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Substrate Specificity and Expression of Three 2,3-Dihydroxybiphenyl 1,2-Dioxygenases from *Rhodococcus globerulus* Strain P6

David B. McKay,[1]† Matthias Prucha,[1]‡ Walter Reineke,[2] Kenneth N. Timmis,[1] and Dietmar H. Pieper[1]*

Department of Environmental Microbiology, GBF—German Research Center for Biotechnology, D-38124 Braunschweig,[1] and Chemical Microbiology, Bergische Universität Wuppertal, D-42097 Wuppertal,[2] Germany

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Rhodococcus globerulus **strain P6 contains at least three genes, *bphC1*, *bphC2*, and *bphC3*, coding for 2,3-dihydroxybiphenyl 1,2-dioxygenases; the latter two specify enzymes of the family of one-domain extradiol dioxygenases. In order to assess the importance of these different isoenzymes for the broad catabolic activity of this organism towards the degradation of polychlorinated biphenyls (PCBs), the capacities of recombinant enzymes expressed in *Escherichia coli* to transform different chlorosubstituted dihydroxybiphenyls formed by the action of *R. globerulus* P6 biphenyl dioxygenase and biphenyl 2,3-dihydrodiol dehydrogenase were determined. Whereas both BphC2 and BphC3 showed similar activities for 2,3-dihydroxybiphenyl and all monochlorinated 2,3-dihydroxybiphenyls, BphC1 exhibited only weak activity for 2′-chloro-2,3-dihydroxybiphenyl. More highly chlorinated 2′-chlorosubstituted 2,3-dihydroxybiphenyls were also transformed at high rates by BphC2 and BphC3 but not BphC1. In *R. globerulus* P6, BphC2 was constitutively expressed, BphC1 expression was induced during growth on biphenyl, and BphC3 was not expressed at significant levels under the experimental conditions. Although we cannot rule out the expression of BphC3 under certain environmental conditions, it seems that the contrasting substrate specificities of BphC1 and BphC2 contribute significantly to the versatile PCB-degrading phenotype of *R. globerulus* P6.**

Polychlorinated biphenyls (PCBs) have been of public and scientific concern for several decades because of their persistence in the environment, their ability to bioaccumulate, and their potential carcinogenicity. Commercially produced PCB mixtures such as Aroclor, Clophen, and Kaneclor typically contain 60 to 80 of the 209 theoretically possible PCB congeners, which differ in the degree and position of chlorination.

A promising approach for dealing with PCB contamination is bioremediation, because a number of biphenyl-degrading organisms are capable of transforming PCB congeners. These organisms belong to both gram-negative and gram-positive genera and catabolize biphenyl to benzoate and 2-hydroxypenta-2,4-dienoate via the so-called upper pathway, which consists of four enzymes: biphenyl 2,3-dioxygenase (BphA), 2,3-dihydro-2,3-dihydroxybiphenyl-2,3-dehydrogenase (BphB), 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC), and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BphD) (7).

To a significant extent, the spectrum of PCB congeners that can be transformed by an organism is determined by the specificity of the biphenyl 2,3-dioxygenase, the enzyme which catalyzes the first step in the upper pathway. Studies on various biphenyl 2,3-dioxygenases have revealed considerable differences in their congener selectivity patterns, as well as their preference for the attacked ring (4, 12, 22, 28, 38, 44).

Though certain PCBs serve as substrates for biphenyl dioxygenases, PCB-degrading organisms do not usually use PCBs as an energy source but rather cometabolically catabolize the substrates. Not surprisingly, metabolites of the upper pathway may be formed as dead-end products by the action of the upper-pathway enzymes (6, 14, 36).

Knowledge of metabolic activities of upper-pathway enzymes downstream of biphenyl dioxygenase would be of interest in order to further our understanding of the capacity of upper pathways. For example, Seah et al. (35) have shown that the *bphD* gene products of *Burkholderia* sp. strain LB400 and *Rhodococcus globerulus* strain P6 differ significantly in their kinetic properties for chlorinated substrate derivatives. However, interpretation of the potential for transformation of PCBs may be complicated by the existence of isoenzymes. In recent years, information indicating that various microorganisms contain multiple metabolic pathways or isoenzymes involved in the degradation of PCBs has accumulated. For example, *Rhodococcus* sp. strain RHA1 contains two distinct PCB degradation systems (23, 39); one is preferentially involved in biphenyl degradation, whereas the other is involved in the degradation of ethylbenzene. Six extradiol dioxygenase genes were recently identified in this organism (33), and three are expressed when the organism is grown on biphenyl. A total of seven *bphC* genes have been found in *Rhodococcus erythropolis* strain TA421 (24, 27), and three have been found in *R. globerulus* strain P6 (3). Thus, the presence of multiple extradiol dioxygenases seems to be common in rhodococcal strains and is thought to contribute to the versatility of this group of bacteria in the degradation of haloaromatic compounds.

Two of the BphC enzymes of *R. globerulus* P6, namely, BphC2 and BphC3, were the first reported members of a new

* Corresponding author. Mailing address: Bereich Mikrobiologie, AG Biodegradation, Gesellschaft für Biotechnologische Forschung mbH, Mascheroder Weg 1, D-38124 Braunschweig, Germany. Phone: 49/(0)531/6181-467. Fax: 49/(0)531/6181-411. E-mail: dpi@gbf.de.

† Present address: Faculty of Science, University of the Sunshine Coast, Maroochydore DC, Queensland 4558, Australia.

‡ Present address: Affymetrix GmbH, D-85399 Hallbergmoos/Munich, Germany.

family of single-domain extradiol dioxygenases (11), whereas BphC1, whose gene was shown to be localized downstream of a *bphA1A2A3A4B* gene cluster (28), belongs to the family of two-domain extradiol dioxygenases. A third member of the family of single-domain extradiol dioxygenases has been characterized from the naphthalenesulfonate-degrading bacterium *Sphingomonas* sp. strain BN6 (19), and one of the BphC enzymes of TA421 also belongs to this family (33). The BN6 enzyme was recently shown to be capable of distal cleavage of 3-chlorocatechol (32), a property that distinguishes this enzyme from all other extradiol cleavage enzymes. Due to their importance in the catabolism of biphenyls, the genes encoding various 2,3-dihydroxybiphenyl dioxygenases have been cloned and sequenced, but there is a paucity of detailed analysis, particularly with regard to their activity towards halogenated 2,3-dihydroxybiphenyls.

In this paper we report the purification of the three isoenzymes of *R. globerulus* strain P6, an analysis of their capacities to transform halogenated 2,3-dihydroxybiphenyls, and their expression characteristics.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* strain MV1190 harboring pJA6X, pJA94, or pJA32, which express the extradiol dioxygenases BphC1, BphC2, and BphC3, respectively, of *R. globerulus* strain P6, were described previously (2). *E. coli* strain BL21(DE3)(pLys) harboring either pAIA15 (containing *bphC* of *Burkholderia* sp. strain LB400) or pT7-5RW (containing *dbfB* of *Sphingomonas wittichii* strain RW1) has also been described (16, 38). *Pseudomonas putida* KT2442 (13) was used as the host of pDM9, a derivative of the broad-host-range vector pBBR1MCS (25), which contains *bphA1A2A3A4B* of *R. globerulus* P6. pDM9 was produced by partially digesting pDM7 (28) with *Sac*I, followed by religation. Growth conditions for KT2442 and derivatives were as previously described (28). *R. globerulus* P6 was grown as described by Asturias and Timmis (3) with biphenyl (1 g/liter) or succinate (10 mM) as sole carbon source or in Luria-Bertani (LB) broth.

Biosynthesis of chemicals used in this study. For the preparation of chlorosubstituted 2,3-dihydroxybiphenyls, *P. putida* KT2442(pDM9) expressing BphA1A2A3A4B of *R. globerulus* strain P6 was grown on LB agar plates containing 500 μg of chloramphenicol per ml as previously described (28). Harvested cells were washed twice and resuspended in phosphate buffer (50 mM, pH 7.5) to a final optical density (A_{546}) of 5. Cell suspensions (20 ml) supplemented with individual PCB congeners (0.1 mM) were incubated in 100-ml Erlenmeyer flasks sealed with Teflon-coated screw caps on a shaking water bath (140 rpm, 30°C) for 5 h. The cell-free supernatant fluid containing chlorosubstituted 2,3-dihydroxybiphenyl was used directly for enzymatic tests.

Larger amounts of 2′-chloro-2,3-dihydroxybiphenyl were prepared from 2-chlorobiphenyl by resting cells of *E. coli* BL21(DE3)(pLysS) harboring pAIA13 (37). Resting cells were prepared as described previously (37). A total of 2 liters of a culture with an optical density (A_{546}) of 2 was incubated with 400 mg of 2-chlorobiphenyl dissolved in 2 ml of dimethyl sulfoxide. The reaction was monitored with a Shimadzu high-pressure liquid chromatography system (LC-10AD liquid chromatograph, DGU-3A degasser, SPD-M10A diode array detector, and FCV-10AL solvent mixer) equipped with a SC125/LiChrospher (Bischoff, Leonberg, Germany) 5-μm column. The aqueous solvent system (flow rate, 1 ml/min) contained 0.01% (vol/vol) H_3PO_4 (87%) and 60% (vol/vol) methanol. After 3 h of incubation, the cells were removed by centrifugation and the supernatant fluid was acidified to pH 4.0 with HCl. Dimethyl sulfoxide was removed by extraction with *n*-hexane (three times, 50 ml each), and residual *n*-hexane was subsequently removed under a stream of nitrogen. The supernatant was then passed over an Isolute ENV+ column (Separtis GmbH, Grenzach-Wyhlen, Germany), and the catechol derivative was eluted from the column by methanol (10 ml). The eluate was evaporated to dryness, and 2′-chloro-2,3-dihydroxybiphenyl was separated from 2,3-dihydroxybiphenyl (formed as a by-product during the enzymatic reaction) by preparative high-pressure liquid chromatography on a Vertex column (250 by 16 mm filled with 5 μM LiChrosorb) with the solvent system described above at a flow rate of 6 ml/min. The eluate was supplemented with NaCl and extracted three times with equal volumes of ethyl acetate. After evaporation to dryness, approximately 21 mg of highly pure 2′-chloro-2,3-dihydroxybiphenyl was obtained. 3′- and 4′-chloro-2,3-dihydroxybiphenyl were prepared as described previously (29).

Preparation of cell extracts. Cell extracts were prepared from 0.1 to 1.5 liters of cells grown in baffled Erlenmeyer flasks. Harvested cells of *E. coli* were resuspended in potassium phosphate buffer (50 mM, pH 7.5), and disrupted with a French press (Aminco, Silver Spring, Md.). Cell debris was removed by centrifugation at 100,000 × *g* for 1 h at 4°C. Cell extracts from *R. globerulus* P6 were prepared as previously described (2).

Enzyme assays. 2,3-Dihydroxybiphenyl dioxygenase activity was determined in 50 mM K/Na-phosphate (pH 7.5) with 0.1 mM 2,3-diydroxybiphenyl as the substrate. Kinetic parameters were determined at substrate ranges of 1 to 1,000 μM (2,3-dihydroxybiphenyl and 2′-, 3′-, or 4′-chloro-2,3-dihydroxybiphenyl), 20 to 5,000 μM (catechol, 3-methylcatechol, and 3-chlorocatechol), and 0.2 to 20 mM (4-methylcatechol) by using previously described extinction coefficients of reaction products (catechol, ε_{375} = 36,000 M^{-1} cm^{-1}; 3-methylcatechol, ε_{388} = 13,800 M^{-1} cm^{-1}; 4-methylcatechol, ε_{382} = 28,100 M^{-1} cm^{-1}; 3-chlorocatechol, ε_{378} = 33,000 M^{-1} cm^{-1}) (10, 19). Extinction coefficients of ring cleavage products formed from 2,3-dihydroxybiphenyl or 2′-, 3′-, or 4′-chloro-2,3-dihydroxybiphenyl were as follows: ε_{434} = 21,700 M^{-1} cm^{-1}; ε_{393} = 26,800 M^{-1} cm^{-1}, ε_{438} = 26,100 M^{-1} cm^{-1}, and ε_{439} = 23,900 M^{-1} cm^{-1}, respectively. Inhibition by 3,4-dihydroxybiphenyl was determined at 2,3-dihydroxybiphenyl concentrations between 1 and 100 μM and inhibitor concentrations up to 2 mM. Substrate inhibition was calculated as previously described (20). Kinetic data were calculated from the initial velocities with the Michaelis-Menten equation by nonlinear regression by using KaleidaGraph (Synergy Software).

Specific activities are expressed as micromoles of product formed per minute per milligram of protein at 25°C. Protein concentrations were determined by the Bradford procedure (8). k_{cat} values were calculated based on deduced molecular masses of the BphC1, BphC2, and BphC3 subunits of 32.1, 20.8, and 21.1 kDa, respectively (2). Activities were usually calculated based on the protein content. Because BphC proteins were purified aerobically (see below), purified enzyme preparations can be assumed to be partially inactivated due to oxidation or loss of active-site Fe^{2+}. The Fe^{2+} content of purified BphC1 and -C2 was quantified as previously described (15) by using enzyme concentrations of 1 to 5 mg/ml in a total volume of 0.3 ml.

The stability of the enzymes in the presence of oxygen was calculated by incubation of enzyme solutions corresponding to 5 mU/ml under standard assay conditions and monitoring the remaining activity after different intervals as previously described (43). Partition ratios were determined spectrophotometrically under standard conditions using 0.5 to 10 mU/ml of enzyme and substrate concentrations of 100 to 200 μM and calculated by dividing the amount of product formed by the amount of enzyme added to the assay.

Purification of extradiol dioxygenases. Purifications were usually performed under aerobic conditions with cell extracts containing 0.9 to 1.3 g of protein in 21 to 27 ml. BphC2 was purified anaerobically as described for the DbfB protein (16), following the detailed protocol described below for aerobic purification. Column chromatography was carried out on a Pharmacia fast protein liquid chromatography system (Amersham Biosciences, Freiburg, Germany). BphC2 and BphC3 were precipitated from cell extracts by ammonium sulfate precipitation. The precipitates that were collected at 35 to 55% (BphC2) or 35 to 58% (BphC3) saturation were redissolved in 10 ml of Tris HCl (pH 7.5) containing 0.24 M ammonium sulfate.

Proteins were eluted from the HiLoad 26/10 phenyl-Sepharose high-performance column by a linear gradient of $(NH_4)_2SO_4$ (0.24 to 0 M) in Tris HCl (20 mM, pH 7.5) over 60 ml, followed by 90 ml of Tris HCl (20 mM, pH 7.5). All BphC-containing protein fractions eluted in fractions without ammonium sulfate (after 110 to 130 ml). Active fractions (16 ml of BphC1-containing eluate with a specific activity of 5.1 U/mg of protein and a protein content of 136 mg, 10 ml of BphC2-containing eluate with a specific activity of 14 U/mg of protein and a protein content of 98 mg, or 8 ml of BphC3-containing eluate with a specific activity of 12.2 U/mg of protein and a protein content of 9 mg) were applied to a MonoQ HR10/10 column (Amersham Bioscience). BphC1 was eluted by stepwise gradients of 0 to 0.4 M NaCl in Tris HCl (50 mM, pH 7.5) over 50 ml and 0.4 to 0.5 M over another 50 ml, whereas BphC2 and BphC3 were eluted by linear gradients of NaCl (0 to 0.24 M over 100 ml for BphC2 and 0 to 0.3 M over 140 ml for BphC3) followed by isocratic elution at 0.24 or 0.3 M NaCl, respectively. Active fractions (8 ml of BphC1-containing eluate with a specific activity of 6.2 U/mg of protein and a protein content of 39 mg, 6 ml of BphC2-containing eluate with a specific activity of 65 U/mg of protein and a protein content of 43 mg, or 4 ml of BphC3-containing eluate with a specific activity of 18.8 U/mg of protein and a protein content of 4.3 mg) were concentrated in each case to a final volume of 0.8 ml by using Ultrafree-PF filters (nominal molecular weight limit, 10,000; Millipore). The samples were then applied to HiLoad 26/60 Superdex 200

Prep Grade gel filtration column and eluted with Tris HCl (20 mM, pH 7.5) containing 20 mM NaCl. Two or three of the most active fractions were pooled and concentrated to final volumes of 1 ± 0.2 ml.

Transformation of chlorosubstituted 2,3-dihydroxybiphenyls. For qualitative analysis of the transformation of chlorosubstituted 2,3-dihydroxybiphenyls, cell-free supernatants obtained after separate incubation of *P. putida* KT2442 (pDM9) with PCB congeners 1 to 39, were incubated for 30 min with BphC1, BphC2, or BphC3 containing cell extracts corresponding to an activity against 2,3-dihydroxybiphenyl (0.1 mM) of 200 mU in a volume of 1 ml. Yellow coloration indicated that the supernatant contained a chlorosubstituted 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA). Absorption spectra were recorded between 350 and 500 nm. Only incubation mixtures where a significant increase in absorbance between 390 and 440 nm (>0.3) was observed were further analyzed, and it was assumed that a significant proportion of the original PCB congener had been transformed into the dihydroxy derivative. On this basis, the dihydroxy derivatives of 17 PCB congeners were further analyzed for their transformation by cell extracts containing BphC of LB400, DbfB of RW1, or BphC1, -C2, or -C3. Cell extracts corresponding to an activity of 70 mU against 2,3-dihydroxybiphenyl were used in a total of 1 ml.

Electrophoretic methods and Western blotting (immunoblotting). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Miniprotean II (Bio-Rad, Munich, Germany) essentially as described by Laemmli (26). The acrylamide concentrations for the concentrating and separating gels were 4 and 12.5% (wt/vol), respectively. The protein bands were stained by Coomassie brilliant blue R250. Molecular mass standards (Bio-Rad) were lysozyme (14.4 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (31.0 kDa), albumin (45.0 kDa), bovine serum albumin (66.2 kDa), and phosphorylase *b* (97.4 kDa). Prestained standards (Gibco-BRL, Eggenstein, Germany) were included prior to Western blotting.

Transfer of electrophoretically separated bands to a nitrocellulose membrane (Bio-Rad) was performed according to the method of Towbin et al. (42). After being blocked in phosphate-buffered saline (PBS) supplemented with 5% skim milk powder overnight at 4°C, the membrane was incubated with hybridoma cell culture supernatant or purified antibodies for 1 h at room temperature, followed by five washing steps in PBST (PBS supplemented with 0.05% Tween 20) to remove unbound antibodies. The blotting membrane was then incubated with peroxidase-conjugated goat anti-mouse antibody. Detection was carried out with 4-chloro-1-naphthol (41) or ECL Plus Western blotting detection reagents (Amersham Biosciences) according to the manufacturer's protocols. Densitometric analysis was performed with ImageQuant (Molecular Dynamics, Krefeld, Germany).

Preparation of monoclonal antibodies. Purified proteins were diluted in PBS buffer to a final concentration of 0.5 mg/ml. The protein solution was mixed with an equal amount of Freund's incomplete adjuvant, and 20 μl of the emulsion was injected subcutaneously into BALB/c mice. Two booster injections were given at 2-week intervals. Lymphocytes were harvested from popliteal lymph nodes and fused with the mouse myeloma cell line X63-Ag 8.536 by using polyethylene glycol as previously described (30). Hybridomas were selected in hypoxanthine-azaserine medium.

In a primary screening, all supernatants from the hybridomas were tested in enzyme-linked immunosorbent assays (ELISAs) with purified BphC1, BphC2, or BphC3. Supernatants collected from 69 hybridomas displaying strong reactivity with BphC1 (23 clones), BphC2 (40 clones), or BphC3 (6 clones) and no obvious cross-reactivity were further evaluated in Western blotting experiments. The supernatants of 34 clones reacted selectively with denatured BphC1 (6 clones), BphC2 (25 clones), or BphC3 (3 clones), of which 14 supernatants showed strong reaction with cell extracts of *E. coli* expressing BphC1 (2 clones) BphC2 (10 clones), or BphC3 (2 clones). Three stable, positive, non-cross-reacting clones were selected by subcloning twice by using the limiting-dilution technique (17, 40).

ELISA. Purified protein (5 μg) or BphC-containing cell extracts (20 μg) were diluted in 0.1 ml of 100 mM $NaHCO_3$ (pH 9.6), transferred to microtiter plates (Maxisorb; Nunc Corp.), and incubated at room temperature for 2 h. Plates were then washed with PBST, blocked for 1 h with 5% (wt/vol) skim milk powder in PBS (17), and incubated with 50 μl of hybridoma culture supernatant for 1 h at room temperature. Unbound antibodies were removed by washing the plates three times with PBST, and plates were then incubated for 1 h with peroxidase-conjugated goat anti-mouse antibodies at a dilution of 1:1,500 in PBS. Unbound conjugate was removed by washing as described above. Color development was achieved by adding *ortho*-phenylenediamine. Absorbance was determined after 15 min at 490 nm by an ELISA plate reader. Antibody isotyping was performed by ELISA with isotype-specific anti-immunoglobulins (ImmunoPure monoclonal antibody isotyping kit I; Pierce), following the manufacturer's instructions.

Production and purification of antibodies. Monoclonal hybridoma cell lines were propagated on petri dishes and finally in 2-liter bottles containing up to 600 ml of fresh OptiMEM medium (Gibco-BRL) incubated at 37°C in 5% CO_2. After 14 days of incubation, the cell-free supernatant was applied to a HiTrap protein A-Sepharose column (Amersham Biosciences) and the monoclonal antibodies were purified and concentrated according to the protocol of the manufacturer.

BIAcore analysis. A BIAcore biosensor 2000 (BIAcore, Freiberg, Germany) was used to quantify the expression of BphC proteins. Prior to immobilization of the antibodies on the biosensor chips, the carboxyl groups of the CM5 matrix were activated by derivatization with EDC-NHS [50 mM N-hydroxy-succinimide–200 mM *N*-ethyl-*N*′((dimethylamino)propyl)-carbodiimide]. Antibodies were immobilized on the CM5 carboxymethylated dextran matrix via amino coupling. Immobilization was performed in 20 mM sodium acetate buffer (pH 5). Typically, antibodies corresponding to 9,000 to 15,000 resonance units were immobilized. The system was calibrated by passing solutions of purified BphC1, BphC2, or BphC3 (100 μl containing 0.01 to 1 μg of protein) dissolved in HBS buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P-20) over the immobilized antibodies. The proteins could be quantified by this procedure with an accuracy of ±10%. Binding was determined by measuring the increase in resonance units. BphC proteins in cell extracts were quantified by using 100 μl of cell extracts containing 30 μg of total protein.

RESULTS AND DISCUSSION

Purification of the three 2,3-dihydroxybiphenyl dioxygenases from *R. globerulus* P6. The three isoenzymes were purified to homogeneity in three (BphC1) or four (BphC2 and BphC3) steps, and all three proteins were apparently homogeneous, as judged by the presence of a single band for each enzyme in SDS-PAGE (data not presented). The molecular masses of purified BphC1, BphC2, and BphC3, as determined by SDS-PAGE, were 34, 28, and 29 kDa, respectively, which is in good agreement with previous data obtained with cell extracts or purified BphC2 (2).

The highest specific activity observed for the three isoenzymes was that of BphC2 after purification under anaerobic conditions (up to 58 U/mg). In contrast, when the same enzyme was purified under aerobic conditions, a maximum specific activity of 30 U/mg was measured. Although the three isoenzymes were clearly oxygen sensitive, rapid purification and storage of the enzymes as a concentrated solution resulted in sufficiently high specific activities of 7.5, 30, and 20 U/mg for BphC1, BphC2, and BphC3, respectively, to allow a detailed biochemical characterization.

Substrate specificities. Kinetic data obtained with 2,3-dihydroxybiphenyl as the substrate were in close agreement with previously published results obtained with cell extracts (3) or purified BphC2 (2). BphC1 was shown to have a significantly lower K_m than either BphC2 or BphC3. All three isoenzymes exhibited substrate inhibition, although inhibition was strongly pronounced only in the case of BphC2. The catalytic efficiencies (k_{cat}/K_m) of the three isoenzymes also differed significantly when the low Fe^{2+} content of the BphC1 preparation was taken into account (Table 1), with BphC1 being approximately 10-fold more efficient than BphC2 or -C3.

All three isoenzymes displayed poor catalytic activity with catechol as the substrate and hence have a preference for bicyclic substrates. BphC1 showed a catalytic efficiency (k_{cat}/K_m) 4 orders of magnitude lower than that observed with 2,3-dihydroxybiphenyl as the substrate. BphC2 and BphC3 were even less active on catechol and were strongly substrate inhibited. 4-Methylcatechol, like catechol, was transformed very inefficiently by all three isoenzymes, with k_{cat}/K_m values 5

TABLE 1. Kinetic constants of 2,3-dihydroxybiphenyl 1,2-dioxygenases BphC1, BphC2, and BphC3

Substrate and enzyme	k_{cat} (s^{-1})	K_m (μM)	K_{ss}[a] (μM)	k_{cat}/K_m (s^{-1} mM^{-1})
2,3-Dihydroxybiphenyl				
BphC1	4.8 ± 0.1[d]	1.0 ± 0.1	3,300 ± 900	4,800
BphC2	16.5 ± 2.1	25.6 ± 5.1	340 ± 50	640
BphC3	9.8 ± 0.3	29.4 ± 3.1	2,860 ± 550	330
3-Methylcatechol				
BphC1	13.6 ± 0.9	106 ± 12	770 ± 100	130
BphC2	9.8 ± 2.8	1,230 ± 370	1,560 ± 650	8.0
BphC3	4.4 ± 1.5	1,940 ± 820	1,360 ± 680	2.3
Catechol				
BphC1	0.92 ± 0.17	2,400 ± 800	27,000 ± 11,000	0.38
BphC2[b]	0.016			
BphC3[b]	0.016			
4-Methylcatechol				
BphC1	0.064 ± 0.015	2,800 ± 780	13,500 ± 4,500	0.023
BphC2	0.36 ± 0.06	93,000 ± 19,000	Not observed	0.004
BphC3	0.24 ± 0.7	61,000 ± 21,000	Not observed	0.004
3-Chlorocatechol				
BphC1	ND[c]			
BphC2[b]	0.06			
BphC3[b]	0.04			

[a] K_{ss}, substrate inhibition constant (20).
[b] Because of a high K_m value and pronounced substrate inhibition, kinetic parameters could not be accurately determined. Therefore, the maximal turnover rate observed at a substrate concentration of 1 mM (for catechol), 0.5 mM (for transformation of 3-catechol by BphC2), or 0.1 mM (for transformation of 3-chlorocatechol by BphC3) is given.
[c] ND, not determined.
[d] Mean ± standard deviation.

orders of magnitude lower than the corresponding values with 2,3-dihydroxybiphenyl. In contrast, 3-methylcatechol was a good substrate for all isoenzymes, and BphC1 displayed a higher maximal turnover with 3-methylcatechol than with 2,3-dihydroxybiphenyl. However, turnover rates of this substrate by BphC2 and BphC3 were only approximately 10% of those determined with 2,3-dihydroxybiphenyl as the substrate. As the K_m values of the enzymes with 3-methylcatechol were in all cases more than 1 order of magnitude higher than those with 2,3-dihydroxybiphenyl, their catalytic efficiencies with 3-methylcatechol were rather low.

Both BphC2 and BphC3 transformed 3-chlorocatechol into a yellow ring cleavage product and thus resemble BphC1 of *Sphingomonas* sp. strain BN6 (19). Thus, all these extradiol dioxygenases of family I.1 (single-domain enzymes) can cleave 3-chlorocatechol in a distal fashion (31) and avoid the formation of an acylchloride. The formation of acylchloride was previously assumed to be the reason for fast enzyme inactivation during substrate turnover due to suicide inactivation by formation of covalent enzyme-product derivatives (5), and it was proposed that such an inactivation also accounts for problems in the degradation of 3-chlorobiphenyl (1). However, recent analysis of inactivation of BphC of LB400 revealed that, at least in this case, inactivation is reversible and obviously due to fast oxidation of catalytically active Fe(II) (43). This raises doubts that the previously proposed mechanism of inactivation is valid at all. Thus, it remains unclear whether the presence or induction of a single-domain extradiol dioxygenase capable of avoiding the formation of an acylchloride is of general advantage in the degradation of chlorinated aromatic derivatives.

There was no detectable transformation of 3,4-dihydroxybiphenyl by any of the isoenzymes, but this compound acted as a competitive inhibitor of 2,3-dihydroxybiphenyl transformation (data not shown). Inhibition constants for BphC1, BphC2, and BphC3 were 80 ± 50, 5 ± 1, and 55 ± 6 μM (mean ± standard deviation), respectively, indicating that the affinity of BphC2 for 3,4-dihydroxybiphenyl is an order of magnitude higher than that of BphC1 and BphC3.

Turnover of chlorinated 2,3-dihydroxybiphenyls by the three isoenzymes of *R. globerulus* P6. As chlorinated derivatives of 2,3-dihydroxybiphenyl were not commercially available, they were prepared by incubating individual PCB congeners with *P. putida* KT2442(pDM9) cells expressing *bphA1A2A3A4* and *bphB* from *R. globerulus* strain P6. Incubation of the crude reaction mixtures with BphC1, BphC2, or BphC3 showed that 17 chlorosubstituted 2,3-dihydroxybiphenyls were transformed into chlorosubstituted HOPDAs by at least one isoenzyme in amounts sufficient to allow identification and quantitation of the dominant product in each mixture. Identification of chlorosubstituted 2,3-dihydroxybiphenyls (Table 2) is based on the known regioselectivity of attack of BphA from *R. globerulus* P6 (28, 44) (preference order: unchlorinated ring > meta-chlorinated ring > para-chlorinated ring > ortho-chlorinated ring) and the absorption spectrum of HOPDAs formed by BphC-catalyzed transformation (HOPDAs having an absorption maximum at 390 to 400 nm bear a chlorosubstituent at the ortho position of the aromatic ring [37]). Even though the formation of other dihydroxybiphenyls as side products cannot be excluded in some cases, this experiment allowed us to iden-

TABLE 2. Transformation of chlorosubstituted 2,3-dihydroxybiphenyls by 2,3-dihydroxybiphenyl 1,2-dioxygenases

Biphenyl congener	Dominantly formed 2,3-dihydroxybiphenyl	λ_{max} (nm)[g]	Relative activity (%) of enzyme (strain)[a]				
			BphC1 (P6)	BphC2 (P6)	BphC3 (P6)	DbfB (RW1)	BphC (LB400)
2-Chloro	2′-Chloro[a]	392 ± 2	3.5	57	50	3	1.2
3-Chloro	3′-Chloro[a]	434 ± 2	64	71	95	47	36
4-Chloro	4′-Chloro[a]	437 ± 2	61	84	77	65	51
2,3-Dichloro	2′,3′-Dichloro[c,d]	392 ± 2	1.5	25	57	0.5	1
2,4-Dichloro	2′,4′-Dichloro[c,d]	392 ± 2	6.5	33	63	0.8	1.1
2,5-Dichloro	2′,5′-Dichloro[c,d]	392 ± 2	4	5.5	27	1.8	2.0
3,4-Dichloro	3′,4′-Dichloro[d]	440 ± 2	31	59	49	28	32
3,5-Dichloro	3′,5′-Dichloro[d]	436 ± 2	51	15	49	12	37
2,3′-Dichloro	2′,5-Dichloro[b]	395 ± 5	0.4	3	5	0.3	1.2
3,3′-Dichloro	3′,5-Dichloro[e]	412 ± 5	3.5	2	8	4.5	3
2,4′-Dichloro	2′,4-Dichloro[a]	396 ± 5	7	6	14	4	0.5
3,4′-Dichloro	4′,5-Dichloro[a]	415 ± 5	1.5	4	9	2	4
2,3,4-Trichloro	2′,3′,4′-Trichloro[c,d]	392 ± 2	1.8	2.5	9	0.2	0.5
2,3,5-Trichloro	2′,3′,5′-Trichloro[c,d]	393 ± 2	1.2	2	21	0.2	2.5
2,4,5-Trichloro	2′,4′,5′-Trichloro[c,d]	393 ± 2	2.5	0.9	3	1.1	1.5
3,4,5-Trichloro	3′,4′,5′-Trichloro[d]	420 ± 5	26	11	7	14	12
3,4,2′-Trichloro	3′,4′,5-Trichloro[f]	434 ± 2	29	11	9	11	17

[a] The identity of the dihydroxybiphenyl dominantly formed was deduced from data in the literature (28, 44). *R. globerulus* P6 biphenyl dioxygenase was shown to produce exclusively or dominantly one dioxygenation product (>95%).

[b] *R. globerulus* was shown to dominantly form 2′,5-dichlorodihydroxybiphenyl (80%) and minor amounts of 3′,5-dichlorodihydroxybiphenyl (44).

[c] The absorption maximum of the ring cleavage product (392 to 396 nm) indicates the presence of a chlorosubstituent at the ortho position of the aromatic ring (37), and thus, the BphA-mediated dioxygenation occurred on the unsubstituted aromatic ring.

[d] The BphA-mediated dioxygenation reaction on di- or trichlorobiphenyls with substitutions on one aromatic ring can be assumed to have occurred on the unchlorinated ring, as proven for monochlorinated biphenyls (28).

[e] As neither dechlorination nor 4,5-dioxygenation has been reported for *R. globerulus* P6 BphA, a dominant 5,6-dioxygenation can be assumed.

[f] The absorption maximum of the ring cleavage product (434 nm) indicates the absence of a chlorosubstituent at the ortho position of the aromatic ring (37), and thus, the BphA-mediated dioxygenation occurred on the less chlorinated aromatic ring.

[g] Mean ± standard error.

tify differences in substrate specificity of the different BphC isoenzymes.

In addition to BphC1, BphC2, and BphC3 from *R. globerulus* strain P6, which belong to the extradiol dioxygenase subfamilies I.3B and I.1 (11), we tested a 2,2′,3-trihydroxybiphenyl dioxygenase (DbfB) from the dibenzofuran degrader *S. wittichii* strain RW1 and 2,3-dihydroxybiphenyl-1,2-dioxygenase (BphC) from *Burkholderia* sp. strain LB400 (belonging to subfamilies I.3.C and I.3.A, respectively) for their activity against chlorosubstituted 2,3-dihydroxybiphenyls (Table 2). While all five enzymes transformed 3′- and 4′-chloro-2,3-dihydroxybiphenyl at rates similar to those observed with 2,3-dihydroxybiphenyl, only BphC2 and BphC3 showed high activities with 2′-chloro-2,3-dihydroxybiphenyl. In general, the single-domain enzymes BphC2 and, in particular, BphC3, showed higher activities for 2′-chlorosubstituted 2,3-dihydroxybipenyls than the two-domain dioxygenases (Table 2). Only some trichlorinated 2,3-dihydroxybiphenyls were transformed faster by the two-domain enzymes.

To gain more detailed insight into possible catalytic differences of the *R. globerulus* P6 BphC isoenzymes, their kinetic properties with monochlorosubstituted 2,3-dihydroxybiphenyls were investigated.

Both BphC2 and BphC3 transformed all three monochlorinated dihydroxybiphenyls with k_{cat} values similar to that of the unsubstituted 2,3-dihydroxybiphenyl (variation was only threefold). Similarly, BphC1 transformed both 3′- and 4′-chloro-2,3-dihydroxybiphenyl with k_{cat} values approximately the same as that for the unsubstituted analogue. However, BphC1 transformed 2′-chloro-2,3-dihydroxybiphenyl at only 5% of the rate of unsubstituted 2,3-dihydroxybiphenyl. BphC1 generally exhibited much lower K_m values than did BphC2 or BphC3, even with 2′-chloro-2,3-dihydroxybiphenyl as the substrate (Table 3).

Like BphC2 and BphC3 of *R. globerulus* P6, the single-domain extradiol dioxygenase BphC1 of *Sphingomonas* sp. strain BN6 shows a high K_m value for 2,3-dihydroxybiphenyl (32). It therefore seems that the single-domain enzymes are characterized by relatively low substrate affinities. In contrast, the two-domain dioxygenases usually have relatively high substrate affinities, with K_m values in the lower micromolar range (10, 18, 34). To our knowledge, transformation of chlorosubstituted dihydroxybiphenyls has been assessed so far only with the 2,3-dihydroxybiphenyl dioxygenase of *Comamonas testosteroni* B-356 (18) and in more detail for the dioxygenase of *Burkholderia* sp. strain LB400 (9), both belonging to the family I.3.A dioxygenases. Whereas the B-356-derived enzyme resembles BphC1 of *R. globerulus* P6 in having a high affinity for 4′-chloro-2,3-dihydroxybiphenyl and reaction rates similar to those for the unchlorinated substrate, the LB400-derived enzyme was characterized by a low affinity specifically for 3′- and 4′-chloro-2,3-dihydroxybiphenyl. Like BphC1, BphC of LB400 showed a relatively slow turnover of 2′-chloro-2,3-dihydoxybiphenyl.

Maximal turnover rates were calculated from enzymes purified aerobically. In the case of BphC2, we found that the enzyme lost about 50% of its activity during purification. However, the activity of BphC1 was significantly lower than those of other purified two-domain enzymes, which are typically in the range of 80 to more than 400 U/mg of protein (10, 14, 16, 21).

TABLE 3. Kinetic constants of 2,3-dihydroxybiphenyl 1,2-dioxygenases BphC1, BphC2, and BphC3 with monochloro-2,3-dihydroxybiphenyls

Substrate and enzyme	k_{cat} (s^{-1})	K_m (μM)	K_{ss}[a] (μM)	k_{cat}/K_m (s^{-1} mM^{-1})	Partition ratio
2,3-Dihydroxybiphenyl					
BphC1b[b]	4.8 ± 0.1[d]	1.0 ± 0.1	3,300 ± 900	4,800	1,410 ± 240
BphC2[c]	16.5 ± 2.1	25.6 ± 5.1	340 ± 50	640	2,250 ± 200
BphC3	9.8 ± 0.3	29.4 ± 3.1	2,860 ± 550	330	1,930 ± 110
2′-Chloro-2,3-dihydroxybiphenyl					
BphC1[b]	0.25 ± 0.01	1.2 ± 0.1	5,550 ± 650	170	470 ± 70
BphC2[c]	7.5 ± 0.4	18.7 ± 1.0	$>10^5$	400	650 ± 80
BphC3	3.5 ± 0.4	18.3 ± 1.5	$>10^5$	190	850 ± 90
3′-Chloro-2,3-dihydroxybiphenyl					
BphC1[b]	4.0 ± 0.1	0.45 ± 0.05	5,300 ± 360	8,900	460 ± 90
BphC2[c]	12.0 ± 1.0	9.7 ± 1.1	$>10^5$	1,200	1,120 ± 60
BphC3	8.5 ± 2.0	9.9 ± 0.9	$>10^5$	860	1,190 ± 110
4′-Chloro-2,3-dihydroxybiphenyl					
BphC1[b]	4.5 ± 0.2	1.5 ± 0.1	6,100 ± 1,300	3,000	270 ± 20
BphC2[c]	18.8 ± 1.5	10.6 ± 1.1	$>10^5$	1,800	2,490 ± 420
BphC3	8.3 ± 0.3	12.0 ± 0.7	$>10^5$	690	1,050 ± 80

[a] K_{ss}, substrate inhibition constant (20).
[b] Values are given relative to protein content. As BphC1 contains only about 12% ± 4% Fe^{2+} per subunit, the values of k_{cat}, k_{cat}/K_m, and the partition ratio can be assumed to be underestimated by a factor of 6 to 12.
[c] Values are given relative to protein content. As BphC2 contains about 70% ± 5% Fe^{2+} per subunit, the values of k_{cat}, k_{cat}/K_m, and the partition ratio can be assumed to be underestimated by a factor of 1.4.
[d] Mean ± standard deviation.

Determination of the Fe^{2+} content of aerobically purified BphC1 and -C2 showed that whereas the BphC2 preparation had an Fe^{2+} content of 70% ± 5% (assuming one Fe^{2+} per subunit), the BphC2 preparation had an Fe^{2+} content of only 12% ± 4% and therefore comprises a significant amount of inactive enzyme. Thus, maximal turnover numbers (as well as catalytic efficiencies and partition ratios) are higher by a factor of 6 to 12 when related to iron content (and thus to the active enzyme) than when determined based on the protein content. Nevertheless, BphC2 and BphC3 can transform mono-, chloro-, and unsubstituted 2,3-dihydroxybiphenyl equally well, whereas BphC1 has relatively poor activity for the 2′-chloro derivative and various more highly chlorinated 2′-chloro-2,3-dihydroxybiphenyls compared to its activity with 2,3-dihydroxybiphenyl. However, taking into account the catalytic efficiency, BphC1 is much more efficient than BphC2 and -C3 on 3′- or 4′-chloro-2,3-dihydroxybiphenyl. Thus, overall, expression of a small-subunit dioxygenase is advantageous for effective transformation of 2′-chloro-2,3-dihydroxybiphenyl and possibly various other 2′-chloro-2,3-dihydroxybiphenyls and particularly important for microorganisms like *R. globerulus* strain P6, which transform 2-chlorosubstituted biphenyls without simultaneous dechlorination (44). In contrast, biphenyl dioxygenase of *Burkholderia* sp. strain LB400 catalyzes dehalogenation of various 2-chlorosubstituted biphenyls, thus avoiding (at least in some cases) the formation of 2′-chlorosubstituted biphenyls (38).

The stability of the three BphC enzymes was evaluated by determining their half-lives in air-saturated buffer. Whereas BphC1 showed a half-life of 43 ± 4 min, inactivation of BphC2 and -C3 was considerably faster, with half-lives of 4.5 ± 0.5 and 7.2 ± 0.8 min, respectively. All three enzymes were susceptible to enhanced inactivation during the turnover of substrates. Generally, partition ratios were lower than those previously reported for BphC of LB400 (9). As was observed for the LB400 enzyme, BphC1 showed the highest partition ratio with 2,3-dihydroxybiphenyl as a substrate (taking into account the iron content of the preparation, a partition ratio of 9,000 to 18,000 can be assumed) whereas partition ratios with chlorinated 2,3-dihydroxybiphenyls were 12 to 33% of that with 2,3-dihydroxybiphenyl. Significantly less pronounced differences in partition ratios were observed for BphC2 and -C3. Both enzymes showed partition ratios with 2,3-dihydroxybiphenyl of approximately 2,000 and with 2′-chloro-2,3-dihydroxybiphenyl of 30% to 40% of that value, again indicating the suitability of these enzymes for supporting degradation of specifically 2-chlorosubstituted biphenyls.

Expression of BphC proteins. Levels of the three different BphC proteins in cells of *R. globerulus* strain P6 growing on

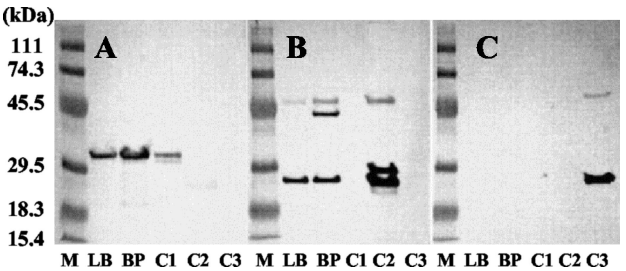

FIG. 1. Western blot analysis of the expression of BphC proteins. Three monoclonal antibodies specific for BphC1 (AkXIII) (A), BphC2 (AkXIV) (B), and BphC3 (AkXV) (C) were used, and cell extracts of *R. globerulus* P6 grown on either complex medium (LB medium) or biphenyl or of *E. coli* cells expressing BphC1 (C1), BphC2 (C2) or BphC3 (C3) (always corresponding to 20 μg of protein) were analyzed. The antibodies were not cross-reactive, since for each antibody, only control cell extracts of *E. coli* expressing each of the antibody's cognate protein gave positive bands (lanes C1, C2, and C3). M, molecular mass marker.

complex (LB) medium or biphenyl were initially determined by SDS-PAGE followed by immunoblotting (Fig. 1). Three monoclonal antibodies, AkXIII, AkXIV, and AkXV, specific for BphC1, BphC2, and BphC3, respectively, were used. These antibodies were not cross-reactive, since for each antibody, only control cell extracts of *E. coli* expressing each of the antibody's cognate protein gave positive bands (Fig. 1, lanes C1, C2, and C3).

Expression of BphC1 and BphC2 was observed in cells of *R. globerulus* P6 grown on biphenyl as the sole carbon source as well as LB medium. The BphC3 protein was not observed in cells grown on either medium.

One apparent anomaly in the experiment is the presence of multiple bands in one of the gels (Fig. 1B) in which the BphC2-detecting antibody was used. In the case of cells of *R. globerulus* P6, a band corresponding to a molecular mass of approximately 48 kDa—a mass approximately twice that of the subunit mass of BphC2—is present and is ascribed to a dimer of that protein. In the case of the control *E. coli* extract expressing BphC2, a band just above the one corresponding to the subunit is also present. Examination of the sequence reveals an alternative start codon upstream of what is assumed to be the normal start codon, which could account for the additional band of a slightly higher molecular mass.

During growth on biphenyl, *R. globerulus* P6 (Fig. 1B, lane BP) produced a protein with an apparent molecular mass of 42 kDa. This band does not correspond to the dimeric form of the enzyme, whose band can be clearly seen above that of the 42-kDa form. We ascribe this 42-kDa band to a protein other than BphC2 which cross-reacts with the antibody for this protein.

Expression of the BphC proteins was quantified by BIAcore analysis during growth of *R. globerulus* P6 on biphenyl, succinate, and LB medium (Fig. 2). BphC3 was never detected at any significant level (BphC3 was also not induced during growth on benzoate [data not shown]). During growth on LB or succinate, BphC1 was expressed at a constitutive low level of 1 to 2 μg/mg of soluble protein. During growth on biphenyl, in the initial phases of growth, BphC1 was present at around 5 μg/mg of soluble protein, and the level gradually rose over the growth phase of the organism, to around 20 μg/mg, which is around 20 times the level observed in cells grown on the other media.

Due to cross-reactivity of the BphC2-specific antibody AkXIV with a protein of a molecular mass of 42 kDa, all cell extracts were simultaneously analyzed by Western blotting for the expression of both BphC2 and the 42-kDa protein. The 42-kDa protein was observed only in cells grown on biphenyl and not those grown on the other media, and in all phases of growth, this protein accounted for approximately 40% ± 5% of the total signal intensity as determined by densitometric analysis. Taking this into account, BphC2 was expressed at high levels, independent of the growth substrate (>5 μg/mg of protein). BphC2 expression was slightly increased during growth on biphenyl. Thus, BphC2 not only can but actually does support BphC1 in transformation of chlorodihydroxybiphenyls when *R. globerulus* strain P6 is confronted with PCBs. The function of BphC3, if any, remains to be established. Asturias et al. (2) had previously reported the presence of two distinct 2,3-dihydroxybiphenyl dioxygenase activities in biphenyl-grown *R. globerulus* P6 but could not determine if the dominant activity is due to BphC2 or BphC3. Expression analysis in this study now provides evidence that this activity is due to BphC2 and that BphC2 is of major importance for biphenyl degradation and PCB cometabolism in *R. globerulus* P6. In accordance with the expression studies, succinate-grown cells of P6 exhibited a significant 2,3-dihydroxybiphenyl dioxygenase activity of 0.12 U/mg of protein.

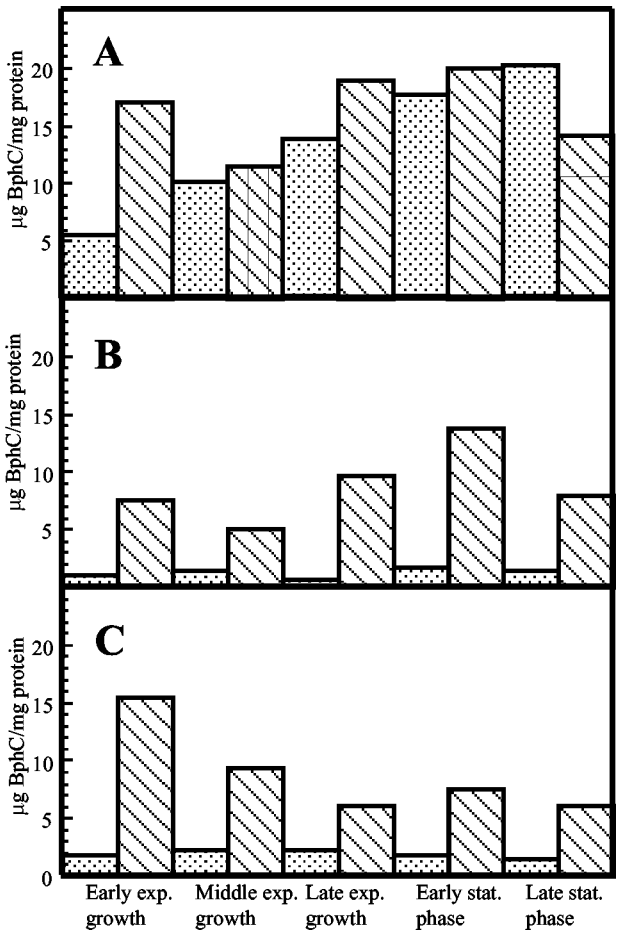

FIG. 2. BIAcore analysis of the expression of 2,3-dihydroxybiphenyl dioxygenases BphC1 (dotted bars) and BphC2 (hatched bars) during growth of *R. globerulus* P6 on biphenyl (A) or succinate (B) or in LB broth (C). The BphC content was analyzed in cell extracts (corresponding to 0.3 mg of protein per ml) during different phases of growth (early exponential growth phase, when cultures had reached less than 20% of maximal optical density; mid-exponential growth phase, when cultures had reached 50% ± 10% of maximal optical density; late exponential growth phase, when cultures had reached 80% ± 10% of maximal optical density; early stationary phase, less than 2 h after cultures had reached maximal optical density; late stationary phase, more than 2 h after cultures had reached maximal optical density). The BphC2 content in panel A was calculated assuming 40% of the signal intensity to be due to cross-reaction with a 42-kDa protein. The data are means of two independent experiments, and variation between respective data points of the experiments was always less than 15%.

Kosono et al. (24) has suggested that, besides the BphC1, -C2, and -C3 proteins, *R. globerulus* strain P6 harbors genes with a high similarity to the *bphC1*, *-C4*, *-C5*, *-C6*, and *-C7* genes of *R. erythropolis* strain TA421, indicating that *R. globerulus* strain P6 may contain eight or more *bphC* genes. However, the 42-kDa protein described above does not correspond in mass to the dimeric form of the single-domain enzyme or to

the monomeric form of the two-domain enzyme. Given that all BphC enzymes characterized thus far are only of these two molecular mass forms, the identity of this protein is unclear. In any case, given the differences in substrate specificities, kinetic parameters, and expression of BphC1 and -C2, it seems highly likely that the different isoenzymes contribute to the broad substrate range of the P6 strain.

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