Titre: Title:	Revisiting the regiospecificity of burkholderia xenovorans LB400 biphenyl dioxygenase toward 2,2′-dichlorobiphenyl and 2,3,2′,3′-tetrachlorobiphenyl
Auteurs: Authors:	Diane Barriault, François Lepine, Mahmood Mohammadi, Sylvain Milot, Nicolas Leberre, & Michel Sylvestre
Date:	2004
Type:	Article de revue / Article
Référence: Citation:	Barriault, D., Lepine, F., Mohammadi, M., Milot, S., Leberre, N., & Sylvestre, M. (2004). Revisiting the regiospecificity of burkholderia xenovorans LB400 biphenyl dioxygenase toward 2,2′-dichlorobiphenyl and 2,3,2′,3′-tetrachlorobiphenyl. Journal of Biological Chemistry, 279(46), 47489-47496. https://doi.org/10.1074/jbc.m406808200

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 46, Issue of November 12, pp. 47489–47496, 2004
Printed in U.S.A.

Revisiting the Regiospecificity of *Burkholderia xenovorans* LB400 Biphenyl Dioxygenase toward 2,2′-Dichlorobiphenyl and 2,3,2′,3′-Tetrachlorobiphenyl*

Received for publication, June 17, 2004, and in revised form, August 19, 2004
Published, JBC Papers in Press, August 31, 2004, DOI 10.1074/jbc.M406808200

Diane Barriault, François Lépine, Mahmood Mohammadi, Sylvain Milot, Nicolas Leberre, and Michel Sylvestre‡

From the Institut National de la Recherche Scientifique, INRS-Institut Armand-Frappier, Laval, Québec H7V 1B7, Canada

2,2′-Dichlorobiphenyl (CB) is transformed by the biphenyl dioxygenase of *Burkholderia xenovorans* LB400 (LB400 BPDO) into two metabolites (*1* and *2*). The most abundant metabolite, *1*, was previously identified as 2,3-dihydroxy-2′-chlorobiphenyl and was presumed to originate from the initial attack by the oxygenase on the chlorine-bearing *ortho* carbon and on its adjacent *meta* carbon of one phenyl ring. 2,3,2′,3′-Tetrachlorobiphenyl is transformed by LB400 BPDO into two metabolites that had never been fully characterized structurally. We determined the precise identity of the metabolites produced by LB400 BPDO from 2,2′-CB and 2,3,2′,3′-CB, thus providing new insights on the mechanism by which 2,2′-CB is dehalogenated to generate 2,3-dihydroxy-2′-chlorobiphenyl. We reacted 2,2′-CB with the BPDO variant p*4*, which produces a larger proportion of metabolite *2*. The structure of this compound was determined as *cis*-3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl by NMR. Metabolite *1* obtained from 2,2′-CB-d_8 was determined to be a dihydroxychlorobiphenyl-d_7 by gas chromatographic-mass spectrometric analysis, and the observed loss of only one deuterium clearly shows that the oxygenase attack occurs on carbons 2 and 3. An alternative attack at the 5 and 6 carbons followed by a rearrangement leading to the loss of the *ortho* chlorine would have caused the loss of more than one deuterium. The major metabolite produced from catalytic oxygenation of 2,3,2′,3′-CB by LB400 BPDO was identified by NMR as *cis*-4,5-dihydro-4,5-dihydroxy-2,3,2′,3′-tetrachlorobiphenyl. These findings show that LB400 BPDO oxygenates 2,2′-CB principally on carbons 2 and 3 and that BPDO regiospecificity toward 2,2′-CB and 2,3,2,′,3′-CB disfavors the dioxygenation of the chlorine-free *ortho-meta* carbons 5 and 6 for both congeners.

The enzymes of the bacterial biphenyl catabolic pathway are very versatile. They can co-metabolically transform several polychlorinated biphenyls (1). The initial reaction of this pathway is catalyzed by the biphenyl dioxygenase (BPDO)[1] (2, 3). The *cis*-(2*R*,3*S*)-dihydroxy-1-phenylcyclohexa-4,6-diene (commonly called *cis*-2,3-dihydro-2,3-dihydroxybiphenyl) generated by the catalytic oxygenation of biphenyl is dehydrogenated by the 2,3-dihydro-2,3-dihydroxybiphenyl-2,3-dehydrogenase (BphB) and the catechol produced is cleaved by the 2,3-dihydroxybiphenyl-1,2-dioxygenase (BphC). The 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid produced is then hydrolyzed by the 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid hydrolase to yield benzoate and 2-hydroxypentanoate (Fig. 1). The substrate specificity of BPDO is crucial, because it limits the range of compounds that can potentially be degraded by the catabolic system. BPDO is a three-component enzymatic system (4–6) (Fig. 1). The first component is an iron-sulfur protein (ISP_{BPH}) that interacts with the substrate to catalyze the addition of molecular oxygen. The second and third components are a flavoprotein reductase and a ferredoxin that are involved in the transfer of electrons from NADH to ISP_{BPH}. BPDO components are coded by *bphA* (α-subunit of ISP_{BPH}), *bphE* (β-subunit of ISP_{BPH}), *bphF* (ferredoxin) and *bphG* (flavoprotein reductase) in *Burkholderia xenovorans* LB400 (7) (also called *Burkholderia fungorum* LB400 or *Pseudomonas cepacia* LB400) (8) and in *Comamonas testosteroni* B-356 (9).

The three symmetrical 2,2′-, 3,3′-, and 4,4′-dichlorobiphenyls are not metabolized equally well by various BPDOs. *C. testosteroni* B-356 BPDO oxygenates 3,3′-dichlorobiphenyl much faster and more efficiently than it does 2,2′-dichlorobiphenyl and 4,4′-dichlorobiphenyl (10). On the other hand, the catalytic activity of *B. xenovorans* LB400 BPDO toward 2,2′-dichlorobiphenyl is much greater than that of B-356 BPDO (10), but 3,3′-dichlorobiphenyl and 4,4′-dichlorobiphenyl are poor substrates for LB400 BPDO (11). This enzyme, in fact, is among the few known BPDOs of natural occurrence that can efficiently metabolize the symmetrical *ortho*-substituted chlorobiphenyl 2,2′-CB and the symmetrical *ortho-meta*-substituted congeners 2,3,2′,3′-CB and 2,5,2′,5′-CB (11, 12).

The major metabolite of 2,2′-CB, representing about 90% of the product, was identified as 2,3-dihydroxy-2′-chlorobiphenyl (2). This metabolite is believed to be produced from the oxygenase attack on the chlorinated side of one of the ring (2, 13) followed by concomitant dehalogenation. This type of oxygenation, leading to elimination of the chlorine atom, is a desirable

* This work was supported by National Science and Engineering Research Council of Canada grants 257566 and 0039579. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: INRS-IAF, 245 Boul Hymus, Pointe-Claire, Québec H9R 1G6, Canada. Tel.: 514-630-8829; Fax: 514-630-8850; E-mail: michel.sylvestre@inrs-iaf.uquebec.ca.

[1] The abbreviations used are: BPDO, biphenyl dioxygenase; BphA, α subunit of BPDO oxygenase component; BphB, 2,3-dihydro-2,3-dihydroxybiphenyl 2,3-dehydrogenase; BphC, 2,3-dihydroxybiphenyl 2,3-dioxygenase; ISP_{BPH}, iron-sulfur protein; 2,2′-CB, 2,2′-dichlorobiphenyl; 3,3′-CB, 3,3′-dichlorobiphenyl; 4,4′-CB, 4,4′-dichlorobiphenyl; 2,5,2′,5′-CB, 2,5,2′,5′-tetrachlorobiphenyl; 2,3,2′,3′-CB, 2,3,2′,3′-tetrachlorobiphenyl; IPTG, isopropyl-β-D-thiogalacto-pyranoside; MES, 2-(*N*-morpholino)ethanesulfonic acid; GC, gas chromatography; MS, mass spectrometry; EI, electron impact; ppm, parts per million; PCB, polychlorinated biphenyl.

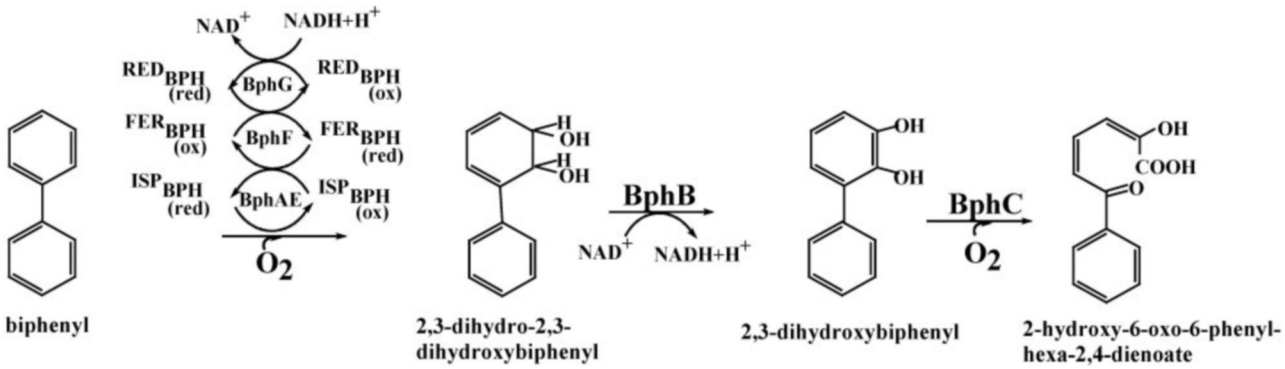

FIG. 1. **Biphenyl catabolic pathway.** The scheme shows the three enzymatic steps used by *B. xenovorans* to transform biphenyl into the yellow *meta*-cleavage metabolite.

feature of engineered enzymes, in that it facilitates downstream degradation of the metabolites. The second metabolite obtained from 2,2′-CB (representing 10% or less of the product) was presumed to be the 5,6-dihydro-5,6-dihydroxy-2,2′-dichlorobiphenyl (2) based on the hypothesis that the enzyme normally catalyzes the oxygenation of vicinal *ortho* and *meta* carbons of biphenyl. Several other reports used these assumptions to interpret the metabolism of 2,2′-CB by engineered enzymes (13–15). In fact, in recent reports (13–15), most of the conclusions drawn about regiospecificity alterations toward 2,2′-CB that occurred after the engineering of BPDOs were based on assumptions that need confirmation.

A single metabolite is produced from 2,5,2′,5′-CB. In this case, the oxygenation occurs on vicinal *meta-para* carbons 3 and 4 (2); this represents a unique mode of oxygenation for BPDOs, which normally oxygenate the biphenyl ring on vicinal *ortho-meta* carbons. The metabolism of 2,3,2′,3′-CB was investigated by Arnett *et al.* (12). They showed that the rate of oxidation of this congener is in the same range as for biphenyl. Two dihydro-dihydroxy-tetrachlorobiphenyls were produced from this congener, but their structure has not been determined unambiguously. Therefore, we do not know whether the regiospecificity of LB400 BPDO favors a *meta-para* or an *ortho-meta* oxygenation for 2,3,2′,3′-CB.

Several reports (11, 14, 16, 30) show that the sequence pattern of a stretch of seven amino acids of the C-terminal portion of BphA called region III strongly influences the range of PCB congeners that the enzyme can oxygenate efficiently, as well as its regiospecificity toward 2,2′-CB. Other amino acids, including residue 377 of LB400 BphA (11, 16) and several other residues that, according to a model of BPDO, have apparently no contact with the substrate (16), seem to act in association with residues of region III to influence the reaction turnover rates and regiospecificity toward chlorobiphenyls. Understanding how these amino acids interact with the substrate will have major effects on the design of strategies to engineer better enzymes. Precise identification of the metabolites generated from BPDO reaction will be essential to reach this goal.

In a recent work, we created the BPDO variant *p*4 by substitution (T335A/F336M). This variant catalyzes the oxygenation of many PCB congeners more efficiently than LB400 BPDO (30). It also exhibited an altered regiospecificity toward 2,2′-CB, producing as a major metabolite the dihydro-dihydroxy-dichlorobiphenyl normally reported as a minor metabolite by LB400 BPDO. However, the fact that the dihydroxy chlorobiphenyl derived from this metabolite was not cleaved by BphC (30) suggested that the oxygenation occurred on the *meta* and *para* carbons 3 and 4 or 4 and 5 of 2,2′-CB rather than on the *ortho* and *meta* carbons 5 and 6. This assumption was confirmed in the present study.

Recent reports have suggested that not all dihydrodiol metabolites resulting from catalytic oxygenation of substituted biphenyl are stable. For example, 3,3′-dihydroxybiphenyl is converted to an unstable dihydrodiol metabolite that readily isomerizes to 3,4-dihydroxy-5-(3′-hydroxyphenyl)-5-cyclohexen-1-one, which is a dead-end metabolite (18). Several hydroxychlorobiphenyls were also found to generate unstable dihydrodiol metabolites (19). In this context, production of 2,3-dihydroxy-2′-chlorobiphenyl from 5,6-dihydro-5,6-dihydroxy-2,2′-dichlorobiphenyl by spontaneous elimination of hydrochloric acid cannot be excluded. In other words, we cannot exclude the possibility that the dehalogenation occurring after LB400 BPDO oxygenation of 2,2′-CB would be a fortuitous reaction resulting from the rearrangement of a dihydro-dihydroxy derivative instead of a reaction involving an interaction between the chlorinated carbon of the substrate with the enzyme active center. Therefore, the assumption that 2,3-dihydroxy-2′-chlorobiphenyl is produced from an oxygenase attack on carbons 2 and 3 of 2,2′-CB would also need to be confirmed.

In this context, the objective of this work was to identify clearly the metabolites generated from 2,2′-CB and 2,3,2′,3′-CB by LB400 BPDO and to get a clear demonstration of the mechanism by which 2,2′-CB is dehalogenated to generate 2,3-dihydroxy-2′-chlorobiphenyl.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and General Protocols—Escherichia coli DH11S (20) was used in this study. Variant *p*4 is an evolved BPDO obtained by simultaneous random mutagenesis of the amino acid residues of region III of LB400 BphA (30). The sequence pattern of *p*4 BphA is identical to that of LB400 BphA except for the mutations T335A and F336M. Plasmids pQE31[LB400-*bphAE*], pQE31[B-356-*bphAE*], and pQE31[*p*4-*bphAE*] were described previously (16, 30). The *E. coli* strains bearing these plasmids expressed His-tagged ISP_{BPH} (16). DNA general protocols were performed as described by Sambrook *et al.* (21).

Chemicals—The chemicals used in this work were of the highest grade available commercially. 2,2′-CB and 2,3,2′,3′-CB were obtained from ULTRAScientific (Kingstown, RI). Biphenyl-d_{10} was obtained from CDN Isotope (Pointe-Claire, PQ, Canada). Chlorination was performed with 425 mg of biphenyl-d_{10} dissolved in 40 ml of hexane. Iron powder (100 mg) was added, and the mixture was cooled in an acetone/dry ice bath and saturated with chlorine gas. The mixture was warmed to room temperature and left overnight in the dark. The mixture was then extracted with 0.1 M NaOH and dried with anhydrous sodium sulfate, and the solvent was evaporated. The residue was then purified by flash chromatography using hexane and on thick layer chromatography plates using cyclohexane as eluent. The fractions were then analyzed by gas chromatography-mass spectrometry to identify those containing 2,2′-dichlorobiphenyl-d_8.

Preparation of Purified Enzyme Preparations and Enzyme Assay Conditions—The procedures to obtain His-tagged purified preparations of BPDO components from recombinant *E. coli* cells have been described previously (3), as was the procedure to obtained His-tagged

purified BphB (22). To produced His-tagged purified 1,2-dihydroxynaphthalene 1,2-dioxygenase, *nahC* was PCR-amplified using the antisense primers *nahC-KpnI/FOR* 5′-GTTCGGTACCCATGAGTAAGCAAGCTGCAG and nahC-KpnI/REV GATGGGTACCTTAACTCAGTTTTACATCCAG from plasmid NAH7 extracted from *Pseudomonas putida* G7 (23). The resulting 900-kb DNA fragment was cloned into the KpnI sites of pQE31. The resulting plasmid pQE31[*nahC*] was transformed into *E. coli* DH11S. The protocols used for expression and purification of the His-tagged enzyme was essentially the one described for B-356 BphC (24).

Production and Purification of Hydroxylated Metabolites from 2,2′-CB and 2,3,2′,3′-CB—2,2′-CB and 2,3,2′,3′-CB Metabolites were obtained from either whole-cell suspensions of *E. coli* [pDB31*bphFG*] + [pQE31*bphAE*] induced with isopropyl-β-D-thiogalacto-pyranoside (IPTG) or produced enzymatically using reconstituted BPDOs prepared from His-tagged purified components. For whole-cell reactions, Log-phase cells in Luria Bertani broth (21) were induced for 2 h with 0.5 mM IPTG. Induced cells were harvested by centrifugation, washed, and suspended to an optical density at 600 nm of 2.0 in M9 medium (21) containing 0.5 mM IPTG. This cell suspension was distributed by portions of 250 ml in 1-liter Erlenmeyer flasks. Each cell suspension received 100 μl of a 50 mM acetone solution of 2,2′-CB or 2,3,2′,3′-CB. The suspensions were incubated for 3 h at 37 °C with shaking. Cell suspensions were extracted at neutral pH with ethyl acetate. A similar protocol was used to produce 3,4-dihydroxy-2,2′-dichlorobiphenyl from 3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl, except that the suspensions were prepared using *E. coli* [pQE31*bphB*] as described previously (22).

Purified enzyme preparations were also used to prepare 2,2′-CB metabolites. The reaction conditions were then set as follows. The BPDO assays were performed in 200 μl of a 100 mM MES buffer, pH 6.5, as described previously (3) and the reaction was initiated by adding 100 nmol of 2,2′-CB dissolved in acetone. The enzyme-specific activities were determined spectrophotometrically by measuring the consumption of NADH at 340 nm (3) or by GC/MS analysis, by measuring the substrate depletion. The BphB assay was performed in 50 mM bicine buffer, pH 9.0, at 37 °C using between 200 ng and 50 μg of enzyme. Each reaction mixture (total volume 200 μl) contained 1.0 mM NAD^+. The reaction was initiated by adding different amounts of a purified preparation of the 3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl. The reaction mixture was extracted with three volumes of ethyl acetate.

In all cases, the ethyl acetate was evaporated under a stream of nitrogen and the residues were dissolved in a mixture of water/methanol/acetonitrile (50:25:25, v/v/v). The solution was injected onto a semi-preparative Zorbax-ODS reversed phase column (9.4 × 25 cm). The column was equilibrated with water/methanol/acetonitrile (50:25:25). The compounds were eluted with a linear gradient to methanol/acetonitrile (80:20) in 30 min at 1 ml/min. The Agilent model 1314 variable wavelength detector was set at 276 nm, which was previously determined to be the maximal wavelength of the dihydro-dihydroxy-dichlorobiphenyl metabolite of 2,2′-CB (2) and the dihydro-dihydroxy-tetrachlorobiphenyl metabolite of 2,3,2′,3′-CB (12). Using this system, the three metabolites generated from 2,2′-CB and the two metabolites generated from 2,3,2′,3′-CB by *E. coli* [pDB31*bphFG*] + [pQE31*bphAE*] eluted as distinct peaks. The peak corresponding to each metabolite was collected, the solvent phase was evaporated in a vacuum, and the residual aqueous phase was extracted with ethyl acetate. The purity of each compound was confirmed by GC/MS analyses of their butylboronate or trimethylsilyl derivatives (16, 25). Proton NMR spectra were obtained at the NMR spectrometry center of INRS-Institut Armand-Frappier with a Brucker 500-mHz spectrometer. The analyses were carried out in deuterated acetone, acetonitrile, and dimethyl sulfoxide at room temperature.

Analysis of 2,2′-CB-d_8 Metabolites—The 2,2′CB-d_8 metabolites were produced by catalytic oxygenation using a reconstituted purified preparation of LB400 BPDO according to the protocols described above for 2,2′-CB metabolites. The metabolites were analyzed by GC/MS analysis using a Hewlett Packard HP6980 series gas chromatograph interfaced with an HP5973 mass selective detector (Agilent Technologies). The mass selective detector was operated in electron impact (EI) mode and used a quadrupole mass analyzer. Under these sets of conditions, the instrument resolution is 0.1 atomic mass units, which is well sufficient to clearly distinguish between two compounds of atomic masses differing by a single atomic mass unit.

RESULTS

Production of 2,2′-CB Metabolites by LB400 and p4 BPDOs—The His-tagged purified preparations of LB400 BPDO used in this investigation had specific activities ranging from 120 to 150 nmol/min/mg of ISP_{BPH} when biphenyl was the substrate compared with 350–400 nmol/min/mg for the specific activity of *p*4 BPDO. When 2,2′-CB was the substrate, the specific activities, based on substrate depletion, were between 35 and 55 nmol/min/mg ISP_{BPH} for LB400 BPDO and 70 and 90 nmol/min/mg ISP_{BPH} for the various enzyme preparations of *p4* BPDO used in the study.

When His-tagged purified preparations of LB400 BPDO were used to catalyze the oxygenation of 2,2′-CB, two metabolites (metabolites *1* and *2*) were detected by GC/MS analysis of their butylboronate derivatives. The spectral features and GC retention time of butylboronate-derived metabolite *1* (shown in Fig. 2) were identical to those of the metabolite generated from 2-chlorobiphenyl by a coupled reaction catalyzed by LB400 BPDO plus BphB (data not shown). These features comprised a molecular ion at *m/z* 286, with diagnostically important ions at 230 $[M - 56]^+$ and an ion of low abundance at *m/z* 194 $[M - 56 - 36]^+$. Based on published data for butylboronate-derived 2,3-dihydroxy-4′-chlorobiphenyl, the major fragmentation sequence involved to the loss of the *n*-butyl moiety from the molecular ion with proton transfer $[M - 56]^+$ followed by elimination of hydrochloric acid $[M - 56 - 36]^+$ (26). These features, along with those from the literature, identified metabolite *1* as 2,3-dihydroxy-2′-chlorobiphenyl.

The mass spectrum of metabolite *2* is shown in Fig. 2. The spectrum is characterized by major ions at *m/z* 322, 287, 265, 238, and 222, which identified it as a dihydro-dihydroxy-dichlorobiphenyl. Based on previous reports (26), the EI fragmentation of butylboronate-derived dihydro-dihydroxy-dichlorobiphenyl should be initiated by the loss of a chlorine radical to yield an ion at *m/z* 287 $[M^+ - 35]$, whereas the elimination of the *n*-butyl moiety from $M^{+\cdot}$ should give rise to a cyclic oxonium-type ion of *m/z* 265 $[M^+ - 57]$. EI also induced the loss of the neutral C_4H_9BO moiety to generate an ion at *m/z* 238 $[M^+ - 84]$ or the loss of the neutral $nC_4H_9BO_2$ moiety to generate an ion at *m/z* 222 $[M^+ - 100]$.

Together, these data show that metabolites *1* and *2* produced in a ratio of 90:10 correspond exactly to those obtained from a purified preparation of LB400 BPDO by Haddock *et al.* (2). Therefore, metabolite *1* corresponded to 2,3-dihydroxy-2′-chlorobiphenyl, which was identified by NMR analysis by Haddock *et al.* (2). Because metabolite *2* was produced in large amounts when purified preparations of *p*4 BPDO were used to catalyze the oxygenation of 2,2′-CB (Fig. 2), we used this enzyme to produce and purify this compound for further analysis. GC/MS analysis of the high pressure liquid chromatography-purified preparation of metabolite *2* showed that its purity was higher than 99%. This purified dihydro-dihydroxy-dichlorobiphenyl compound was stable for days at 20 °C in ethyl acetate, acetonitrile, or acetone.

When resting cell suspensions of *E. coli* [pQE31*bphFG*] + [pDB31 LB400-*bphAE*] or [pQE31*bphFG*] + [pDB31 *p4-bphAE*] were used to catalyze the oxygenation of 2,2′-CB, the same two metabolites were produced. Their ratio was slightly different from that obtained with purified enzyme preparations (30). Furthermore, in this case, a third metabolite (metabolite *3*) representing ~1–5% of total metabolites was also detected. The molecular weight and mass spectral fragmentation patterns of its butylboronate derivative corresponded to a dihydroxy-dichlorobiphenyl similar to the dihydroxy-monochlorobiphenyl described above. Its mass spectrum showed ions at *m/z* 320 $[M]^{+\cdot}$, *m/z* 264 $[M - 56]^+$, and at *m/z* 228 $[M - 56 - 36]^+$

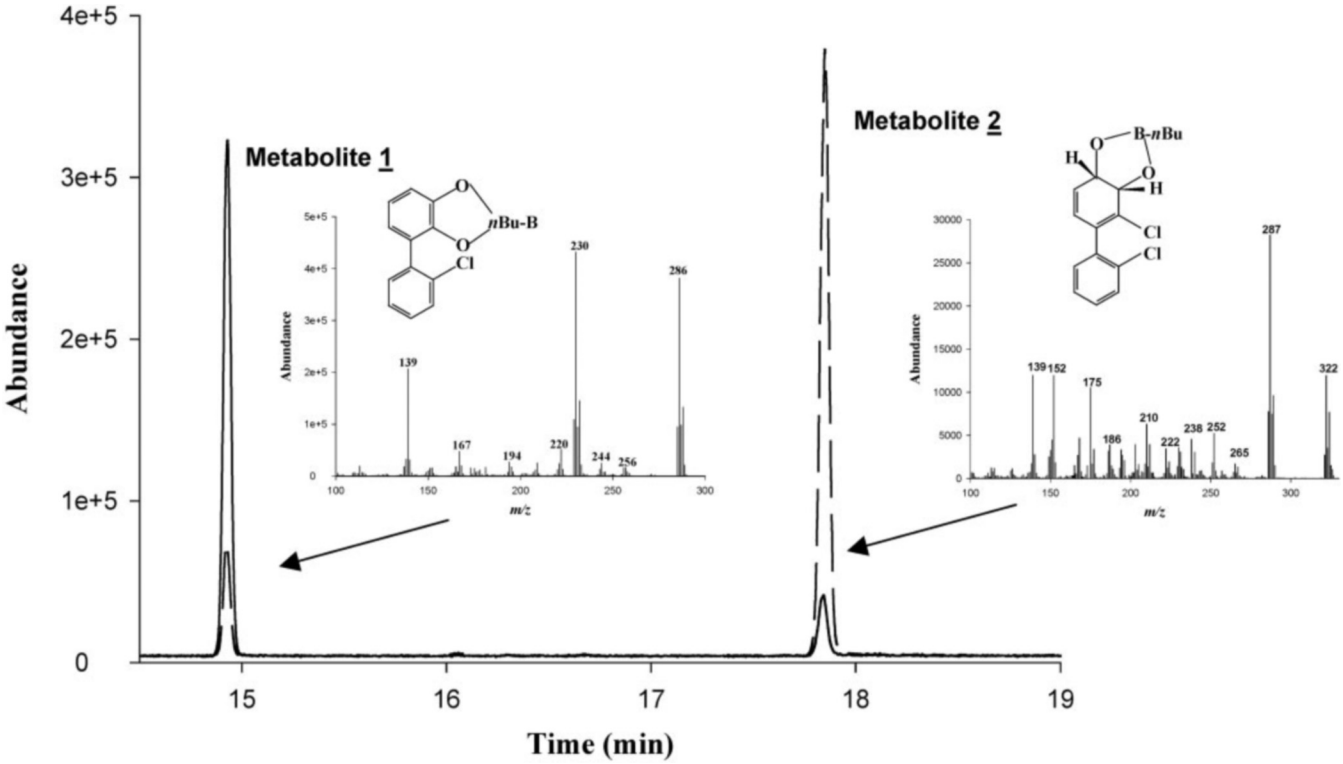

FIG. 2. **Catalytic conversion of 2,2′-CB by LB400 and *p*4 BPDOs showing the ratio of metabolite *1* and *2* generated from 2,2′CB by LB400 BPDO (*solid line*) and *p*4 BPDO (*dashed line*) and the EI spectra of metabolites *1* and *2*.**

(Fig. 3). Metabolite *3* was in fact the oxidized derivative of metabolite *2* (see below).

Conversion of Metabolite 2 by BphB and BphC—When 10–50 μg of His-tagged purified preparations of BphB were used to catalyze the oxidation of 1 to 25 nmol of metabolite *2* in 200 μl of reaction medium, substrate depletion on the order of 5 nmol/min was recorded. However, no dihydroxylated metabolite was detected, and no NADH production was recorded when the assay was monitored by spectrophotometry at 340 nm. There was no substrate depletion from control assays containing all components of the assay medium except the enzyme or containing boiled enzyme, showing that metabolite *2* does not react with a component of the enzyme assay. However, the fact that the substrate disappeared from control assays containing the enzyme without NAD^+ suggests that a reaction occurred between the substrate and the enzyme, causing the substrate to be either irreversibly bound to the protein or transformed into a reactive species that polymerized. We still do not know the precise reaction occurring between the enzyme and metabolite *2*. However, when the amount of enzyme added to 200 μl of reaction medium was reduced to 200 ng, a trace of a dihydroxy-dichlorobiphenyl metabolite was detected by GC/MS from 25 nmol of metabolite *2*. Based on its GC retention time and the mass spectral features of its butylboronate derivative, this metabolite was the same as metabolite *3* that was produced when resting cell suspensions of recombinant *E. coli* [pDB31*bphFG*] + [pQE31 LB400-*bphAE*] or [pDB31*bphFG*] + [pQE31 *p4-bphAE*] were used to catalyze the oxygenation of 2,2′-CB (not shown). Therefore, metabolite *3* was most probably produced by dehydrogenation of metabolite *2* by a nonspecific dehydrogenase present in *E. coli* cells.

When IPTG-induced suspensions of *E. coli* pQE31[LB400-*bphB*] instead of purified BphB, were used to catalyze the oxidation of metabolite *2,* it was converted to its dihydroxy derivative (metabolite *3*) efficiently. IPTG-induced *E. coli* pQE31[LB400-*bphB*] catalyzed the complete conversion of 25 nmol of metabolite *2* to its dihydroxyl derivative in less than 30 min (Fig. 3).

Based on the NMR spectra of metabolite *2* (see below), metabolite *3* was identified as 3,4-dihydroxy-2,2′-dichlorobiphenyl. When metabolite *3* was the substrate for BphC, no metabolite was detected either by GC/MS analysis or by spectrophotometric monitoring of the yellow *meta*-cleavage metabolite at wavelengths ranging from 380 to 430 nm. Furthermore, the substrate was recovered entirely. This is similar to previous observations that BphC was unable to cleave 3,4-dihydroxy-2,5,2′,5′-tetrachlorobiphenyl. In previous work, we also found that 3,4-dihydroxy-2,5,2′,5′-tetrachlorobiphenyl could be cleaved by 1,2-dihydroxynaphthalene dioxygenase (27), the enzyme that catalyzes the step corresponding to BphC in the naphthalene catabolic pathways. Likewise, 1,2-dihydroxynaphthalene dioxygenase was able to convert 3,4-dihydroxy-2,2′-dichlorobiphenyl into a yellow metabolite that was monitored at 380 nm (data not shown).

Identification of the Dihydro-dihydroxy-dichlorobiphenyl Metabolite 2—Proton NMR analysis of metabolite *2* identified it as *cis*-3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl (structure shown in Fig. 4). In deuterated acetone, the spectrum shows two different sets of signals that can be attributed to two different conformations of the molecule in a 1:2 ratio. These two conformations correspond to two rotamers caused by restricted rotation around the biphenyl linkage as a result of the steric hindrance of the two *ortho* chlorines. That these two sets of signals are indeed the result of two rotamers can be demonstrated by changing the solvent from deuterated acetone to deuterated chloroform, which caused the ratio to change to 1:1. In the higher field portion of the spectrum, the two rotamers show signals at 7.48 (m, H_1), 7.41–7.37 (m, H_{2-3}), and 7.36–7.22 (m, H_4) ppm, corresponding to the aromatic protons (Fig. 4) in a pattern characteristic of 2-chloro substituted phenyl ring. In the lower field portion of the spectrum of the most abundant rotamer are signals characteristic of aliphatic pro-

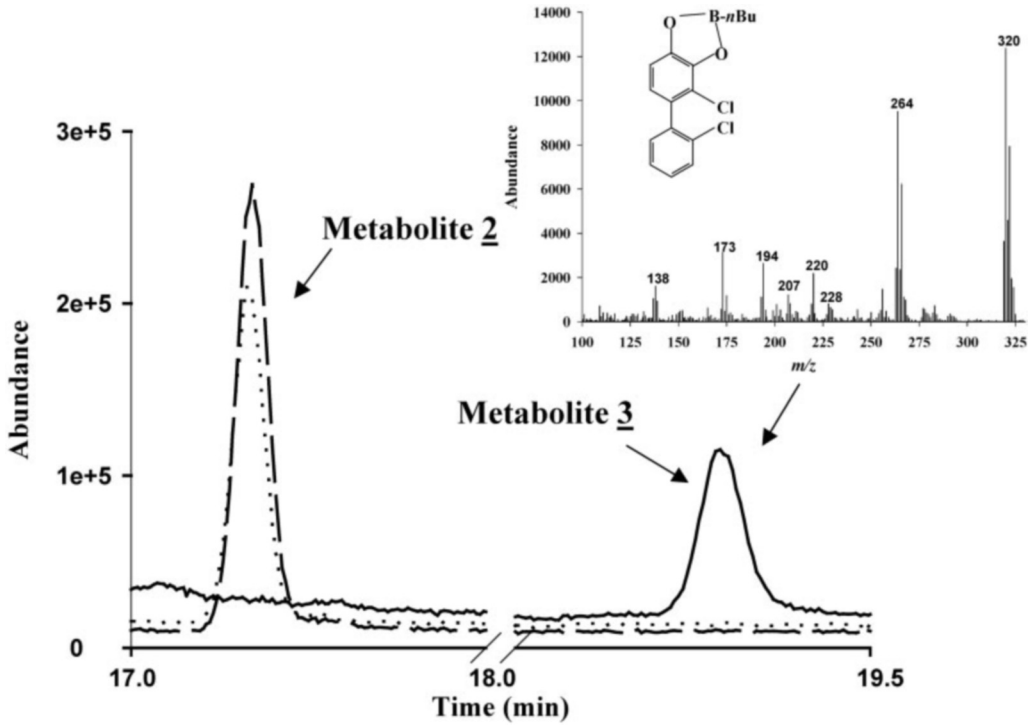

FIG. 3. **Catalytic conversion of metabolite *2* to metabolite *3* by *E. coli* cells expressing BphB.** *Solid curve*, total ion chromatogram of an ethyl acetate extract of an IPTG-induced *E. coli*[pQE31 *bphB*] suspension in M9 medium incubated for 15 min with 25 nmol of metabolite *2*; *dotted line*, control of an IPTG-induced *E. coli*[pQE31] suspension in M9 medium incubated for 15 min with 25 nmol of metabolite *2*; *dashed line*, control of M9 medium containing 25 nmol of metabolite *2* but without cells. The mass spectrum of metabolite *3* and its structure are also shown.

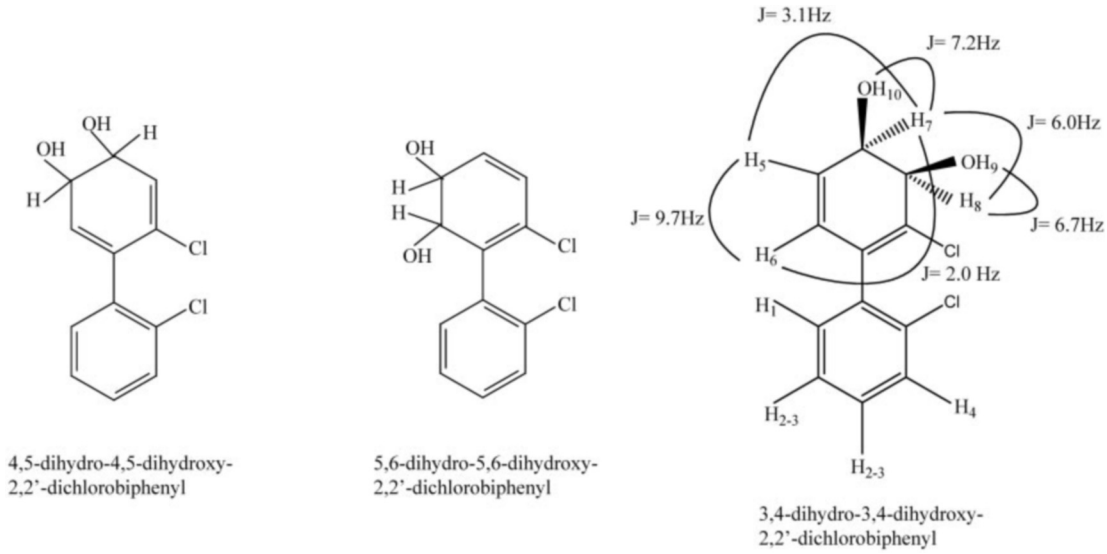

FIG. 4. **Structure of the three dihydro-dihydroxy isomers that could theoretically be generated by catalytic oxygenation of 2,2′-CB and the NMR coupling constants of 3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl isolated from the reaction.**

tons at 5.94 (dd, H_5), 5.80 (dd, H_6), 4.6 (m, H_7), and 4.24 (dd, H_8), in addition to the two hydroxylic protons at 4.43 (d, H_9) and 4.14 ppm (d, H_{10}), respectively. The two olefinic protons H_5 at 5.94 and H_6 at 5.80 ppm are coupled to each other (J = 9.7 Hz), and H_5 is coupled to H_7 (J = 3.1 Hz). H_7 is coupled, through long-range coupling, with H_6 (J = 2.0 Hz) and to the adjacent H_8 (J = 6.0 Hz) (Fig. 4). This latter value is characteristic of protons in *cis* configuration, confirming that the two hydroxyl groups are also in a *cis* configuration, as observed in reactions performed by oxygenases. Finally, the olefinic proton H_6 at 5.80 ppm shows a positive nuclear Overhauser effect when the aromatic protons of the other ring are irradiated. The less abundant rotamer presented the same aliphatic protons at 5.91 (dd), 5.84 (dd), 4.64 (m), and 4.23 (dd) and the same hydroxylic protons at 4.40 (d) and 4.08 (d), with coupling constants similar to those encountered in the major rotamer. Only 3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl could account for such features. In 4,5-dihydro-4,5-dihydroxy-2,2′-dichlorobiphenyl, the olefinic protons would not be coupled to each other, and in 5,6-dihydro-5,6-dihydroxy-2,2′-dichlorobiphenyl, there would be no nuclear Overhauser effect between the olefinic protons and the aromatic protons of the other ring (Fig. 4). Thus, the minor metabolite resulting from the catalytic oxygenation of 2,2′-CB by LB400 BPDO (which corresponded to the major metabolite produced by *p*4 BPDO variant) resulted from an oxygenase attack on carbons 3 and 4 rather than 5 and 6, as originally presumed.

All together, the data show that metabolites *1* and *2*, which were the sole metabolites generated by LB400 and *p*4 BPDOs when purified enzyme preparations were used to catalyze the oxygenation of 2,2′-CB, were 2,3-dihydroxy-2′-chlorobiphenyl and 3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl, respectively. Metabolite *3*, which was obtained when recombinant cells of *E. coli* [pQE31 *bphAE* + pDB31 *bphFG*] were used to catalyze the oxygenation of 2,2′-CB, was 3,4-dihydroxy-2,2′-dichlorobiphenyl, resulting from oxidation of *cis*-3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl.

Use of Deuterated 2,2′-CB to Identify the Carbons that Interact with the Oxygenase Active Site—The fact that *cis*-3,4-dihydro-3,4-dihydroxy-2,2′-chlorobiphenyl and not 5,6-dihydro-5,6-dihydroxy-2,2′-dichlorobiphenyl was produced by catalytic oxygenation of 2,2′-CB shows that BPDO can attack at positions other than *ortho* and *meta*. This raises the question whether 2,3-dihydroxy-2′-chlorobiphenyl was truly generated through an oxygenase attack on carbons 2 and 3, as originally postulated. To understand clearly the impact of structural changes on the regiospecificity of engineered BPDOs toward *ortho*-substituted chlorobiphenyls, it was mandatory that we clearly identify the carbons that interact with the enzyme catalytic center during oxygenation. To achieve this, we used 2,2′-CB-d_8. The preparation of 2,2′-CB-d_8 used in this work was synthesized chemically according to the procedure described under "Experimental Procedures." Of the various purified fractions obtained, one contained 3.7 mg of 2,2′-dichlorobiphenyl-d_8 that was found to be 94% pure; the residual impurities were 2-chlorobiphenyl-d_9 (2.9%), 2,4′-dichlorobiphenyl-d_8 (1.7%), and 4,4′-dichlorobiphenyl-d_8 (1.6%). The percentages of these various compounds in the final product was determined by GC/MS according to the intensity of their respective molecular ions and their identity by their relative retention times on a 30-m DB-5 capillary column.

When this preparation of 2,2′-CB-d_8 was used as substrate with a purified preparation of LB400 BPDO, metabolites *1* and *2* were detected in a ratio of ~80:20. The mass spectral features of the butylboronate-derived metabolite *1* (Fig. 5) are identical to the mass spectrum of the butylboronate-derived metabolite *1* of 2,2′-CB shown in Fig. 2, except that the fragmentation ions were displaced by a value of *m/z* +7. Thus the diagnostically important ions were found at *m/z* 293 $[M]^+$, at *m/z* 237 $[M - 56]^+$, and at *m/z* 201 $[M - 56 - 36]^+$. These spectra show that a single deuterium atom was lost from 2,2′-CB-d_8 during the oxygenation reaction, and this could have occurred only if carbons 2 and 3 had reacted with the activated dioxygen. If the oxygen had reacted with carbons 5 and 6 followed by a rearrangement, the substrate would have lost 2 deuterium atoms (Fig. 5). The EI fragmentation pattern of the deuterated 2,3-dihydroxy-2-chlorobiphenyl would then have been displaced by a value of *m/z* +6 compared with the metabolite obtained from nondeuterated 2,2′-CB. Data conclusively show that the oxygenation occurred on carbons 2 and 3 and not on carbons 5 and 6.

Metabolism of 2,3,2′,3′-CB by LB400 BPDO—The metabolites produced from 2,3,2′,3′-CB by LB400 BPDO have never been identified precisely. In a previous report, two metabolites were generated from this congener in a ratio of 10–15:85–90 (12). The mass spectral fragmentation patterns of their butylboronate derivatives were typical of tetrachlorinated dihydrodihydroxybiphenyl and show ions at *m/z* 390 $[M]^{+\cdot}$, *m/z* 355 $[M - 35]^+$, *m/z* 333 $[M - 57]^+$, *m/z* 306 $[M - 84]^+$, and *m/z* 290 $[M - 100]^+$ (Fig. 6). The only two possibilities are 5,6-dihydro-5,6-dihydroxy-2,3,2′,3′-tetrachlorobiphenyl and 4,5-dihydro-4,5-dihydroxy-2,3,2′,3′-tetrachlorobiphenyl. These metabolites were purified by high pressure liquid chromatography. NMR analysis of the major metabolite identified it as *cis*-4,5-dihydro-4,5-dihydroxy-2,3,2′,3′-tetrachlorobiphenyl (structure shown in Fig. 7), showing that this congener is oxygenated principally on the chlorine-free vicinal *meta-para* carbons rather than the *ortho-meta* carbons.

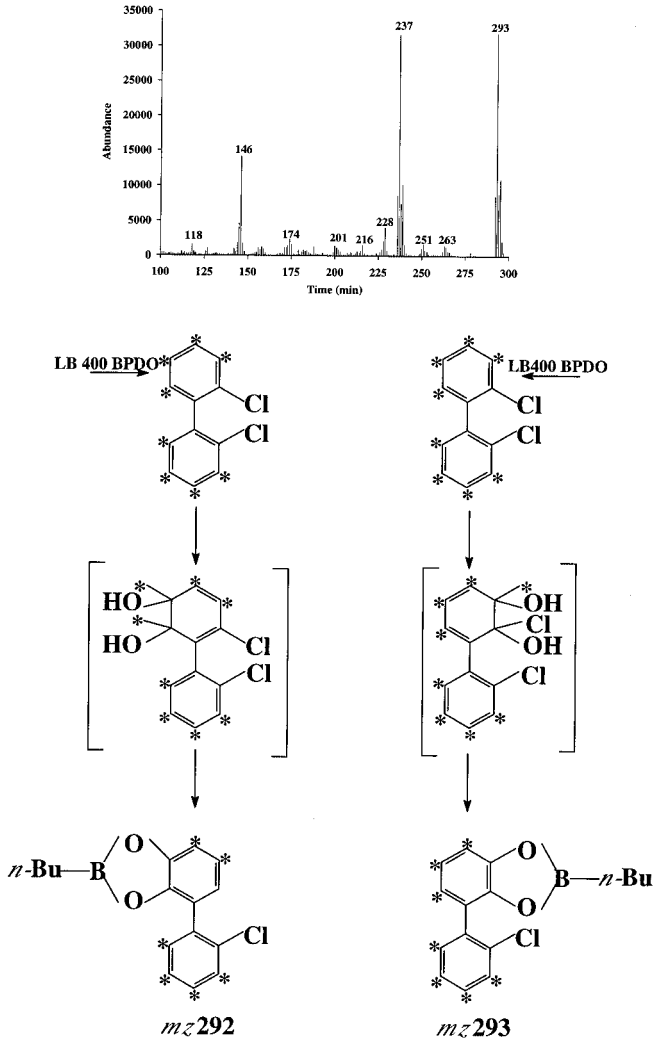

FIG. 5. **MS spectrum of metabolite *1* from 2,2′-CB-d_8 and scheme representing the molecular mass of its butyl boronate derivative depending on the pathway used for its formation.** *Asterisks* represent deuterium atoms.

In deuterated chloroform, the NMR spectrum of the major metabolite shows two different sets of signals in a 58:42 ratio that corresponds also to two rotamers, as seen with 3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl. The high field portion of the spectrum shows signals at 7.47 (d, J = 8.2Hz, H_1), 7.21 (m, H_2), and 7.08 ppm (d, J = 7.5 H_3) for the major isomer and at 7.47 (d, J = 8.2 Hz, H_1), 7.21 (m, H_2), and 7.15 ppm (d, J = 7.3 Hz, H_3), for the minor one, in a pattern characteristic of 2,3-dichloro substituted phenyl ring.

In the lower field portion of the spectrum, the most abundant rotamer shows three aliphatic protons at 5.89 (broad s, H_4), 4.75 (m, H_5) and 4.31 ppm (d, J = 5.4 Hz, H_6) (Fig. 7). In deuterated acetonitrile, the two hydroxylic protons are detected at 3.74 (J = 5.0 Hz, H_7) and 3.53 (J = 7.2 Hz, H_8) ppm. Likewise, for the minor rotamer, there were signals of aliphatic protons at 6.01 (d, J = 3.7 Hz, H_4), 4.63 (dd, J = 4.0 Hz and J = 6.0 Hz, H_5), and 4.51 ppm (d, J = 6.0 Hz, H_6). In deuterated acetonitrile, its two hydroxylic protons appeared at 3.79 (J = 7.2 Hz) and 3.48 ppm (J = 7.2Hz). As for 3,4-dihydro-3,4-

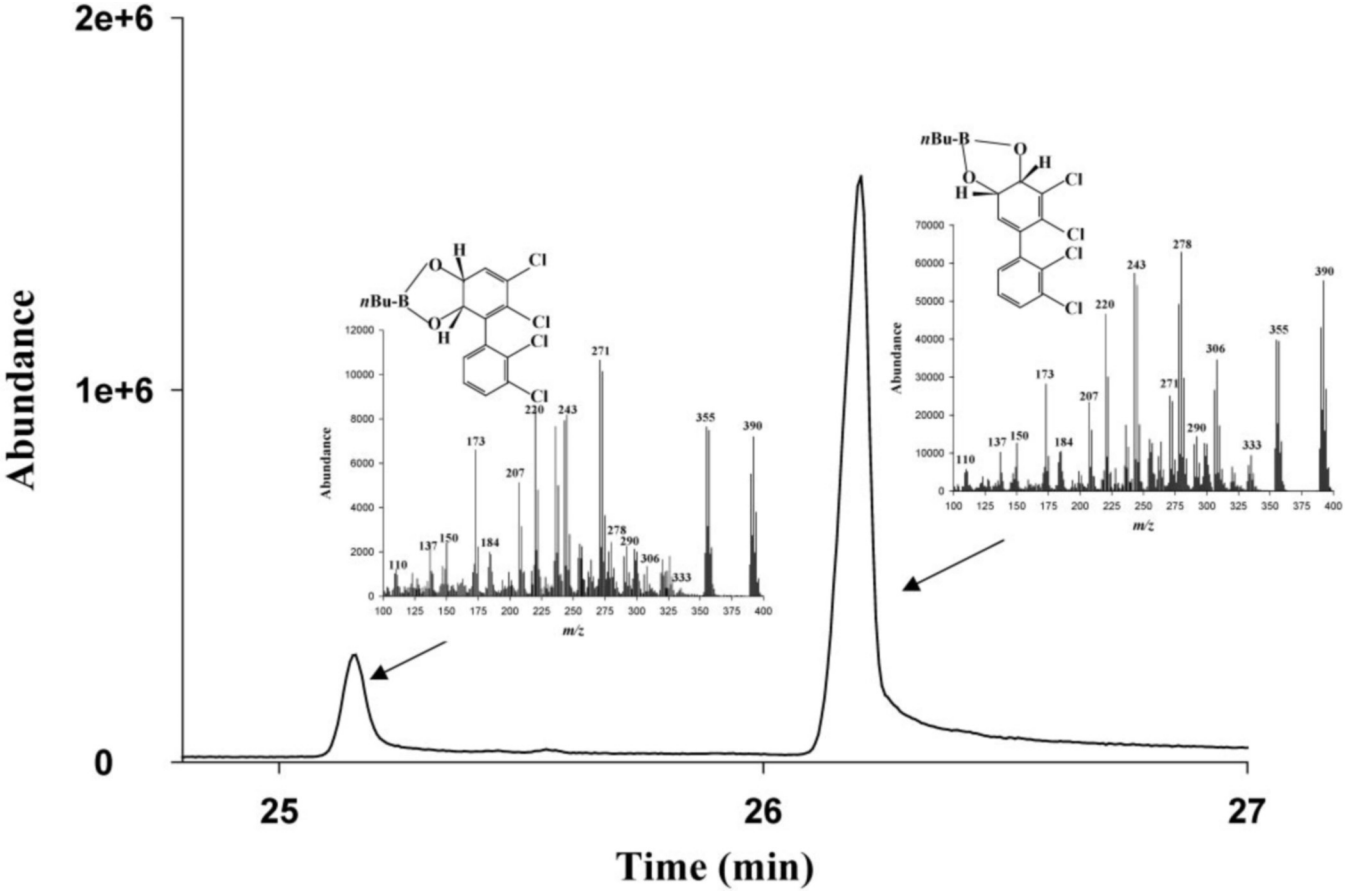

FIG. 6. **Catalytic conversion of 2,3,2′,3′-CB by LB400 BPDO showing the ratio of the two metabolites that are produced, their EI spectra, and their chemical structure.**

dihydroxy-2,2′-dichlorobiphenyl, the coupling value between the protons on the carbons bearing the hydroxyl groups is characteristic of a *cis* configuration, for both the major and minor rotamer, confirming that the two hydroxyl group are also in a *cis* configuration. Finally, the single olefinic proton H_4 at 5.89 ppm shows a positive nuclear Overhauser effect when the aromatic protons of the other ring are irradiated. Only 4,5-dihydro-4,5-dihydroxy-2,3,2′,3′-tetrachlorobiphenyl could account for such features. In 5,6-dihydro-5,6-dihydroxy-2,3,2′,3′-dichlorobiphenyl, there would be no nuclear Overhauser effect between the olefinic proton and the aromatic protons of the other ring. Thus, the major metabolite resulting from the catalytic oxygenation of 2,3,2′,3′-CB by LB400 BPDO resulted from an oxygenase attack on carbons 4 and 5.

Other evidence confirmed that 2,3,2′,3′-CB was oxygenated principally on carbons 4 and 5. As for *cis*-3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl, a high pressure liquid chromatography-purified preparation of *cis*-4,5-dihydro-4,5-dihydroxy-2,3,2′,3′-tetrachlorobiphenyl slowly disappeared from the reaction medium when a purified preparation of BphB was used to catalyze its oxidation, but no metabolite was recovered (data not shown). Furthermore, when this metabolite was the substrate for a coupled reaction using purified BphB plus BphC, no yellow metabolite was obtained. However, when a purified preparation of BphB was used to catalyze the dehydrogenation of *cis*-5,6-dihydro-5,6-dihydroxy-2,3,2′,3′-tetrachlorobiphenyl, it was converted to the corresponding hydroxy derivative, which was then converted to a yellow metabolite when BphC was used to catalyze its oxidation (data not shown).

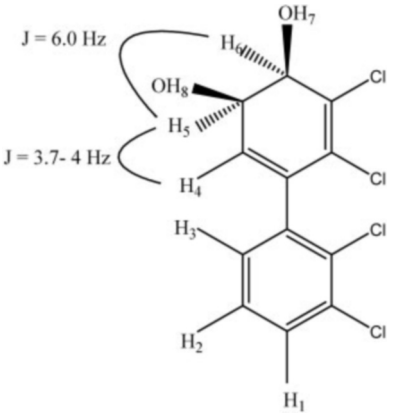

FIG. 7. **Structure and NMR coupling constants of the minor rotamer of 4,5-dihydro-4,5-dihydroxy-2,3,2′,3′-tetrachlorobiphenyl in deuterated chloroform.**

DISCUSSION

The metabolism of 2,2′-CB by LB400 BPDO was examined by Haddock *et al.* (2). They identified 2,3-dihydroxy-2′-chlorobiphenyl as the major metabolite from 2,2′-CB, suggesting that the chlorine-substituted *ortho* carbon was oxygenated during the catalytic reaction. The minor metabolite was presumed to result from an oxygenation occurring on carbons 5 and 6, at the other *ortho* and *meta* positions.

In this investigation, we confirmed the assumption made by Haddock *et al.* (2) that the dehalogenation occurring during the catalytic oxygenation of 2,2′-CB by LB400 BPDO is the result

of an oxygenase attack on carbons 2 and 3 of the phenyl ring. A reaction mechanism has already been proposed for this. The reaction would be similar to the oxygenolytic dehalogenation occurring during catalytic oxygenation of chlorobenzoates (2, 28, 29). In this case, 2-chloro-[*cis*-2,3-dihydroxy-1-[2′-chlorophenyl]-cyclohexa-4,6 diene] (or commonly 2,3-dihydro-2,3-dihydroxy-2,2′-dichlorobiphenyl) is believed to be unstable and to spontaneously lose HCl in a manner similar to the dehydration reaction responsible for the re-aromatization of the *cis*-diol structure to catechol at low pHs.

Furthermore, we identified the other minor metabolite resulting from LB400 BPDO oxygenation of 2,2′-CB as *cis*-3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl instead of 5,6-dihydro-5,6-dihydroxy-2,2′-dichlorobiphenyl, showing that 2,2′-CB can take two orientations inside the catalytic pocket, whereby the active iron faces the vicinal *ortho-meta* carbons of the chlorinated side of the phenyl ring, or it can also face the vicinal *meta-para* carbons 3 and 4. No metabolite corresponding to an oxygenation of the vicinal *ortho-meta* carbons 5 and 6 or of the vicinal *meta-para* carbons 4 and 5 of the nonchlorinated side of the ring was detected. It is noteworthy that the more relaxed *p*4 BPDO active center presented a significantly altered regiospecificity toward 2,2′-CB to favor catalytic oxygenation of the vicinal *meta-para* carbons 3 and 4 but was unable to oxygenate the vicinal carbons 5 and 6. This suggests that interactions between amino acid residues other than region III of BphA and one or both of the *ortho*-chlorines prevent the nonchlorinated vicinal *ortho-meta* carbons of the phenyl ring from occupying a position that would allow them to interact with the activated dioxygen. This is supported by the fact that 2,3,2′,3′-CB is oxygenated preferentially on the *meta-para* carbons 4 and 5 rather than the *ortho-meta* carbons 5 and 6 by both LB400 and *p*4 BPDOs. Based on the fact that BPDOs normally introduce the dioxygen on vicinal *ortho-meta* carbons, these would have been the favored carbons for oxygenation if the chlorine substituents had not influenced the orientation of 2,3,2′,3′-CB molecule inside the catalytic pocket.

BPDO regiospecificity toward each individual chlorobiphenyl congener is critical to determine whether further enzymes of the biphenyl catabolic pathway will metabolize it. Although BphB was found to catalyze the oxidation of *cis*-3,4-dihydro-3,4-dihydroxy-2,2′,5,5′-tetrachlorobiphenyl to corresponding catechol (25), BphC was unable to cleave 3,4-dihydroxybiphenyl and its chlorinated congeners (27). In contrast, we found that BphB does not oxidize *cis*-3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl very efficiently. Our data suggest that this metabolite reacts with the enzyme to either irreversibly bind to it or to generate a reactive species that polymerizes. Together, these data show the importance of considering any alteration to regiospecificity occurring during the engineering processes directed to evolve enhanced PCB degrading BPDOs. On the other hand, the observations that *E. coli* cells expressing BphB can oxidize *cis*-3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl to its dihydroxy metabolite and that 1,2-dihydroxy-naphthalene dioxygenase, the equivalent of BphC in the naphthalene catabolic pathway, cleaves 3,4-dihydroxy-2,2′-dichlorobiphenyl indicate that *cis*-3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl would not accumulate as a dead-end compound in the environment.

According to a model based on naphthalene dioxygenase crystal structure, it was recently demonstrated that BPDO residues that had not been predicted to be influential can play a major role in BPDO specificity (17). The present report provides a further example that the enzyme-substrate interactions that determine BPDO regiospecificity toward chlorobiphenyls are complex. Identification of the amino acid residues that interact with the substrate and determination of their mechanism of interaction will be essential to engineer evolved BPDO exhibiting an extended PCB degrading potency. Ongoing work aims at identifying these amino acid residues.

REFERENCES

1. Abramowicz, D. A. (1994) *Res. Microbiol.* **145,** 42–46
2. Haddock, J. D., Horton, J. R., and Gibson, D. T. (1995) *J. Bacteriol.* **177,** 20–26
3. Hurtubise, Y., Barriault, D., and Sylvestre, M. (1996) *J. Biol. Chem.* **271,** 8152–8156
4. Haddock, J. D., and Gibson, D. T. (1995) *J. Bacteriol.* **177,** 5834–5839
5. Haddock, J. D., Pelletier, D. A., and Gibson, D. T. (1997) *J. Ind. Microbiol. Biotechnol.* **19,** 355–359
6. Hurtubise, Y., Barriault, D., Powlowski, J., and Sylvestre, M. (1995) *J. Bacteriol.* **177,** 6610–6618
7. Denef, V. J., Park, J., Tsoi, T. V., Rouillard, J. M., Zhang, H., Wibbenmeyer, J. A., Verstraete, W., Gulari, E., Hashsham, S. A., and Tiedje, J. M. (2004) *Appl. Environ. Microbiol.* **70,** 4961–4970
8. Erickson, B. D., and Mondello, F. J. (1992) *J. Bacteriol.* **174,** 2903–2912
9. Sylvestre, M., Sirois, M., Hurtubise, Y., Bergeron, J., Ahmad, D., Shareck, F., Barriault, D., Guillemette, I., and Juteau, J. M. (1996) *Gene* **174,** 195–202
10. Hurtubise, Y., Barriault, D., and Sylvestre, M. (1998) *J. Bacteriol.* **180,** 5828–5835
11. Mondello, F. J., Turcich, M. P., Lobos, J. H., and Erickson, B. D. (1997) *Appl. Environ. Microbiol.* **63,** 3096–3103
12. Arnett, C. M., Parales, J. V., and Haddock, J. D. (2000) *Appl. Environ. Microbiol.* **66,** 2928–2933
13. Seeger, M., Camara, B., and Hofer, B. (2001) *J. Bacteriol.* **183,** 3548–3555
14. Suenaga, H., Watanabe, T., Sato, M., Ngadiman, and Furukawa, K. (2002) *J. Bacteriol.* **184,** 3682–3688
15. Zielinski, M., Backhaus, S., and Hofer, B. (2002) *Microbiology* **148,** 2439–2448
16. Barriault, D., Plante, M. M., and Sylvestre, M. (2002) *J. Bacteriol.* **184,** 3794–3800
17. Zielinski, M., Kahl, S., Hecht, H. J., and Hofer, B. (2003) *J. Bacteriol.* **185,** 6976–6980
18. Sondossi, M., Barriault, D., and Sylvestre, M. (2004) *Appl. Environ. Microbiol.* **70,** 174–181
19. Francova, K., Mackova, M., Macek, T., and Sylvestre, M. (2004) *Environ. Pollut.* **127,** 41–48
20. Lin, J. J., Smith, M., Jessee, J., and Bloom, F. (1992) *BioTechniques* **12,** 718–721
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
22. Sylvestre, M., Hurtubise, Y., Barriault, D., Bergeron, J., and Ahmad, D. (1996) *Appl. Environ. Microbiol.* **62,** 2710–2715
23. Simon, M. J., Osslund, T. D., Saunders, R., Ensley, B. D., Suggs, S., Harcourt, A., Suen, W. C., Cruden, D. L., Gibson, D. T., and Zylstra, G. J. (1993) *Gene* **127,** 31–33
24. Hein, P., Powlowski, J., Barriault, D., Hurtubise, Y., Ahmad, D., and Sylvestre, M. (1998) *Can. J. Microbiol.* **44,** 42–49
25. Barriault, D., Vedadi, M., Powlowski, J., and Sylvestre, M. (1999) *Biochem. Biophys. Res. Commun.* **260,** 181–187
26. Massé, R., Messier, F., Ayotte, C., Lévesque, M. F., and Sylvestre, M. (1989) *Biomed. Environ. Mass Spectrom.* **18,** 27–47
27. Barriault, D., Durand, J., Maaroufi, H., Eltis, L. D., and Sylvestre, M. (1998) *Appl. Environ. Microbiol.* **64,** 4637–4642
28. Fetzner, S., Müller, R., and Lingens, F. (1992) *J. Bacteriol.* **174,** 279–290
29. Nakatsu, C. H., and Wyndham, R. C. (1993) *Appl. Environ. Microbiol.* **59,** 3625–3633
30. Barriault, D., and Sylvestre, M. (2004) *J. Biol. Chem.* **279,** 47480–47488