of a plastic-backed thin-layer silica gel 60 F_{254} plate (EM Science, Gibbstown, NJ, USA) and air-dried. The plate was developed with 100% *n*-heptane to remove unreacted biphenyl from the origin. The origin, containing the reaction product, was then excised, and the radioactivity present was measured by liquid scintillation counting. The assay was optimized for buffer type, concentration, pH, and incubation time. The activity of BPDO in cell extracts and following each purification step was determined by the same procedure. One unit of activity equals the amount of protein that produces 1 nmol of product per min.

Cytochrome *c* reductase activity

The NADH:cytochrome *c* oxidoreductase assay [61] was used to analyze $reductase_{BPH\text{-}B1}$ and $ferredoxin_{BPH\text{-}B1}$ activity. For assaying $reductase_{BPH\text{-}B1}$, the reaction mixture (1.0 ml total volume) contained 50 mM MES buffer (pH 7.0), 300 μM NADH, 87 μM horse heart cyctochrome *c* (type III; Sigma) and appropriate amounts of $reductase_{BPH\text{-}B1}$ (1–20 μg protein depending on the purity of the protein). For assaying $ferredoxin_{BPH\text{-}B1}$, the reaction mixture (1.0 ml total volume) contained the same components as above, plus 1 μg of purified $reductase_{BPH\text{-}B1}$ and appropriate amounts of $ferredoxin_{BPH\text{-}B1}$ (1–10 μg protein depending on the purity of the protein). The activity was determined by monitoring the increase in absorbance at 550 nm. An extinction coefficient of 21,000 M^{-1} cm^{-1} for reduced minus oxidized cytochrome *c* was used in calculating the activity [61]. One unit of activity was defined as the amount of protein that reduced 1 μmol of cyctochrome *c* per min.

Flavin determination

Identification and quantification of the flavin present in $reductase_{BPH\text{-}B1}$ was carried out by high pressure liquid chromatography (HPLC) as previously described [54], and from the absorbance of the FAD (ε_{460} = 11,300 $M^{-1}cm^{-1}$, [62]). After thermal denaturation of the protein at 100°C for 5 min, protein was removed by centrifugation at 10,000×g for 5 min at 4°C, and the supernatant was analyzed. HPLC analyses were performed with a Waters Associates HPLC system (600E solvent delivery system, U−6 K injector, model 910 photodiode array detector, and Millennium Chromatography Manager software). Separations were carried out on a Beckman Ultrasphere reverse-phase column (4.6 mm × 25 cm) with a mobile phase of methanol-water (20:80) at a flow rate of 1 ml/min.

Spectroscopy

Electron paramagnetic resonance (EPR) spectra of $oxygense_{BPH\text{-}B1}$ and $ferredoxin_{BPH\text{-}B1}$ were recorded at 77 K in a Bruker model ESP 300 spectrometer (ESR Facility, University of Iowa) as isolated (oxidized) and following reduction with an excess of sodium dithionite. The settings were as follows: 5 mW microwave power, 3,650-G centerfield, 9.29-GHz microwave modulation frequency, 42-s sweep time ad 1.0×10^5 receiver gain. The absorbance spectrum of each protein was recorded under an argon atmosphere on a Beckman DU7500 or an Aminco DW-2000 spectrophotometer as isolated and during reduction with NADH and catalytic amounts of the required electron transfer proteins.

Iron and acid-labile sulfide

Iron and acid-labile sulfide were determined by published methods [3, 63].

Protein determinations

Protein concentrations were determined by the method of Bradford [9] using bovine serum albumin as the standard.

Molecular weight determinations

The native molecular weights of $reductase_{BPH\text{-}B1}$ and $ferredoxin_{BPH\text{-}B1}$ were determined by Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrometry (Biflex III, Bruker Daltonics, Manning park Billerica, MA). The native molecular weight of $oxygenase_{BPH\text{-}B1}$ in BTGD buffer was determined by dynamic light scattering at 6°C [24] using a DYNAPRO Instrument from Protein Solutions. The subunit molecular weights were determined by MALDI mass spectrometry and sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE [39]) on 12.5% gels with Low Molecular Weight standards (Bio-Rad Laboratories). Samples were boiled for 6 min immediately prior to loading gels.

N-terminal amino acid sequence analysis

The N-terminal amino acid sequences of $reductase_{BPH\text{-}B1}$, $ferredoxin_{BPH\text{-}B1}$, and the α and β subunits of $oxygenase_{BPH\text{-}B1}$ were determined by Edman degradation on an automated sequencer (Applied Biosystems, Foster City, CA, USA) at the University of Iowa Molecular Analysis Facility after SDS-PAGE and electroblotting

of the purified proteins onto a polyvinylidene difluoride membrane (ProBlott; Applied Biosystems).

Protein crystallization

Initial crystallization screenings were carried out with the Crystal Screen II, Crystal Screen, Crystal SaltRx and PEG/Ion kits (Hampton Research, Riverside, CA, USA) using the hanging drop vapor-diffusion method [4]. The crystallization drops, containing 2 µl protein solution and 2 µl precipitant solution, were equilibrated against 0.75 ml of precipitant solution. Details of crystallization conditions for each protein are given in the Results and discussion.

Data collection and processing

X-ray diffraction data was collected on the IMCA-CAT beamline 17-ID at the Advanced Photon Source at Argonne National Laboratories. The data for reductase$_{BPH-B1}$ were indexed and integrated using Mosflm [49] and scaled using Scala [14]. The large cell edge of ferredoxin$_{BPH-B1}$ required a synchrotron light source to obtain high flux and small oscillation angle data frames. The data for ferredoxin$_{BPH-B1}$ and oxygenase$_{BPH-B1}$ were processed and scaled with the software packaged*TREK [46]. Initial phases were generated by the program AMoRe [43] using ferredoxin$_{NAP-9816-4}$ and naphthalene dioxygenase (1NDO.pdb) as the starting models. Structural refinement is currently underway for all three components.

Whole cell biotransformations

For biotransformation studies, *E. coli* BL21(DE3) star(pT7-7/*bphA4A3*)(pET101D/*bphA1fA2f*) was grown at 30°C in a Biostat B-10 fermentor as described above for purification of oxygenase$_{BPH-B1,}$ and harvested by centrifugation (14,000×g at 4°C for 15 min). The cells (66 g) were washed twice with 50 mM KH_2PO_4 buffer (pH 7.5) (phosphate buffer). Biotransformations were performed by resuspending cells to a final concentration of 4.5 g (wet weight) per 100 ml of phosphate buffer. Solid substrates (naphthalene, biphenyl, or phenanthrene) were added to a final concentration of 0.1% (wt/vol). Cultures (50 ml) were incubated at 30°C with shaking (150 rpm) for 36–48 h. Culture supernatants were collected and extracted as previously described [51], and products were analyzed by GC-MS following trimethylsilyl (TMS) derivatization with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce, Rockford, IL) as described by the manufacturer.

Large scale biotransformations of benzo[a]pyrene were performed in three 2.8-l Fernbach flasks with 400 ml resuspended cells (18 g) in each flask. Benzo[a]pyrene (30 mg in 2 ml of acetone) and 0.4 g sodium pyruvate were added to 400 ml cell suspensions and each flask was incubated on a rotary shaker (200 rpm) at 30°C for 16 h.

Cultures were then extracted three times with an equal volume of sodium hydroxide-washed ethyl acetate [51]. The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated at 32°C under reduced pressure prior to analysis.

Preparative thin layer chromatography (PLC) was performed on 2 mm Silica Gel 60 F_{254} plates (EM Science), which were developed in solvent (chloroform: acetone, 80:20, vol/vol). Fractions separated by PLC were extracted with methanol.

HPLC was performed with a Hewlett Packard LC/ESI System, consisting of a VYDAC C-18 column (4.6 mm by 25 cm) with a solvent program composed of a methanol-water linear gradient (40–90%, vol/vol, 20 min, 90% methanol, 20 min). The flow rate was 0.7 ml/min. The absorbance spectra of a major component (95%) and a minor component (5%) separated by HPLC were determined using Beckman DU7500 spectrophotometer. The elemental composition of the major component was determined by high resolution mass spectrometry.

Results and discussion

Reductase$_{BPH-B1}$ and ferredoxin$_{BPH-B1}$ were resolved by ion exchange chromatography of cell extracts prepared from *E. coli* BL21(DE3)(pT7-7/*bphA3A4*). This procedure yielded a yellow fraction (reductase$_{BPH-B1}$), which eluted at 0.3 M KCl and a brown fraction (ferredoxin$_{BPH-B1}$) eluting at 0.43 M KCl.

Reductase$_{BPH-B1}$

Purified reductase$_{BPH-B1}$ was obtained by a four-step procedure utilizing ion-exchange and hydrophobic interaction chromatography (Table 1). The purified enzyme gave a single band when analyzed by SDS-PAGE (Fig. 2). Properties of the protein are summarized in Table 2 and are similar to the properties reported for the flavoprotein reductase components of the BPDOs from *Burkholderia xenovorans* LB400 [10] and *Comamonas testosteroni* B-356 [30, 31]. The molecular weight of reductase$_{BPH-B1}$ as determined by Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrometry was 43,173, which is consistent with

Table 1 Purification of BPDO $_{BPH-B1}$ components from recombinant *E. coli* strains[a]

Purification step	Amount of protein (mg)	Activity[a]		Yield (%)	Purification (fold)
		Units/mg	Units		
Reductase$_{BPH-B1}$					
Crude cell extract	3,230	0.6[b]	2,040	100	
Q-Sepharose	703	2.0	1,410	69	3
Butyl-Sepharose	97	10.8	1,050	52	17
Hydroxyapatite	42	16.4	686	34	26
Phenyl-Sepharose high performance	5.8	62.3	361	18	99
Ferredoxin$_{BPH-B1}$					
Crude cell extract	3,790	0.8[c]	3,000	100	
Q-Sepharose	734	3.3	2,410	80	4
Octyl-Sepharose	318	9.4	2,050	68	12
Hydroxyapatite	80	17.2	1,370	46	22
Oxygenase$_{BPH-B1}$					
Crude cell extract	3,980	28.9[d]	115,000	100	
Q-Sepharose	461	149	68,600	60	5
Butyl-Sepharose	231	162	37,500	33	6
Hydroxyapatite	82	294	24,000	20	10

[a] Reductase$_{BPH-B1}$ and ferredoxin$_{BPH-B1}$ were purified from *E. coli* BL21(DE3)(pT7-7/*bphA3A4*), and oxygenase$_{BPH-B1}$ was purified from *E. coli* BL21star(pET101D/*bphA1fA2f*)

[b] One unit of reductase$_{BPH-B1}$ activity is defined as the amount of enzyme required to reduce one µmol of cytochrome *c* per min in the presence of excess ferredoxin$_{BPH-B1}$

[c] One unit of ferredoxin$_{BPH-B1}$ activity is defined as the amount of enzyme required to reduce one µmol of cytochrome *c* per min in the presence of excess reductase$_{BPH-B1}$

[d] One unit of oxygenase$_{BPH-B1}$ activity is defined as the amount of enzyme required to produce

the value (43,447) predicted from the nucleotide sequence of the *bphA4* gene (G. J. Zylstra, unpublished data). The results indicate that reductase$_{BPH-B1}$ consists of a single subunit. The N-terminal amino acid sequence of purified reductase$_{BPH-B1}$ (Table 2) was

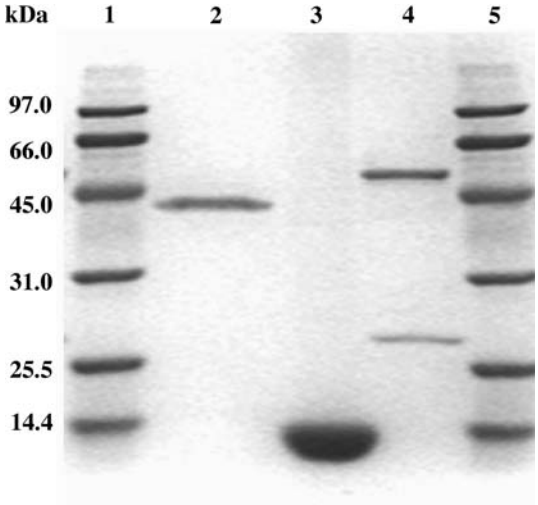

Fig. 2 SDS-PAGE of purified BPDO system components. Lanes 1 and 5, molecular weight standards; lane 2, reductase$_{BPH-B1}$ (4 µg of protein); lane 3, ferredoxin$_{BPH-B1}$(8 µg of protein); lane 4, oxygenase$_{BPH-B1}$ (5 µg of protein). Proteins were stained with Coomassie brilliant blue R250

identical to the sequence deduced for *bphA4* (G. J. Zylstra, unpublished data). The absorbance spectrum of the purified protein had absorbance maxima at 378 nm and 450 nm with a shoulder at 475 nm (Fig. 3a). The extinction coefficient at 450 nm was 9.82 $mM^{-1}cm^{-1}$. This peak was bleached when anaerobically titrated with NADH. A linear decrease in absorbance was observed to terminate when 10 nmol NADH had been added to 9.3 nmol of reductase$_{BPH-B1}$ (Fig. 3a). Addition of FAD or FMN to assay mixtures did not stimulate activity, and the FAD (0.93 mol of FAD/mol) content of reductase$_{BPH-B1}$ indicated that the flavin site was almost fully occupied in the purified preparation. No iron or acid-labile sulfur was detected from the purified protein.

Cytochrome *c* reductase activity of reductase$_{BPH-B1}$ was dependent on the presence of ferredoxin, but it was not specific for ferredoxin$_{BPH-B1}$. Similar activities were obtained in the presence of ferredoxin$_{BPH-B1}$ or ferredoxin$_{TOL-F1}$ [57]. With ferredoxin$_{2NT-JS42}$ (from the 2-nitrotoluene dioxygenase system of *Acidovorax* sp. strain JS42 [45]), ferredoxin$_{NAP-9816-4}$ (from the naphthalene dioxygenase system of *Pseudomonas* sp. strain NCIB 9816-4 [28]), and ferredoxin$_{BPH-LB400}$ (from the BPDO system of *B. xenovorans* LB400 [26]), activities were 65, 46, and 21% of the activity with

Table 2 Properties of BPDO $_{BPH-B1}$ components

Properties	Reductase$_{BPH-B1}$	Ferredoxin $_{BPH-B1}$	Oxygenase $_{BPH-B1}$
Molecular mass (kDa)[a]	43.2	11.5	217
Subunit molecular mass (kDa)[b] (from deduced amino acid sequence[c])	44.1, (43.447)	10.8, (11.657)	α 47.8, (50.917) β 21.9, (20.653)
Subunit structure	Monomer	Monomer	Heterotrimer
Iron (g-atoms per mol)[a]	–[d]	2.10	2.43/αβ
Acid labile sulfur (g-atoms per mol)[a]	–	1.86	1.81/αβ
FAD (mol per mol)[a]	0.93	–	–
Absorption spectra, λ_{max} (nm)	378, 450	326, 463, 570 (shoulder)	332, 457, 550 (shoulder)
Extinction coefficients ($mM^{-1}cm^{-1}$)(λ_{max}) (nm)	8.65 (378), 9.82 (450)	15.3 (326), 7.62 (463)	12.3/αβ (332), 7.22/αβ (457)
EPR[a] (reduced), g_x g_y g_z	NA	2.02, 1.90, 1.82	2.02, 1.91, 1.73
Gene designation	*bphA4*	*bphA3*	*bphA1fA2f*
N-terminal amino acid sequence[a]	MRSIAIVGANLAGGRAV-	SNKLRLCQVA-	α, SSDATLVDTVN- β, SSEQIPVTPDD-

The molecular mass determined from the deduced amino acid sequence was used in all calculations

NA not applicable

[a] Determined as described in Materials and methods

[b] Determined by SDS-PAGE

[c] G. J. Zylstra, unpublished data

[d] None detected

ferredoxin$_{BPH-B1}$, respectively. Reductase$_{BPH-B1}$ was also capable of substituting for the native reductase components of toluene dioxygenase [56], naphthalene dioxygenase [27], BPDO (from LB400 [10]), 2-nitrotoluene dioxygenase, and nitrobenzene dioxygenase [45] systems in assays with the native substrates (data not shown). NADH was the preferred electron donor for reductase$_{BPH-B1}$. The cytochrome *c* reduction and BPDO activities were reduced to 2 and 9%, respectively, when NADH was substituted by NADPH.

Ferredoxin$_{BPH-B1}$

Ferredoxin$_{BPH-B1}$ was purified to apparent homogeneity by a three-step procedure utilizing ion exchange and hydrophobic interaction chromatography (Table 1). The purified protein gave a single band when analyzed by SDS-PAGE (Fig. 2). The molecular mass of ferredoxin$_{BPH-B1}$ determined by MALDI-TOF was consistent with the deduced molecular mass derived from the sequence of the *bphA3* gene (Table 2). The N-terminal amino acid sequence of ferredoxin$_{BPH-B1}$ (Table 2) was identical to the amino acid sequence predicted from the nucleotide sequence of *bphA3* (G. J. Zylstra, unpublished data).

The absorbance spectrum of purified ferredoxin$_{BPH-B1}$ was typical of a Rieske-type iron-sulfur protein with absorbance maxima at 326 nm and 463 nm with a broad shoulder in the region of 570 nm. The absorbance at 463 nm (ε, 7.62 $mM^{-1}cm^{-1}$) was bleached under anaerobic conditions in the presence of NADH and catalytic amounts of reductase$_{BPH-B1}$ (Fig. 3b) with the appearance of new peaks at 435 nm and 520 nm. Full reduction of 1 mol of ferredoxin$_{BPH-B1}$ occurred with the uptake of 0.5 mol of NADH (Fig. 3b, inset), indicating that ferredoxin$_{BPH-B1}$ accepts single electrons. Oxidized ferredoxin$_{BPH-B1}$ did not give an EPR spectrum. However, signals with g-values of 2.02, 1.90 and 1.82 were observed after reduction with sodium dithionite. The absorbance and EPR spectra are characteristic of a Rieske [2Fe-2S] center and this was confirmed by the presence of approximately 2 g atoms each of iron and acid-labile sulfur in the isolated protein. The properties of ferredoxin$_{BPH-B1}$ are similar to those reported for the ferredoxin components of the *B. xenovorans* LB400 [26] and *C. testosteroni* B-356 [31] BPDO systems and the toluene dioxygenase system from *Pseudomonas putida* F1 [55].

Oxygenase$_{BPH-B1}$

Oxygenase$_{BPH-B1}$ was purified 10-fold with 20% recovery of the activity present in the crude cell extract (Table 1). Hydrophobic interaction with a Butyl-Sepharose column enhanced the purity as shown by SDS-PAGE, although almost half the original dioxygenase activity was lost in this step (Table 1). The enzyme consisted of large (α) and small (β) subunits (Fig. 2) with molecular masses of 50,643 (α subunit) and 20,556 (β subunit), which were determined by MALDI mass

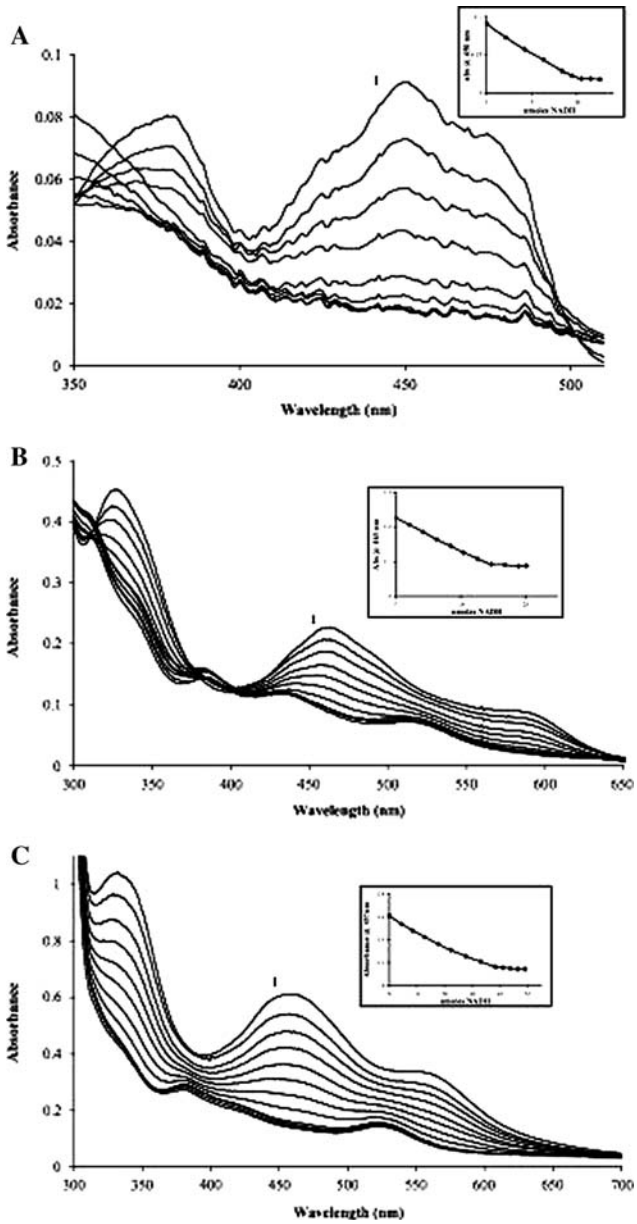

Fig. 3 Oxidized and reduced spectra of purified BPDO components. **a** Anaerobic reduction of reductase $_{BPH\text{-}B1}$ by NADH. The cuvet contained, in a final volume of 1.0 ml of 50 mM MES buffer, pH 7.0, reductase $_{BPH\text{-}B1}$ (9.3 nmol). Curve 1 shows the absorption spectrum of oxidized reductase $_{BPH\text{-}B1}$ under anaerobic conditions. The remaining curves show spectra obtained after separate 2.1 nmol or 1.05 nmol additions of NADH. The inset shows the decrease in absorption at 450 nm after successive additions of NADH. **b** Anaerobic reduction of ferredoxin $_{BPH\text{-}B1}$ by NADH in the presence of reductase $_{BPH\text{-}B1}$. The cuvet contained, in a final volume of 1.0 ml of 50 mM MES buffer, pH 7.0, ferredoxin $_{BPH\text{-}B1}$ (29.7 nmol) and catalytic amounts of reductase $_{BPH\text{-}B1}$ (1.9 nmol). Curve 1 shows the absorption spectrum of oxidized ferredoxin $_{BPH\text{-}B1}$ under anaerobic conditions. The remaining curves show spectra obtained after separate 2.1 nmol additions of NADH. The inset shows the decrease in absorption at 463 nmol after successive additions of NADH. **c** Anaerobic reduction of oxygenase $_{BPH\text{-}B1}$ by NADH in the presence of reductase$_{BPH\text{-}B1}$ and ferredoxin$_{BPH\text{-}B1}$. The cuvet contained, in a final volume of 1.0 ml of 50 mM MES buffer, pH 7.0, oxygenase$_{BPH\text{-}B1}$ (84.7 nmol), and catalytic amounts of reductase$_{BPH\text{-}B1}$ (2.8 nmol) and ferredoxin$_{BPH\text{-}B1}$ (2.9 nmol). Curve 1 shows the absorption spectrum of oxidized oxygenase$_{BPH\text{-}B1}$ under anaerobic conditions. The remaining curves show spectra obtained after separate 4.3 nmol additions of NADH. The inset shows the decrease in absorption at 457 nm after successive additions of NADH

spectrometry. A molecular mass of 217,600 was obtained for the native enzyme, indicating an $\alpha_3\beta_3$ subunit conformation. These values and the N-terminal amino acid sequences of the oxygenase$_{BPH\text{-}B1}$ α and β subunits (Table 2) were consistent with the deduced amino acid sequences for *bphA1fA2f* (G. J. Zylstra, unpublished data). The oxygenase$_{BPH\text{-}B1}$ α subunit reacted with a monoclonal antibody specific for the toluene dioxygenase α subunit from *P. putida* F1, but did not react with anti-F1 β subunit antibody [40] or antibodies [44] specific for naphthalene dioxygenase from *Pseudomonas* sp. NCIB 9816-4 (data not shown). These results suggest that oxygenase$_{BPH\text{-}B1}$ is a member of the Toluene/Biphenyl family of dioxygenases [21], although the N-terminal sequence analysis indicates that it is quite different in sequence from BPDOs from *B. xenovorans* LB400 [25], *Pseudomonas pseudoalcaligenes* KF707 [59] and *C. testosteroni* B-356 BPDOs [30].

The properties of oxygenase$_{BPH\text{-}B1}$ (Table 2) indicate that it contains a Rieske [2Fe-2S] cluster. The iron and acid-labile sulfide contents of oxygenase$_{BPH\text{-}B1}$ were 2.43 and 1.81 mol each per mol αβ heterodimer. Addition of exogenous ferrous iron was required for maximal activity (data not shown), suggesting that iron was lost during the purification and may explain why the oxygenase component contained less iron than expected for a heterotrimer. The absorbance spectrum of oxidized oxygenase$_{BPH\text{-}B1}$ showed peaks at 332 nm (ε, 12.29) and 457 nm (ε, 7.22 $mM^{-1}cm^{-1}$) with a broad shoulder at 550 nm (Fig. 3c). When reduced anaerobically with NADH in the presence of catalytic amounts of reductase$_{BPH\text{-}B1}$ and ferredoxin$_{BPH\text{-}B1}$, 0.5 mol of NADH was required to reduce each αβ heterodimer of oxygenase$_{BPH\text{-}B1}$ (Fig. 3c). The EPR spectrum of reduced oxygenase$_{BPH\text{-}B1}$ gave g values of 2.01, 1.91 and 1.73, further confirming the presence of a Rieske center.

The results in Fig. 3c show that electrons are transferred from NADH to oxygenase$_{BPH\text{-}B1}$ via reductase$_{BPH\text{-}B1}$ and ferredoxin$_{BPH\text{-}B1}$. In addition, biphenyl 2,3-dihydrodiol was the only product detected by autoradiography when oxygenase$_{BPH\text{-}B1}$ was combined with reductase$_{BPH\text{-}B1}$ and ferredoxin$_{BPH\text{-}B1}$. No products were detected in the absence of added NADH or electron transfer components. It is of interest to note that a His-tagged BPDO purified from *C. testosteroni* B-356

oxidizes biphenyl to dihydrodiols at the 2,3- and 3,4-positions [30].

The effects of substituting various reductases and ferredoxins from other dioxygenase systems on the biphenyl oxidizing activity of oxygenase$_{BPH-B1}$ are shown in Table 3. The results show that none of the alternative ferredoxin components transferred electrons to oxygenase$_{BPH-B1}$. However, reductase$_{BPH-B1}$ could be replaced by all four of the other tested reductases (Table 3). Together, ferredoxin$_{BPH-B1}$ and reductase$_{BPH-B1}$ were capable of transferring electrons to the oxygenase components of the naphthalene dioxygenase, 2-nitrotoluene dioxygenase, and nitrobenzene dioxygenase systems, but not BPDO (from LB400) or toluene dioxygenase (data not shown). These results indicate that both reductase$_{BPH-B1}$ and ferredoxin$_{BPH-B1}$ are quite flexible in their redox partner interactions, but that oxygenase$_{BPH-B1}$ is highly selective: only ferredoxin$_{BPH-B1}$ could transfer electrons to oxygenase$_{BPH-B1}$.

Table 3 BPDO $_{BPH-B1}$ activity: oxygenase$_{BPH-B1}$ in the presence of various reductase and ferredoxin components

Reductase[a]	Ferredoxin[b]	Relative activity (%)[c]
Rd_{BPH-B1}	Fd_{BPH-B1}	100
Rd_{TOL-F1}	Fd_{TOL-F1}	0
$Rd_{NAP-9816-4}$	$Fd_{NAP-9816-4}$	0
$Rd_{2NT-JS42}$	$Fd_{2NT-JS42}$	0
$Rd_{BPH-LB400}$	$Fd_{BPH-LB400}$	0
Rd_{TOL-F1}	Fd_{BPH-B1}	120
$Rd_{NAP-9816-4}$	Fd_{BPH-B1}	84
$Rd_{2NT-JS42}$	Fd_{BPH-B1}	54
$Rd_{BPH-LB400}$	Fd_{BPH-B1}	100
Rd_{BPH-B1}	Fd_{TOL-F1}	0
Rd_{BPH-B1}	$Fd_{NAP-9816-4}$	0
Rd_{BPH-B1}	$Fd_{2NT-JS42}$	0
Rd_{BPH-B1}	$Fd_{BPH-LB400}$	0

[a] Reductase components: Rd_{TOL-F1}, toluene dioxygenase reductase from *P. putida* F1[56], $Rd_{NAP-9816-4}$, naphthalene dioxygenase reductase from *Pseudomonas* sp. strain NCIB 9816-4 [27], $Rd_{2NT-JS42}$, 2-nitrotoluene dioxygenase reductase from *Acidovorax* sp. strain JS42 [45], $Rd_{BPH-LB400}$, BPDO reductase from *B. xenovorans* strain LB400 [10]

[b] Ferredoxin components: Fd_{TOL-F1}, toluene dioxygenase ferredoxin from *P. putida* F1 [57], $Fd_{NAP-9816-4}$, naphthalene dioxygenase ferredoxin from *Pseudomonas* sp. strain NCIB9816-4 [28], $Fd_{2NT-JS42}$, 2-nitrotoluene dioxygenase ferredoxin from *Acidovorax* sp. strain JS42 [45], $Fd_{BPH-LB400}$, BPDO ferredoxin from *B. xenovorans* strain LB400 [26]

[c] Reaction mixtures (0.25 ml) contained 58 μg oxygenase$_{BPH-B1}$ and excess amounts of reductase and ferredoxin components in 50 mM MES buffer (pH 6.9), with the addition of 400 μM NADH, 400 μM ferrous ammonium sulfate, 1 μM FAD, and 160 μM [^{14}C]biphenyl. Activity was determined by the standard assay with a 4 min incubation time and is reported relative to activity in the presence of purified reductase$_{BPH-B1}$ and ferredoxin$_{BPH-B1}$

Substrate preference

The activity of oxygenase$_{BPH-B1}$ with toluene and naphthalene as substrates was detected by the formation of [^{14}C]naphthalene 1,2-dihydrodiol and [^{14}C]toluene 2,3-dihydrodiol from ^{14}C-labeled substrates. The activity with [^{14}C]biphenyl was 189 U/mg protein, while those with [^{14}C] naphthalene and [^{14}C] toluene were 17.6 U/mg protein (9.3% of the activity with biphenyl) and 31.1 U/mg protein (16.5% of the activity with biphenyl), respectively, indicating that naphthalene and toluene are poor substrates for oxygenase$_{BPH-B1}$ compared to biphenyl.

E. coli expressing the *bphA3A4A1fA2f* genes oxidized biphenyl and naphthalene to their respective *cis*-2,3- and 1,2-dihydrodiols, which were identical to authentic compounds previously isolated from *S. yanoikuyae* B1 [22] and *P. putida* strain 119 [33]. Phenanthrene was oxidized to two *cis*-dihydrodiol isomers corresponding to the *cis*-1,2- and 3,4-dihydrodiols, which is also consistent with previous results with *S. yanoikuyae* B1 [34]. When the recombinant *E. coli* was incubated with benzo[a]pyrene, less than two percent of the substrate was converted to a major (compound 1) and a minor (compound 2) dihydrodiol with the absorption spectra shown in Fig. 4. The spectra are identical to the *cis*-benzo[a]pyrene 9,10-dihydrodiol and *cis*-benzo[a]pyrene 7,8-dihydrodiol formed by *S. yanoikuyae* B1 [41]. A recombinant BPDO formed by DNA shuffling of the oxygenase genes from *P. pseudoalcaligenes* KF707 and *B. xenovorans* LB400 oxidizes a variety of heterocyclic aromatic substrates to *cis*-dihydrodiols [42]. However, this strain has not been reported to oxidize polycyclic aromatic hydrocarbons. Based on the results reported here, the recombinant BPDO encoded by *bphA3A4A1fA2f* has a substrate preference that is comparable to the biphenyl-induced BPDO from *S. yanoikuyae*, with biphenyl as the preferred substrate.

The BPDO induced during growth of *S. yanoikuyae* with biphenyl has been purified (C. L. Yu and D. T. Gibson, unpublished) and the N-terminal amino acid sequences of its α and β subunits are identical to the sequences shown in Table 2 for the α and β subunits of the dioxygenase purified from the recombinant strain. These results show that the *bphA1fA2f* genes encode a BPDO with specificity for polycyclic aromatic hydrocarbons. However, they do not rule out the possibility that other *bphA1A2* genes in the B1 genome may encode dioxygenases with similar overlapping substrate specificities. The presence of four different αβ pairs encoding Rieske non-heme iron oxygenases has recently been reported for *Mycobacterium vanbaalenii*

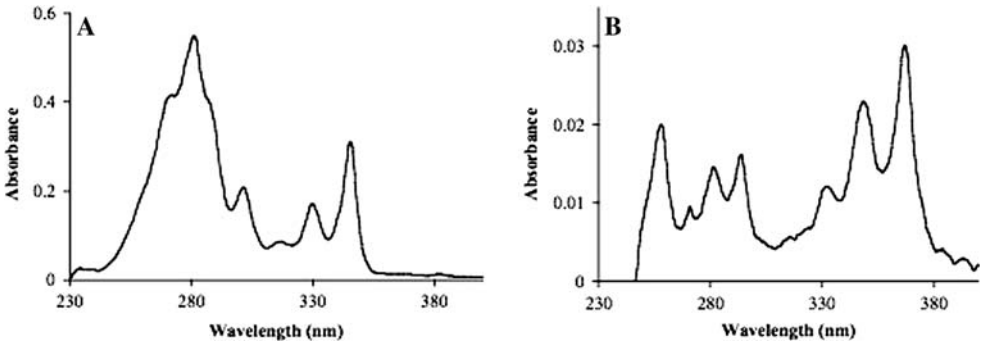

Fig. 4 Absorption spectra of **a** *cis*- 9,10-dihydroxy-dihydrobenzo[a]pyrene; **b** *cis*- 7,8-dihydroxy-dihydrobenzo[a]pyrene formed from benzo[*a*]pyrene by *E. coli* BL21(DE3)star (pAlter-Ex2/*bphA3A4*)(pET101D/*bphA1fA2f*)

PYR-1. One of these, *nidA3B3*, encodes an enzyme with a similar substrate preference to the B1 BPDO [38].

Protein crystallization

Reductase$_{BPH-B1}$ (20 mg/ml) in 50 mM potassium phosphate buffer (pH 6.8) was crystallized in 0.1 M HEPES (pH 7.0), 2.2 M ammonium sulfate. Small crystals of the reductase$_{BPH-B1}$ grew after several weeks at 6°C. Diffraction-grade crystals (Fig. 5a) were obtained after macroseeding under the same conditions. The crystals initially diffracted beyond 2 Å; however they quickly degraded due to radiation damage in the beam. A complete data set to 2.5 Å was obtained. Indexing and analysis of the space group indicated that the crystal lattice is of the I422 space group. Cell dimensions were calculated to be $a = b = 121.2$ Å and $c = 136.5$ Å.

Ferredoxin$_{BPH-B1}$ (33 mg/ml) in 50 mM potassium phosphate buffer (pH 6.8), was crystallized in 0.1 M citric acid (pH 4.2), 1.6 M ammonium sulfate. Oval shaped crystals with a strong red-brown color formed after 2 weeks at 6°C (Fig. 5b). The ferredoxin$_{BPH-B1}$ crystals diffracted to 2.0 Å and were in the hexagonal space group $P6_522$. The cell dimensions were $a = b = 62.1$ and $c = 238.4$ Å and $\alpha = \beta = 90°$ $\gamma = 120°$. The crystal structure of the ferredoxin component of BPDO from LB400 has been determined [13]. The results show that the histidine ligands of the [2Fe-2S] cluster are exposed near the surface of the protein. The structure of ferredoxin$_{BPH-B1}$ is currently being determined and it will be interesting to see if the B1 component has similar properties.

Oxygenase$_{BPH-B1}$ (20 mg/ml) crystallized in 20% (w/v) polyethylene glycol 3350, 1 M NaCl and 0.1 M $ZnCl_2$ at 6°C. Reddish hexagonal crystals formed after several weeks at 6°C (Fig. 5c). The oxygenase$_{BPH-B1}$ crystals diffracted to 1.75 Å and were members of the space group $P3_121$ with cell dimensions of $a = b = 134.95$ $c = 219.88$ Å and $\alpha = \beta = 90°$ $\gamma = 120°$. A detailed study of the kinetic properties and crystallization of a recombinant oxygenase from *C. testosteroni* B-356 BPDO has been reported [32]. Although kinetic properties were not determined in the present study, the structure of

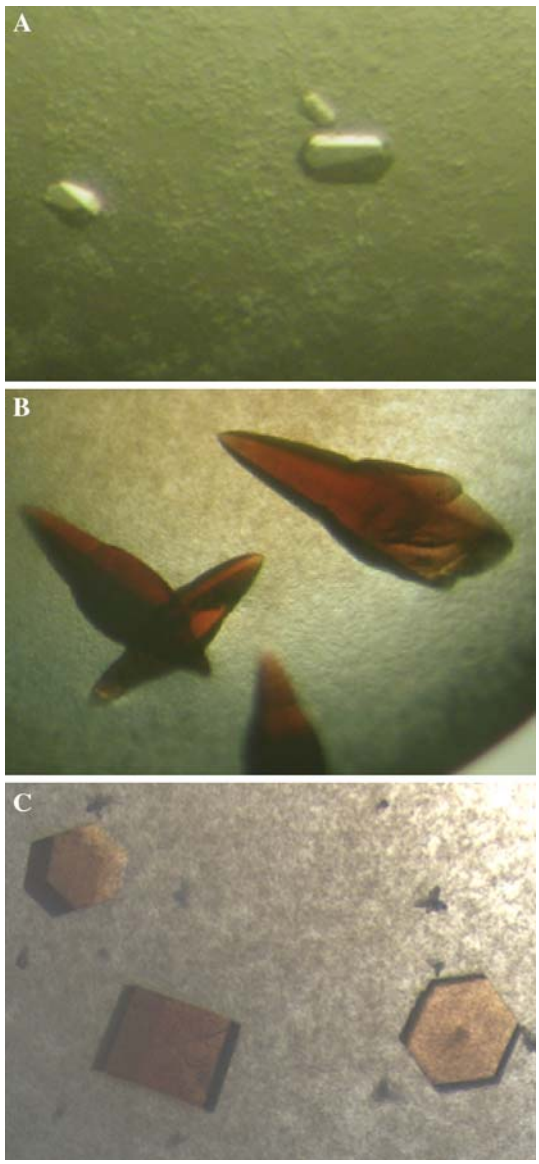

Fig. 5 Diffraction-grade crystals of reductase$_{BPH-B1}$ (**a**) ferredoxin$_{BPH-B1}$ (**b**) oxygenase$_{BPH-B1}$ (**c**)

oxygenase$_{BPH-B1}$ has been determined [18]. The active site of the enzyme has a large enough pocket to accommodate polycyclic aromatic hydrocarbons as large as benzo[a]pyrene. A review of the known structures of Rieske dioxygenases has been published [17]. Most of the published work on BPDOs from other organisms has focused on their ability to oxidize polychlorinated biphenyls (reviewed in [19, 47]), and at this time the ability of these enzymes to oxidize polycyclic aromatic hydrocarbons larger than phenanthrene has not been reported.

Conclusions

The results reported in this study indicate that the BPDO components purified from *E. coli* expressing *bphA3A4* and *bphA1fA2f* are the same as the components induced during the growth of *S. yanoikuyae* B1 with biphenyl. The substrate range of the cloned dioxygenase has not been fully explored. However the formation of the benzo[a]pyrene *cis*-9,10- and 7,8-dihydrodiols is consistent with this view.

The properties of reductase$_{BPH-B1}$, ferredoxin$_{BPH-B1}$ and oxygenase$_{BPH-B1}$ are remarkably similar to the Rieske dioxygenase systems purified from *B. xenovorans* LB400 [10, 25, 26] and *C. testosteroni* B-356 [30, 31]. However, biphenyl-grown cells of these organisms have not been reported to oxidize the wide range of substrates demonstrated for B1. The crystal structure of oxygenase$_{BPH-B1}$ provides further confirmation of the ability of the enzyme to accommodate large PAHs in the active site [17, 18].

The single known ferredoxin-reductase pair from strain B1 functioned as an effective electron shuttle system for the dioxygenase, which lends further support to the proposed use of one set of redox partners for the six known dioxygenases in this strain [2, 12, 37]. However, based on results presented here, oxygenase$_{BPH-B1}$ is quite specific in its interaction with ferredoxin$_{BPH-B1}$. Ferredoxins from the toluene, naphthalene, and 2-nitrotoluene dioxygenase systems as well as ferredoxin$_{BPH-LB400}$ were incapable of productive interaction with oxygenase$_{BPH-B1}$ (Table 3). Taken together, these results suggest that that there will be a conserved ferredoxin docking site on the surface of the six diverse dioxygenases in strain B1 that will allow specific binding of ferredoxin$_{BPH-B1}$. In contrast, the choice of reductase partner for ferredoxin$_{BPH-B1}$ was much less specific (Table 3). The crystal structures of the protein components will provide the basis for future protein-protein interaction studies to address these questions. This study paves the way for a wide range of structure-function studies of this versatile and useful enzyme.

Acknowledgments This work was supported by US Public Health Service grants GM29909 to D.T.G. and GM62904 to S.R. from the National Institute of General Medical Sciences. C.L.Y was supported by National Science Foundation Engineering Research Centers Program grant EEC-0310689. D.F. and E.N.B. were supported by fellowships from the University of Iowa Center for Biocatalysis and Bioprocessing. Use of the IMCA-CAT beamline 17-ID at the Advanced Photon Source was supported by the companies of the Industrial Macromolecular Crystallography Association through a contract with the Center for Advanced Radiation Sources at the University of Chicago. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38. We thank Lisa Keefe, Kevin Battaile and Irina Koshelev for help with data collection at the IMCA-CAT facility. We thank the University of Iowa Protein Crystallography Facility for the use of dynamic light scattering equipment, and Garry Buettner and Sean Martin for determining EPR spectra at the University of Iowa ESR Facility.

References

1. Akhtar MN, Boyd DR, Thompson NJ, Koreeda M, Gibson DT, Mahadevan V, Jerina DM (1975) Absolute stereochemistry of the dihydroanthracene-*cis*- and -*trans*-1,2-diols produced from anthracene by mammals and bacteria. J Chem Soc Perkin 1:2506–2511
2. Bae M, Kim E (2000) Association of a common reductase with multiple aromatic terminal dioxygenases in *Sphingomonas yanoikuyae* strain B1. J Microbiol 38:40–43
3. Beinert H (1983) Semi-micro methods for analysis of labile sulfide and of labile sulfide plus sulfane sulfur in unusually stable iron-sulfur proteins. Anal Biochem 131:373–378
4. Bergfors TM (1999) Protein crystallization: techniques, strategies, and tips. International University Line, La Jolla
5. Boyd DR, Sheldrake GN (1998) The dioxygenase-catalysed formation of vicinal *cis*-diols. Nat Prod Rep 15:309–324
6. Boyd DR, Sharma ND, Harrison JS, Kennedy MA, Allen CCR, Gibson DT (2001) Regio- and stereo-selective dioxygenase-catalysed *cis*-dihydroxylation of fjord-region polycyclic arenes. J Chem Soc Perkin Trans I:1264–1269
7. Boyd DR, Sharma ND, Agarwal R, Resnick SM, Schocken MJ, Gibson DT, Sayer JM, Yagi H, Jerina DM (1997) Bacterial dioxygenase-catalyzed dihydroxylation and chemical resolution routes to enantiopure *cis*-dihydrodiols of chrysene. J Chem Soc, Perkin Trans 1:1715–1723
8. Boyd DR, Sharma ND, Modyanova LV, Carroll JG, Malone JF, Allen CCR, Hamilton JTG, Gibson DT, Parales RE, Dalton H (2002) Dioxygenase-catalyzed *cis*-dihydroxylation of pyridine-ring systems. Can J Chem 80:589–600
9. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
10. Broadus RM, Haddock JD (1998) Purification and characterization of the NADH:ferredoxin$_{BPH}$ oxidoreductase component of biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400. Arch Microbiol 170:106–112
11. Cerniglia CE, Morgan J, Gibson DT (1979) Bacterial and fungal oxidation of dibenzofuran. Biochem J 180:175–185
12. Cho O, Choi KY, Zylstra GJ, Kim Y-S, Kim S-K, Lee JH, Sohn H-Y, Kwon G-S, Kim YM, Kim E (2005) Catabolic role of a three-component salicylate oxygenase from *Sphingomonas yanoikuyae* B1 in polycyclic aromatic hydrocarbon degradation. Biochem Biophys Res Commun 327:656–662

13. Colbert CL, Couture MM-J, Eltis LD, Bolin JT (2000) A cluster exposed: structure of the Rieske ferredoxin from biphenyl dioxygenase and the redox properties of Rieske Fe-S proteins. Structure 8:1267–1278
14. Collaborative (Computational Project Number (1994) The CCP4 suite: programs for protein crystallography. Acta Crystallogr D Biol Crystallogr 50:760–763
15. Davis RW, Botstein D, Roth JR (1980) Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor
16. Eaton SE, Resnick SM, Gibson DT (1996) Initial reactions in the oxidation of 1,2-dihydronaphthalene metabolism by *Sphingomonas yanoikuyae* strains. Appl Environ Microbiol 62:4388–4394
17. Ferraro DJ, Gakhar L, Ramaswamy S (2005) Rieske business: structure-function of Rieske non-heme oxygenases. Biochem Biophys Res Commun 338:175–190
18. Ferraro DJ, Brown E, Gakhar L, Yu CL, Parales RE, Gibson DT, Ramaswamy S (2006) Structural basis for multi-ring aromatic hydrocarbon dihydroxylation by biphenyl 2,3-dioxygenase from *Sphingomonas yanoikuyae* B1. Submitted for publication
19. Furukawa K, Suenaga H, Goto M (2004) Biphenyl dioxygenases: functional versatilities and directed evolution. J Bacteriol 186:5189–5196
20. Gibson DT (1999) *Beijerinckia* sp. strain B1: a strain by any other name. J Ind Microbiol Biotechnol 23:284–293
21. Gibson DT, Parales RE (2000) Aromatic hydrocarbon dioxygenases in environmental biotechnology. Curr Opin Biotechnol 11:236–243
22. Gibson DT, Roberts RL, Wells MC, Kobal VM (1973) Oxidation of biphenyl by a *Beijerinckia* species. Biochem Biophys Res Commun 50:211–219
23. Gibson DT, Mahadevan V, Jerina DM, Yagi H, Yeh HJC (1975) Oxidation of the carcinogens benzo[a]pyrene and benzo[a]anthracene to dihydrodiols by a bacterium. Science 189:295–297
24. Habel JE, O JF, Borgstahl GE (2001) Dynamic light-scattering analysis of full-length human RPA14/32 dimer: purification, crystallization and self-association. Acta Crystallogr D Biol Crystallogr 57:254–259
25. Haddock JD, Gibson DT (1995) Purification and characterization of the oxygenase component of biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400. J Bacteriol 177:5834–5839
26. Haddock JD, Pelletier DA, Gibson DT (1997) Purification and properties of ferredoxin$_{BPH}$, a component of biphenyl 2,3-dioxygenase of *Pseudomonas* sp. strain LB400. J Ind Microbiol Biotechnol 19:355–359
27. Haigler BE, Gibson DT (1990) Purification and properties of NADH-ferredoxin$_{NAP}$ reductase, a component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. J Bacteriol 172:457–464
28. Haigler BE, Gibson DT (1990) Purification and properties of ferredoxin$_{NAP}$, a component of naphthalene dioxygenase from *Pseudomonas* sp. strain NCIB 9816. J Bacteriol 172:465–468
29. Hudlicky T, Gonzalez D, Gibson DT (1999) Enzymatic dihydroxylation of aromatics in enantioselective synthesis: Expanding asymmetric methodology. Aldrichimica Acta 32:35–62
30. Hurtubise Y, Barriault D, Sylvestre M (1996) Characterization of active recombinant His-tagged oxygenase component of *Comamonas testosteroni* B-356 biphenyl dioxygenase. J Biol Chem 271:8152–8156
31. Hurtubise Y, Barriault D, Powlowski J, Sylvestre M (1995) Purification and characterization of the *Comamonas testosteroni* B-356 biphenyl dioxygenase components. J Bacteriol 177:6610–6618
32. Imbeault NY, Powlowski JB, Colbert CL, Bolin JT, Eltis LD (2000) Steady-state kinetic characterization and crystallization of a polychlorinated biphenyl-transforming dioxygenase. J Biol Chem 275:12430–12437
33. Jeffrey AM, Yeh HJC, Jerina DM, Patel TR, Davey JF, Gibson DT (1975) Initial reactions in the oxidation of naphthalene by *Pseudomonas putida*. Biochemistry 14:575–583
34. Jerina DM, Selander H, Yagi H, Wells MC, Davey JF, Mahadevan V, Gibson DT (1976) Dihydrodiols from anthracene and phenanthrene. J Am Chem Soc 98:5988–5996
35. Jerina DM, Van Bladeren PJ, Yagi H, Gibson DT, Mahadevan V, Neese AS, Koreeda M, Sharma ND, Boyd DR (1984) Synthesis and absolute configuration of the bacterial *cis*-1,2-, *cis*-8,9-, and *cis*-10,11-dihydrodiol metabolites of benz[a]anthracene formed by a strain of *Beijerinckia*. J Org Chem 49:3621–3628
36. Khan AA, Wang R-F, Cao W-W, Franklin W, Cerniglia CE (1996) Reclassification of a polycyclic aromatic hydrocarbon-metabolizing bacterium, *Beijerinckia* sp. strain B1, as *Sphingomonas yanoikuyae* by fatty-acid analysis, protein pattern-analysis, DNA-DNA hybridization, and 16S ribosomal DNA-sequencing. Int J Sys Bacteriol 46:466–469
37. Kim E, Zylstra GJ (1999) Functional analysis of genes involved in biphenyl, naphthalene, phenanthrene, and *m*-xylene degradation by *Sphingomonas yanoikuyae* B1. J Ind Microbiol Biotechnol 23:294–302
38. Kim S-J, Kweon O, Freeman JP, Jones RC, Adjei MD, Jhoo J-W, Edmondson RD, Cerniglia CE (2006) Molecular cloning and expression of genes encoding a novel dioxygenase involved in low- and high-molecular-weight polycyclic aromatic hydrocarbon degradation in *Mycobacterium vanbaalenii* PYR-1. Appl Environ Microbiol 72:1045–1054
39. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685
40. Lynch NA, Jiang H, Gibson DT (1996) Rapid purification of the oxygenase component of toluene dioxygenase from a polyol-responsive monoclonal antibody. Appl Environ Microbiol 62:2133–2137
41. Mahaffey WR, Gibson DT, Cerniglia CE (1988) Bacterial oxidation of chemical carcinogens: formation of polycyclic aromatic acids from benz[a]anthracene. Appl Environ Microbiol 54:2415–2423
42. Misawa N, Shindo K, Takahashi H, Suenaga H, Iguchi K, Okazaki H, Harayama S, Furukawa K (2002) Hydroxylation of various molecules including heterocyclic aromatics using recombinant *Escherichia coli* cells expressing modified biphenyl dioxygenase genes. Tetrahedron 58:9605–9612
43. Navaza J (1994) AMoRe: an automated package for molecular replacement. Acta Cryst A 50:157–163
44. Parales RE, Emig MD, Lynch NA, Gibson DT (1998) Substrate specificities of hybrid naphthalene and 2,4-dinitrotoluene dioxygenase enzyme systems. J Bacteriol 180:2337–2344
45. Parales RE, Huang R, Yu C-L, Parales JV, Lee FKN, Ivkovic-Jensen MM, Liu W, Lessner DJ, Friemann R, Ramaswamy S, Gibson DT (2005) Purification, characterization, and crystallization of the components of the nitrobenzene and 2-nitrotoluene dioxygenase enzyme systems. Appl Environ Microbiol 71:3806–3814
46. Pflugrath JW (1999) The finer things in X-ray diffraction data collection. Acta Crystallogr D Biol Crystallogr 55:1718–1725

47. Pieper DH (2005) Aerobic degradation of polychlorinated biphenyls. Appl Microbiol Biotechnol 67:170–191
48. Pinyakong O, Habe H, Omori T (2003) The unique aromatic catabolic genes in sphingomonads degrading polycyclic aromatic hydrocarbons (PAHs). J Gen Appl Microbiol 49:1–19
49. Powell HR (1999) The rossmann fourier autoindexing algorithm in MOSFLM. Acta Crystallogr D Biol Crystallogr 55:1690–1695
50. Resnick SM, Torok DS, Gibson DT (1993) Oxidation of carbazole to 3-hydroxycarbazole by naphthalene 1,2-dioxygenase and biphenyl 2,3-dioxygenase. FEMS Microbiol Lett 113:297–302
51. Resnick SM, Torok DS, Lee K, Brand JM, Gibson DT (1994) Regiospecific and stereoselective hydroxylation of 1-indanone and 2-indanone by naphthalene dioxygenase and toluene dioxygenase. Appl Environ Microbiol 60:3323–3328
52. Romine MF, Stillwell LC, Wong K-K, Thurston SJ, Sisk EC, Sensen C, Gaasterland T, Fredrickson JK, Saffer JD (1999) Complete sequence of a 184-kilobase catabolic plasmid from *Sphinomonas aromaticivorans* F199. J Bacteriol 181:1585–1602
53. Schocken MJ, Gibson DT (1984) Bacterial oxidation of the polycyclic aromatic hydrocarbons acenaphthene and acenaphthylene. Appl Environ Microbiol 48:10–16
54. Shames SL, Fairlamb AH, Cerami A, Walsh CT (1986) Purification and characterization of trypanothione reductase from *Crithidia fasciculata*, a newly discovered member of the family of disulfide-containing flavoprotein reductases. Biochemistry 25:3519–3526
55. Subramanian V, Liu T-N, Yeh W-K, Gibson DT (1979) Toluene dioxygenase: purification of an iron-sulfur protein by affinity chromatography. Biochem Biophys Res Commun 91:1131–1139
56. Subramanian V, Liu T-N, Yeh W-K, Narro M, Gibson DT (1981) Purification and properties of NADH-ferredoxin$_{TOL}$ reductase: a component of toluene dioxygenase from *Pseudomonas putida*. J Biol Chem 256:2723–2730
57. Subramanian V, Liu T-N, Yeh W-K, Serdar CM, Wackett LP, Gibson DT (1985) Purification and properties of ferredoxin$_{TOL}$: a component of toluene dioxygenase from *Pseudomonas putida* F1. J Biol Chem 260:2355–2363
58. Tabor S, Richardson CC (1985) A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc Natl Acad Sci USA 82:1074–1078
59. Taira K, Hirose J, Hayashida S, Furukawa K (1992) Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. J Biol Chem 267:4844–4853
60. Takeuchi M, Hamana K, Hiraishi A (2001) Proposal of the genus *Sphingomonas sensu stricto* and three new genera, *Sphingobium*, *Novosphingobium* and *Sphingopyxis*, on the basis of phylogenetic and chemotaxonomic analyses. Int J Syst Evol Microbiol 51:1405–1417
61. Ueda T, Lode ET, Coon MJ (1972) Enzymatic ω-oxidation. VI. Isolation of homogeneous reduced diphosphopyridine nucleotide-rubredoxin reductase. J Biol Chem 247:2109–2116
62. Whitby LG (1953) A new method for preparing flavin-adenine dinucleotide. Biochem J 54:437–442
63. Zabinski R, Munck E, Champion PM, Wood JM (1972) Kinetic and Mössbauer studies on the mechanism of protocatechuic acid 4,5-oxygenase. Biochemistry 11:3212–3219
64. Zylstra GJ, Kim E (1997) Aromatic hydrocarbon degradation by *Sphingomonas yanoikuyae* B1. J Ind Microbiol Biotechnol 19:408–414
65. Zylstra GJ, Kim E, Goyal AK (1997) Comparative molecular analysis of genes for polycyclic aromatic hydrocarbon degradation. Genet Eng 19:257–269