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Genetic and Biochemical Characterization of the Biphenyl Dioxygenase from *Pseudomonas* sp. Strain B4

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Abstract Biphenyl dioxygenase (BPDO), which catalyzes the first step in the bacterial degradation of biphenyl and polychlorinated biphenyls, was characterized in *Pseudomonas* sp. B4. The *bphA* locus containing the four structural genes encoding BPDO were cloned and sequenced. A regulatory gene as well as a putative regulatory sequence were identified upstream of this locus. A transposase-like gene was found within a 1-kb region further upstream, thereby suggesting that the *bphA* locus may be carried on a transposable element. The three components of the BPDO enzyme have been separately overexpressed and purified from *E. coli*. The ferredoxin and terminal dioxygenase components showed biochemical properties comparable to those of two previously characterized BPDOs, whereas the ferredoxin reductase exhibited an unusually high lability. The substrate selectivity of BPDO was examined *in vivo* using resting cell assays performed with mixtures of selected polychlorinated biphenyls. The results indicated that *para*-substituted congeners were the preferred substrates. *In vitro* studies were carried out on a BPDO complex where the reductase from strain B4 was replaced by the more stable isoform from *Comamonas testosteroni* B-356. The BPDO enzyme had a specific activity of 0.26±0.02 μmol $min^{-1}mg^{-1}$ of ISP_{BPH} with biphenyl as the substrate. The 2,3-, 4,4'-, and 2,4,4'-chlorobiphenyls were converted to single dihydrodiols, while 2,4'-dichlorobiphenyl gave rise to two dihydrodiols. The current data also indicated that 2,4,4'-trichlorobiphenyl was a better substrate than the 4,4'-dichlorinated congener.

Key words: Polychlorinated biphenyls, *bph* genes, *Pseudomonas*, biphenyl dioxygenase, enzyme selectivity.

Polychlorinated biphenyls (PCBs) are synthetic compounds composed of a biphenyl molecule bearing from 1 to 10 chlorine atoms. More than 200 PCB congeners exist, many of which are toxic and potential carcinogens [26]. Because these compounds are known to accumulate in the food chain, their dispersion in the environment represents a serious health hazard for humans. A number of aerobic bacteria endowed with PCB degrading ability have been isolated from contaminated soil or sediments. In most cases, these bacteria only utilize PCBs as cometabolic substrates, have a narrow congener specificity, and are only able to degrade a small number of lightly chlorinated PCBs [3, 11, 13]. Genetic studies have shown that all the bacterial species studied so far share a common degradative pathway [9, 23, 29, 30]. PCBs are degraded to chlorobenzoates through four enzymatic steps, the initial attack being catalyzed by biphenyl dioxygenase (BPDO). This enzyme catalyzes the incorporation of two atoms of oxygen into an aryl ring to generate 2,3-dihydro-2,3-dihydroxybiphenyl. BPDO has been purified from three bacterial species including the Gram-negative *Burkholderia cepacia* LB400 [16] and *Comamonas testosteroni* B-356 [18,19] and the Gram-positive *Rhodococcus globerulus* P6 [7]. BPDO is a three-component enzyme composed of a terminal dioxygenase (ISP_{BPH}), ferredoxin (FER_{BPH}), and ferredoxin reductase (RED_{BPH}). The terminal component of the dioxygenase is an $\alpha_3\beta_3$ hexamer containing one [2Fe-2S] Rieske center and one mononuclear Fe^{2+} per pair of αβ subunits. As shown for other ring-hydroxylating dioxygenases, the RED_{BPH} and FER_{BPH} components transfer electrons resulting from NAD(P)H oxidation to the ISP_{BPH} active site, where activation of molecular oxygen and substrate hydroxylation take place [6, 17].

A few bacteria degrading a wide range of PCBs have already been isolated, including *B. cepacia* LB400 [3] and two *Rhodococcus* species [22, 28]. The superior PCB-degrading ability of these strains appears to be due to the broad substrate specificity of the BPDO enzyme, as shown in the case of strain LB400 [16, 24]. The BPDO from LB400 can oxidize poorly degradable PCBs, like di-*ortho*-substituted congeners [14], and exhibits a 3,4-dioxygenase

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activity towards such substrates [16]. In contrast, congeners bearing two chlorine substituents in a *para* position, like 4,4'-dichlorobiphenyl, are poorly transformed, whereas the BPDO from *P. pseudoalcaligenes* KF707, which displays a relatively narrow specificity towards PCBs, efficiently degrades 4,4'-dichlorobiphenyl [12, 14]. Although the BPDOs from *P. pseudoalcaligenes* KF707 and *B. cepacia* LB400 would appear to have quite distinct substrate selectivity towards chlorobiphenyls, they show closely similar amino acid sequences. Only a few amino acid residues of the ISP_{BPH} α subunit have been found to determine the substrate specificity of the enzyme [10, 21, 24].

Accordingly, this report characterized the biphenyl dioxygenase from a *Pseudomonas* strain which was previously selected for its ability to efficiently degrade 4-chlorobiphenyl [8]. The *bphA* locus encoding BPDO was cloned and sequenced. The ISP_{BPH}, FER_{BPH}, and RED_{BPH} components were individually purified in recombinant form, then associated *in vitro*, resulting in the reconstitution of a fully active enzyme. The molecular and catalytic properties of this enzyme were compared to those of previously described enzyme systems.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The following *E. coli* strains were used in this study: DH5α (Life Technologies SARL, Cergy, France); BL21(DE3) [pLysS] (Novagen, Madison, WI, U.S.A.); SG13009[pREP4] (Qiagen SA, Courtaboeuf, France). For plasmid maintenance, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 30 μg/ml; chloramphenicol 30 μg/ml. The *Pseudomonas* sp. strain B4 was isolated from contaminated sediments from the Seine river, France [8]. The bacteria were routinely grown in a Luria-Bertani (LB) broth [27]. For the PCB degradation experiments, strain B4 was grown at 30°C in a biphasic culture [2], consisting of a minimal PAS medium [3] supplemented with 10% (v/v) silicone oil, containing 0.2 g/l biphenyl.

DNA Amplification, Cloning, and Hybridization

The plasmid DNA from *E. coli* was isolated according to published procedures [27] or using a commercial kit (Midi Kit, Qiagen). The genomic DNA from *Pseudomonas* sp. B4 was isolated using a resin-based purification system (genomic-tip 500, Qiagen). Restriction endonuclease reactions, agarose gel electrophoresis, and the transformation of *E. coli* were performed according to previously described protocols [27]. The DNA ligation was carried out with a commercial kit (Roche Diagnostics, Meylan, France). The *bph* genes from *Pseudomonas* sp. B4 were amplified using the following primers, designed based on the sequences of the *P. pseudoalcaligenes* KF707 *bph* genes [30]: 5'-CATA**TG**AGCTCATCAATCAA-3'(BP51) and 5'-*GGATCC***TA**GAAGAACATGCT-3' were used for the *bphA1*-*bphA2* gene pair, 5'-CGCATA**TG**AAATTTACCAGAGTT-3' and 5'-*GGATCC***TCA**TGGCGCCAGATA-3' for *bphA3*, 5'-CATA**TG**ATCGACACCATCGC-3' and 5'-*GGATCC***TCA**ATTCGGTTTG-3'(BP34) for *bphA4* (underlined and italicised letters denote *Nde*I and *Bam*HI sites, respectively, that were introduced into the primers to facilitate cloning. Bold letters indicate the position of the initiation codons or stop anticodons of relevant genes). A 4.1-kb DNA fragment encompassing the *bphA1-A4* region was obtained using primers BP51 and BP34. A 1.0-kb DNA fragment containing a 5'- portion of *bphA1* was also amplified using BP51 and GGCACACTTCGATTTCGTT as the primers. This last fragment, as well as the *bphA3* coding sequence amplified as indicated above, were labelled with digoxigenin (DIG) using DIG-dUTP (Roche Diagnostics), and used as probes in the Southern hybridization experiments. The DNA fragments hybridizing with the probes were revealed using a luminescent detection kit (Roche Diagnostics).

The region upstream of *bphA1* was amplified by an inverse PCR [25] using primers 5'-CATATGAGCTCA-TCAATCAA-3' and 5'-GGGTGGCCGCCAAATCA-3'. The PCR reactions were carried out in a total volume of 50 μl, which contained 100 ng of B4 genomic DNA as the template, 100 μM dNTPs, 100 pmol of each primer, and 2.5 U DNA polymerase. Taq polymerase (Promega France, Charbonnoères) was used for fragments <2 kb, whereas a *Taq/Pwo* enzyme mix (Expand PCR System, Roche Diagnostics) was used for fragments >2 kb. The DNA amplification was performed for 25 cycles as follows; denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and primer extension at 68°C for 3 min.

DNA Sequence Determination and Analysis

The DNA sequence of the region encompassing *orf2*, *orf0*, *bphA1*, *bphA2*, *orf1*, *bphA3*, and *bphA4* (ca. 6.0 kb) was determined by sequencing six overlapping PCR fragments. Any mismatched errors that may have occurred during amplification were tested for by sequencing the 6.0-kb region three times on both strands, using independently amplified fragments. The sequences were analyzed using the LaserGene Program from DNASTAR (Madison, WI, U.S.A.). The 6-kb DNA sequence obtained in this study was submitted to the DDBJ, EMBL, and GenBank databases and assigned accession no. AJ251217.

Plasmid Constructions

The plasmids described below were obtained by cloning the PCR amplified DNA fragments into pGEM-T (Promega) and the subsequent subcloning of *Nde*I-*Bam*HI inserts into pET9a or pET15b (both from Novagen). Plasmid pROD6

was constructed by subcloning the *bphA1A2A3A4* region into pET9a. pROD7 and pROD8 were obtained by subcloning *bphA3* and *bphA3A4* into pET9a, respectively. pROD9 and pROD10 were constructed by subcloning *bphA4* into pET9a (pROD9) and pET15b (pROD10). The pROD10 construct creates a fusion, resulting in a protein bearing an N-terminal His-tag.

Resting-Cell Assays

The PCB degradation ability of strain B4 was determined by resting-cell assays [3]. The bacteria were grown on biphenyl as the sole carbon source, harvested when the turbidity at 600 nm reached 0.8–1.2, washed twice in 50 mM sodium phosphate buffer (pH 7.5), and resuspended to a turbidity of 1.0 in the same buffer. Bacterial samples (1 ml) were transferred to 10-ml screw-cap glass vials, containing PCB mixes made of ten congeners, 0.5 μM each. Two PCB mixes were used (Table 1), the composition of which was identical to that of mixes 1B and 2B given in [3]. Control vials contained cells that had been killed with 0.7% perchloric acid prior to PCB addition. The vials were shaken at 200 rpm for 24 h at 30°C and then extracted with 4 ml hexane. One milliliter of extract was evaporated to dryness and then dissolved in 100 μl acetonitrile before PCB analysis using GC-MS. The percent removal of PCB congeners was calculated relative to the acid-killed control, and results were expressed as means of triplicate resting-cell assays.

Purification of B4 ISP_{BPH} Component

E. coli BL21(DE3)[pLysS][pROD6] was grown in 6 l of terrific broth and incubated for 4 h at 37°C with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The bacteria (55 g, wet weight) were harvested, resuspended in two volumes of a sonication buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 5% (vol/vol) ethanol] and disrupted for 5 min on ice with a Bioblock Scientific ultrasonifier (Fisher Bioblock Scientific, Illkirch, France). The lysate was centrifuged for 10 min at 12,000 ×g and the supernatant fraction was diluted with the same volume of buffer A1 [50 mM Tris-HCl (pH 7.5), 5% (wt/vol) glycerol, 5% (vol/vol) ethanol] before being applied to a 40-ml DEAE-cellulose column (DE52, Whatman International Ltd, Maidstone, U.K.) equilibrated with buffer A1. After extensive washing with the same buffer, the brown ISP_{BPH} fraction was eluted with buffer A1 containing 250 mM NaCl, and further purified using a Kontron FPLC system (Bio-Tek Kontron Instruments, St Quentin Yvelines, France). The protein fraction was adjusted to 40% saturation with ammonium sulfate, then applied to a 30-ml phenyl-Sepharose column (Amersham Pharmacia Biotech, Orsay, France) which was developed with a linear gradient from 40 to 0% ammonium sulfate in buffer A1. The brown ISP_{BPH} fraction was dialyzed against buffer A1, then loaded on a 20-ml column of Q-hyperD (Biosepra SA, Cergy, France). The column was developed with a linear gradient from 0 to 250 mM NaCl in buffer A1. The purified ISP_{BPH} was concentrated to 63 μM by ultrafiltration through a 30-kDa cut-off Omega membrane (Pall-Gelman Laboratory, St Germain-en-Laye, France) and stored in liquid nitrogen.

Purification of B4 FER_{BPH} Component

E. coli BL21(DE3)[pLysS][pROD7] was grown at 37°C in 5 l of LB broth, and then incubated at 26°C with 0.5 mM IPTG for 16 h. The purification procedure was performed at 4°C under argon. The bacteria (17 g, wet weight) were resuspended in three volumes of 50 mM Tris-HCl (pH 8.0) containing 5 mM EDTA, and then disrupted by ultrasonication. The bacterial extract was clarified by centrifugation at 12,000 ×g for 10 min and applied to a 30-ml DEAE-cellulose column (DE52 Whatman) equilibrated with buffer A2 [25 mM Tris-HCl (pH 7.5)]. After washing with buffer A2 containing 100 mM NaCl, the brown FER_{BPH} fraction was eluted with 250 mM NaCl. This fraction was diluted four-fold in buffer A2 and concentrated onto a 5-ml DEAE-cellulose column. The FER_{BPH} fraction was loaded on a 540-ml AcA 54 gel filtration column (Biosepra), equilibrated with buffer A2 containing 200 mM NaCl. The column was developed at a flow rate of 20 ml/h. The FER_{BPH} fraction was concentrated to 189 μM by ultrafiltration through an 8-kDa cut-off Omega membrane, and stored as frozen droplets in liquid nitrogen.

Purification of B4 His-Tagged RED_{BPH} Component

E. coli BL21(DE3)[pLysS][pROD10] was grown in 5 l of LB broth, and then *bphA4* overpression was induced with 0.5 mM IPTG for 6 h at 30°C. The bacteria (10 g, wet weight) were resuspended in four volumes of 50 mM Tris-HCl, pH 8.0, and disrupted by ultrasonication. The lysate was centrifuged at 12,000 ×g for 15 min, then applied to a 10-ml IMAC-Ni^{2+} column (Chelating-Sepharose, Amersham Pharmacia Biotech) equilibrated with 25 mM Tris-HCl (pH 8.0), containing 5 mM imidazole and 0.5 M NaCl. After washing the column successively with five volumes of the same buffer and five volumes of a buffer containing 50 mM imidazole, a yellow fraction containing His-tagged RED_{BPH} was eluted with 25 mM Tris-HCl (pH 8.0) containing 300 mM imidazole. This fraction was dialyzed against 1 l of buffer A3 [25 mM Tris-HCl (pH 7.5), 10% (wt/vol) glycerol], and then loaded on a 2-ml prepacked column of Q-hyperD (Biosepra) equilibrated with buffer A3. The column was developed with a linear gradient from 0 to 800 mM NaCl using a Kontron FPLC system. The purified His-tagged RED_{BPH} was stored in liquid nitrogen.

Purification of the B-356 ht-RED_{BPH} Component

The reductase component of *C. testosteroni* B-356 was purified from *E. coli* strain SG13009[pREP4][pEQ31::*bphG*],

which was designed to overproduce this protein [18]. Briefly, His-tagged B-356 RED_{BPH} was isolated by IMAC on a Ni^{2+}-charged chelating sepharose column under conditions identical to those used for the B4 RED_{BPH} isolation.

Protein Analyses

The proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a Tris-glycine buffer system using a mini slab-gel unit (Hoefer SE200, Amersham Pharmacia Biotech) and stained with Coomassie blue [27]. The absorbance spectra were recorded using a diode-array Hewlett Packard 8452A spectrophotometer. The protein concentration was routinely determined with a BCA colorimetric assay (Pierce, Rockford, IL, U.S.A.). The concentration of the purified BPDO components was also estimated spectrophotometrically using the following absorption coefficients: $\varepsilon_{455}=10{,}100\ M^{-1} \cdot cm^{-1}$ for ISP_{BPH}; $\varepsilon_{460}=4{,}500\ M^{-1} \cdot cm^{-1}$ for FER_{BPH}; and $\varepsilon_{448}=11{,}500\ M^{-1} \cdot cm^{-1}$ for RED_{BPH}. The absorption coefficients of the LB400 enzyme components were used [5,15]. The B-356 RED_{BPH} protein was quantified using $\varepsilon_{450}=11{,}250\ M^{-1} \cdot cm^{-1}$ [18].

BPDO Assays

The *in vitro* enzyme assays were performed on a reconstituted BPDO complex obtained by mixing the purified ISP_{BPH} and FER_{BPH} components from strain B4 with RED_{BPH} from strain B-356. The assays were performed in 90 mM N-morpholinoethanesulfonic acid (MES), pH 6.0, following a protocol adapted from a previously described method [18]. The reaction mixtures (200-µl total volume) contained 500 nmol of NADH, 1.2 nmol of $FeSO_4$, 100 nmol of a substrate (biphenyl or one of the indicated chlorobiphenyls dissolved in 10 µl acetone), 1 nmol of ISP_{BPH}, 1 nmol of the RED_{BPH} component, and 2 nmol of the FER_{BPH} component. The reaction mixtures were incubated at 37°C in Eppendorf tubes under shaking. The reaction was initiated by adding a 10-fold concentrated mixture of the three enzyme components and stopped by two successive extractions using 0.4 ml ethyl acetate. A reaction mixture containing all the components except for ISP_{BPH} was used as the control.

The continuous monitoring of NADH oxidation was performed by fluorometric measurements using a Spex Fluoromax 5051 spectrometer (Jobin Yvon SA, Longjumeau, France). The excitation wavelength was set at 340 nm and the light emission was measured at 459 nm. The reactions were carried out at 37°C in a quartz cuvette maintained under constant agitation. The assay mixture contained the same reagents as for the BPDO assay described above, except that the final volume was 1.5 ml and the NADH content was 200 nmol. The ISP_{BPH} concentration in the assay was 0.67 µM. The reaction was initiated by adding the desired amount of substrate dissolved in 5 µl of acetone. All assays were performed in triplicate. The kinetic parameters were calculated from the initial rates of NADH oxidation measured at biphenyl concentrations ranging from 5 to 100 µM.

GC-MS Analysis

The PCBs and their oxidation products were analyzed by GC-MS using a HP6890/HP5973 apparatus (Agilent Technologies, Les Ulis, France) equipped with a coiled-capillary column (0.25 mm inner diameter, 30 m long) packed with 5% phenyl and 95% dimethylpolysiloxane (HP5-MS, Agilent). Prior to analysis, the oxidation products from the BPDO assays were derivatized as follows; ethyl acetate extracts were dried over sodium sulfate, evaporated under a stream of argon, then dissolved in 20 µl of dimethylformamide: acetone, 1:1 by volume. *n*-Butylboronate (NBB) derivatives of the diol products were generated by reacting samples with 80 µl of NBB reagent for 15 min. For the gas chromatographic separation of PCB mixtures, samples (1 µl) were injected onto the column in the split mode (1/50 ratio) with helium as the carrier gas (flow rate: 50 ml/min). The PCBs were separated by applying a two-step temperature gradient from 60 to 200°C at a rate of 12°C/min, then from 200 to 300°C at a rate of 17°C/min. For analysis of the NBB derivatives, a temperature gradient from 100 to 250°C at 10°C/min was employed. The relative percentages of the products were calculated from the ion peak areas.

Chemicals

Biphenyl, *n*-butylboronate (Supelco reagent), antibiotics, and most other chemicals were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). The PCBs were obtained from Interchim (Montluçon, France), and silicone oil, type 47V20, was from Sodipro (Echirolles, France). The IPTG was purchased from Eurogentec (Seraing, Belgium).

RESULTS AND DISCUSSION

PCB Degradation by Strain B4

Pseudomonas sp. strain B4 was isolated from PCB-contaminated sediments and selected for its ability to degrade 4-chlorobiphenyl [8]. The PCB degradative competence of strain B4 was investigated by performing resting-cell assays using two defined mixtures of ten congeners [3]. The results indicated that the double-*para*-substituted congeners were preferentially attacked by this bacterium (Table 1). Strain B4 poorly degraded PCBs containing more than three chlorines, and was unable to degrade congeners bearing a double *ortho*-substitution. The range of congeners degraded by strain B4 was similar to that found for strain KF707, and relatively narrow compared to that of strain LB400 (Table 1).

Cloning of the *bphA* Locus and Sequence Analysis

Based on PCR amplification, genomic DNA from strain B4 was used as a template to generate two nucleotidic

Table 1. Extent of PCB depletion by strain B4 as compared to strains LB400 and KF707.

Congener	% depletion by strain:		
	LB400[a]	KF707[a]	B4[b]
2,3	100	100	100
2,2'	100	5	0
2,4'	100	100	100
4,4'	15	100	100
2,5,2'	100	0	0
2,5,4'	94	83	19±1.5
2,4,4'	45	93	49±2
2,3,2',3'	94	60	32±5.5
2,3,2',5'	96	0	0
2,5,2',5'	95	0	0
2,5,3',4'	83	0	0
2,4,3',4'	16	24	0
2,4,2",4	38	0	0
3,4,3',4'	0	0	0
2,4,5,2',5'	73	0	0
2,3,4,2',5'	58	0	0
2,4,5,2',3'	38	0	0
2,4,6,2',4' (IS)[c]	0	0	0
2,4,5,2',4',5'	18	0	0

[a]Data published by Erickson and Mondello [10].
[b]Average values from three independent resting-cells assays ±standard deviation. Depletion rates below 10% were considered insignificant.
[c]Internal standard not degraded.

fragments, 0.33 and 1.0 kb in size, corresponding to *bphA3* and the 5'-end portion of *bphA1*, respectively. Hybridization experiments using these two PCR fragments as probes indicated that the *bph* genes of strain B4 were probably organized in a way similar to those found in strain KF707 [30] (data not shown). Next, a 4.1-kb DNA fragment spanning the region between *bphA1* and *bphA4* was amplified and sequenced. The sequence analysis revealed that this fragment was composed of five genes, *bphA1*, *bphA2*, *orf1*, *bphA3*, and *bphA4* (Fig. 1). The predicted properties of these gene products are presented in Table 2. The deduced amino acid sequences of the B4 enzyme components

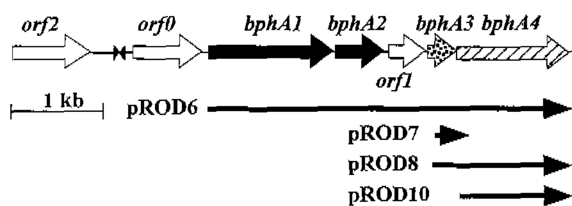

Fig. 1. Genetic organization of the *bphA* locus of *Pseudomonas* sp. B4.
In addition to the four structural *bph* genes encoding BPDO, three open reading frames (*orfs*) are indicated. The position of a potential regulatory site (inversed arrowheads) is also shown upstream of *orf0*. The heavy arrows shown below indicate the DNA fragments used for the overexpression of the BPDO components in *E. coli*.

Table 2. Predicted properties of gene products found in the *bphA* locus of strain B4 and a 2-kb upstream region.

Gene	% GC	Nucleotide size	Predicted protein	Molecular mass (Da)	Isoelectric point
orf2	64.7	600	Transposase	21,616	9.5
orf0	58.0	738	Regulator	27,642	8.1
bphA1	62.1	1,380	$ISP_{BPH}\alpha$	51,512	6.2
bphA2	56.3	564	$ISP_{BPH}\beta$	22,086	5.9
orf1	58.0	417	Unknown	ND[a]	ND
bphA3	56.1	330	FER_{BPH}	11,955	4.6
bphA4	69.0	1,227	RED_{BPH}	42,958	6.1

[a]ND means not determined.

exhibited a high homology with the corresponding subunit sequences of the enzyme from strains KF707 and LB400 [9, 30]. The ferredoxin (BphA3) was identical, whereas the reductase (BphA4) and β subunit of ISP_{BPH} (BphA2) differed by a single residue. In contrast, the α subunit of ISP_{BPH} differed at 11 amino acid positions from the KF707 enzyme and also at 11 other positions from the LB400 enzyme. Most of the nonconserved residues of the B4 ISP_{BPH} α subunit were located in the C-terminal region of the protein (Fig. 2). Previous studies performed on the LB400 BPDO and related enzymes demonstrated that this region of the α subunit contains a limited number of residues at defined positions which determine the selectivity of the enzyme towards PCB congeners [10, 24]. In this respect, the B4 enzyme was more closely related to the KF707 enzyme as it contained four residues conferring the ability to attack PCB congeners with double *para*-substitutions (Asp335, Ile336, Thr338, and Thr341), and lacked an Asn residue in position 377 which confers on the LB400 enzyme the ability to oxidize congeners bearing *ortho*-substituents, like 2,5,2'5'-tetrachlorobiphenyl [21, 24]. However, the B4 ISP_{BPH} α subunit had a Tyr residue in position 277, like

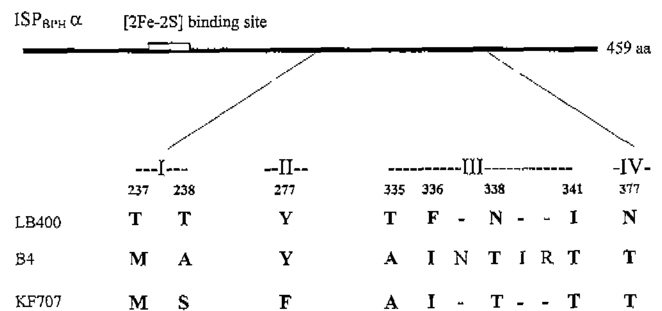

Fig. 2. Schematic representation of the ISP_{BPH} sequence showing amino acid regions known to be involved in PCB congener recognition.
The four relevant sequence regions of the ISP_{BPH} α subunits from strains B4, LB400, and KF707 have been aligned. The numbering refers to the residue positions in the sequences. The dashes denote the conserved residues. The position of the putative [2Fe-2S] binding site is also indicated.

the LB400 enzyme, whereas a Phe occurs at this position in the KF707 enzyme (Fig. 2).

Sequence Analysis of a Region Upstream from the *bphA* Locus

An inverse PCR strategy, as described in the Materials and Methods section, was used to clone a region extending approximately 2 kb upstream from *bphA1*. Two open reading frames were identified in this region, called *orf0* and *orf2*, which exhibited the same transcriptional orientation as *bphA1* (Fig. 1). *orf0* was identical in size (738 bp) and similar in sequence (99.5% identity) to a gene found in the same position in strain KF707, which was recently shown to encode a regulatory protein required for growth on biphenyl [31]. This protein specifically binds a 18-bp regulatory sequence located 92 bp upstream of *orf0*, thereby activating the transcription of its own gene. In strain B4, an identical 18-bp DNA sequence was identified in an equivalent position, suggesting that the *orf0* gene product may play a similar regulatory role.

orf2 was situated 380 bp upstream of *orf0*, and showed a high degree of similarity with an *orf* present in a gene cluster coding for a naphthalene degradation pathway in the *Pseudomonas* species. ORF2 exhibited a 98.2% identity with ORF1 of *P. aeruginosa* K1 (EMBL-GenBank accession number D841469, unpublished) and a 97.7% identity with the *tnpA1* product of *P. stutzeri* AN10 [4]. Based on the deduced amino acid sequence, these *orfs* may encode a transposase [4]. These observations suggest that the *bph* genes of strain B4 may have been acquired by horizontal gene transfer.

Purification and Properties of B4 ISP_{BPH}

ISP_{BPH} was produced from plasmid pROD6 by heterologous overexpression in *E. coli* BL21(DE3)pLysS. Although pROD6 contains the four structural genes of BPDO, only two polypeptides with M_r values corresponding to the α subunit (53,000) and the β subunit (27,000) of ISP_{BPH} were synthesized in appreciable amounts, as judged from an SDS-PAGE analysis of the *E. coli* extract (data not shown). Since FER_{BPH} was successfully overexpressed from another plasmid carrying only *bphA3* (see below), the lack of expression of *bphA3* and *bphA4* from pROD6 may have been due to transcriptional hindrance by the intervening *orf* present between *bphA2* and *bphA3*, or to the possible instability of the >4.1-kb mRNA transcript.

ISP_{BPH} was overproduced as a brown protein, which was purified in three steps and yielded about 1.3 mg of protein per g of cell paste (wet weight). The purified B4 ISP_{BPH} showed two bands on SDS-PAGE (Fig. 3A), with M_r values slightly higher than the values deduced from the sequences of the products of *bphA1* and *bphA2* (53,000 instead of 51,512 for the α subunit, and 27,000 instead of 22,086 for the β subunit). The UV-visible spectrum of the purified ISP_{BPH} exhibited broad maxima around 325 and 455 nm and a shoulder at about 575 nm, which are characteristic of a Rieske-type [2Fe-2S] cluster (data not shown). The protein exhibited characteristics similar to its counterpart in the biphenyl dioxygenases from strain LB400 [15] and *C. testosteroni* B-356 [18].

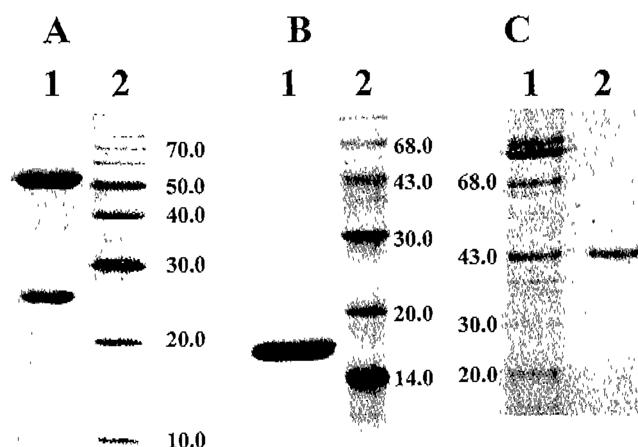

Fig. 3. SDS-PAGE analysis of purified BPDO components. (A) Lane 1, B4 ISP_{BPH}; lane 2, M_r markers; (B) lane 1, B4 FER_{BPH}; lane 2, M_r markers; (C) lane 1, M_r markers; lane 2, His-tagged B4 RED_{BPH}. The marker sizes are indicated in kDa.

Purification and Properties of B4 FER_{BPH}

B4 FER_{BPH} was overexpressed in *E. coli* BL21(DE3)pLysS containing pROD7, leading to accumulation in the cells of a M_r 17,000 polypeptide, half of which was found in insoluble inclusion bodies. By lowering the temperature to 26°C during induction, a greater proportion of the protein was recovered as a holoprotein, although part of it was still insoluble. The ferredoxin was sensitive to oxygen, and thus had to be purified under anaerobic conditions to prevent any oxidative damage. The yield of purified B4 FER_{BPH} was around 2.4 mg/g. The purified B4 FER_{BPH} showed a single band on SDS-PAGE (Fig. 3B). Its molecular mass was about 12 kDa as determined by gel filtration column chromatography, indicating that the ferredoxin was monomeric. The UV-visible spectrum of purified FER_{BPH} showed maxima around 325 and 460 nm and a shoulder at about 575 nm (data not shown). The absorbance ratio A_{460}/A_{280} of FER_{BPH} was 0.21. After reduction with sodium dithionite under anaerobic conditions, new maxima at 384 and 430 nm were observed. These features are typical for proteins containing a Rieske-type [2Fe-2S] cluster. An EPR analysis of the ferredoxin in a reduced form showed a rhombic signal with g-values at 1.82, 1.90, and 2.02, confirming that this protein contains a Rieske-type [2Fe-2S] cluster (data not shown).

Purification of B4 His-Tagged RED_{BPH}

The reductase component was made of a 43-kDa polypeptide, which could not be detected in extracts of *E. coli* carrying

bphA4 on two different expression plasmids, pROD8 or pROD9. When *E. coli* BL21(DE3)pLysS(pROD8) was used, only the BphA3 polypeptide was overproduced, although the expression plasmid carried both *bphA3* and *bphA4*, indicating that the reductase gene was poorly expressed. Using a construction introducing a His-tag fusion at the N-terminus of the *bphA4* gene product (pROD10), a polypeptide with the predicted size became detectable upon an immunoblot analysis of the cell extract with an antibody directed against the hexahistidine peptide (data not shown). Although the recombinant protein was produced at a low level, it was isolated by IMAC affinity chromatography. An additional purification step using anion-exchange chromatography yielded an essentially homogenous preparation, as judged from an SDS-PAGE analysis (Fig. 3C). The yield of purified B4 RED_{BPH} was low (0.03 mg/g cell paste). Its apparent M_r (45,000) was in close agreement with the predicted molecular mass deduced from the BphA4 amino acid sequence, augmented by the 2-kDa His-tag. The purified B4 RED_{BPH} was yellow and displayed an absorbance spectrum with maxima at 278 nm, 376 nm, and 450 nm, the characteristic of a flavoprotein (data not shown). Using similar conditions for expression and purification, the RED_{BPH} component of strain B-356 was obtained in good yields (5 mg/g *E. coli* cells). Hence, the low level of expression of B4 RED_{BPH} in *E. coli* as well as its low recovery upon purification suggest that it is unstable. Except for this trait, the biphenyl dioxygenase from strain B4 exhibited general biochemical properties similar to those of other arene hydroxylating dioxygenases [6].

Catalytic Activity of Reconstituted BPDO Complex

As the reductase component of the B4 BPDO enzyme appeared to be unstable, the RED_{BPH} component of *C. testosteroni* B-356 was purified and combined with the ISP_{BPH} and FER_{BPH} components of the B4 enzyme, and then the catalytic activity of the complex was studied *in vitro*. The reconstituted enzyme thus obtained had a specific activity of 0.26±0.02 μmol/min/mg ISP_{BPH} with biphenyl as the substrate. This value was higher than that previously reported for the LB400 enzyme [0.091 U/mg; [15]], yet lower than that for *C. testosteroni* B-356 [2.4 U/mg; [18]]. Although the bacterial source may explain the differences between the enzymes, other factors related to the purification procedure and the assay conditions may also be invoked. In a recent study where such factors were optimized, the B-356 dioxygenase showed a specific activity of 4.9 U/mg [20].

The BPDO reaction rates were determined at various biphenyl concentrations by the continuous monitoring of NADH oxidation. In the control experiments in which biphenyl was omitted, a low rate of NADH oxidation was observed, due likely to the autooxidation of the FER_{BPH} or RED_{BPH} component. When taking this into account in calculations, a good correlation was observed between the amount of NADH oxidized by the enzyme and the amount of biphenyl transformed into dihydrodiol (data not shown). Hence, the kinetic parameters of the BPDO-catalyzed reaction could be determined from the plots of the NADH oxidation rates versus the biphenyl concentration. The enzyme showed a V_{max} of 110±10 μM/min and an apparent K_m of 84±17 μM. In comparison, the *C. testosteroni* B-356 dioxygenase showed a similar V_{max} (141 μM/min), yet its K_m was one order of magnitude lower (6.2 μM) [20].

Besides biphenyl, B4 BPDO was able to catalyze the dihydroxylation of selected chlorobiphenyls as summarized in Table 3. The oxidation products formed during the *in vitro* assays were extracted and derivatized with *n*-butylboronate prior to a GC-MS analysis. Among the seven potential substrates tested, four yielded single dihydrodiols, one gave two dihydrodiols, and two did not yield significant amounts of oxidation products (Table 3). Accordingly, a tentative assignment of the positions of the hydroxylated carbons is proposed on the basis of previous studies

Table 3. Oxidation products formed by B4 BPDO from biphenyl and selected PCBs.

Substrate	Characteristics of products identified by GC-MS		
	Retention time (min)	Mass[a]	Identification
Biphenyl	16.2	254	2,3-dihydrodiol
2,3-Dichlorobiphenyl	18.8	322	dihydrodiol
2,4'-Dichlorobiphenyl	18.6 (80)[b]	322	dihydrodiol
	18.8 (20)[b]	322	dihydrodiol
4,4'-Dichlorobiphenyl	20.0	322	dihydrodiol
2,4,4'-Trichlorobiphenyl	20.2	356	dihydrodiol
2,5,4'-Trichlorobiphenyl	–	–	no product
2,2',5,5'-Tetrachlorobiphenyl	–	–	no product

[a]Masses of *n* butylboronate derivatives.

[b]2,4'-Dichlorobiphenyl yielded two dihydrodiols. Values in parentheses are percent amounts of products calculated from ion peak area.

[16, 21]: 2,3 for biphenyl and 4,4'-chlorobiphenyl; 2',3' for 2,3- and 2,4,4'-chlorobiphenyl; and 2',3' and 5,6 for the two dihydrodiols generated from the 2,4'-dichlorinated congener. The assignments concerning the dihydrodiols derived from the latter three substrates are more speculative since no characterization of such compounds has yet been reported.

The substrate preference of the B4 enzyme was deduced from the extent of the substrate conversion at the end of the incubation time allotted to each enzyme reaction. Biphenyl and 2,3-dichlorobiphenyl were entirely converted to the corresponding dihydrodiols within 4 and 10 min, respectively. When subjected to an identical BPDO treatment for 45 min, about 70% of the 2,4'-dichlorobiphenyl and 42% of the 2,4,4'-trichlorobiphenyl were dihydroxylated, whereas only around 3% of the 4,4'-dichlorobiphenyl was transformed. These data indicate that the catalytic rate of the enzyme varied widely depending on the substrates, as recently reported for the enzyme from strain LB400 [1]. In addition, when the extent of the oxidation of the 4,4'- and 2,4,4'-substituted congeners was compared, trichlorobiphenyl appeared to be a better substrate. Hence, in some cases, the presence of one additional chlorine substituent in one of the rings would appear to promote a faster catalytic reaction.

Both *in vivo* and *in vitro* assays showed that the B4 BPDO complex preferentially attacked *para*-substituted congeners (Tables 1 and 3). In this respect, it resembles the enzyme from strain KF707 [12], consistent with the fact that the two enzymes have very close amino acid sequences. In addition, the ISP_{BPH} α subunits of the two enzymes share four conserved residues, found to be responsible for the ability of BPDO to dihydroxylate 4,4'-chloro-substituted congeners [10] (Fig. 2).

Some discrepancies were, however, observed between the *in vivo* and *in vitro* activities of the B4 enzyme toward certain chlorobiphenyls (see Tables 1 and 3). Resting-cell assays showed a significant depletion of 2,5,4'-chlorobiphenyl, while no dihydrodiol was detected in *in vitro* assays. Perhaps the catalytic activity of the B4 enzyme towards this congener was too slow to observe the oxidation product. In addition, the *in vitro* assays with the purified B4 enzyme demonstrated that 2,4,4'- and 4,4'-chlorobiphenyls were converted to corresponding dihydrodiols, and indicated that the trichlorinated congener was a better substrate than the dichlorinated one. In contrast, in the resting-cell assays, 4,4'-chlorobiphenyl was more completely degraded than 2,4,4'-chlorobiphenyl. Several possible explanations may account for the lower oxidation rate of 2,4,4'-chlorobiphenyl observed *in vivo*, including competitive inhibition by other substrates, differences of solubility, or membrane permeability. In any case, these observations indicate that the results of *in vivo* assays do not always reflect precisely the substrate preference of a given BPDO enzyme towards individual PCBs.

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