Evolution of the Biphenyl Dioxygenase BphA from *Burkholderia xenovorans* LB400 by Random Mutagenesis of Multiple Sites in Region III*

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It is now established that several amino acids of region III of the biphenyl dioxygenase (BPDO) α subunit are involved in substrate recognition and regiospecificity toward chlorobiphenyls. However, the sequence pattern of the amino acids of that segment of seven amino acids located in the C-terminal portion of the α subunit is rather limited in BPDOs of natural occurrence. In this work, we have randomly mutated simultaneously four residues (Thr335-Phe336-Ile338-Ile339) of region III of *Burkholderia xenovorans* LB400 BphA. The library was screened for variants able to oxygenate 2,2′-dichlorobiphenyl (2,2′-CB). Replacement of Phe336 with Met or Ile with a concomitant change of Thr335 to Ala created new variants that transformed 2,2′-CB into 3,4-dihydroxy-2,2′-dichlorobiphenyl, which is a dead end metabolite that was not cleaved by BphC. Replacement of Thr335-Phe336 with Ala335-Leu336 did not cause this type of phenotypic change. Regiospecificity toward congeners other than 2,2′-CB that were oxygenated more efficiently by variant Ala335-Met336 than by LB400 BPDO was similar for both enzymes. Thus structural changes that altered the regiospecificity toward 2,2′-CB did not affect the metabolite profile of other congeners, although it affected the rate of conversion of these congeners. It was especially noteworthy that both LB400 BPDO and the Ala335-Met336 variant generated 2,5-dihydroxy-2,4,4′,4″-trichlorobiphenyl as the sole metabolite from 2,4,2′,4″-CB and 4,5-dihydroxy-2,5-dihydroxybiphenyl as the major metabolite from 2,3,2′,3′-CB. This shows that 2,4,2′,4″-CB is oxygenated principally onto vicinal ortho-meta carbons 2 and 3 and that 2,3,2′,3′-CB is oxygenated onto para-carbons 4 and 5 by both enzymes. The data suggest that interactions between the chlorine substituents on the phenyl ring and specific amino acid residues of the protein influence the orientation of the phenyl ring inside the catalytic pocket.

Biphenyl dioxygenase (BPDO) catalyzes the first reaction of the biphenyl catabolic pathway. BPDO comprises three components (1, 2); the iron-sulfur oxygenase (ISPBP) made up of an α subunit (M<sub>α</sub> = 51,000) and a β subunit (M<sub>β</sub> = 22,000), the ferredoxin (FER<sub>BP</sub>BP, M<sub>α</sub> = 12,000), and the ferredoxin reductase (RED<sub>BP</sub>BP, M<sub>β</sub> = 43,000). The encoding genes for *Burkholderia xenovorans* LB400 (3), which is also called *Burkholderia* (Pseudomonas) sp. LB400 (4, 5), are bphA (ISP<sub>BP</sub>BP α subunit), bphE (ISP<sub>BP</sub>BP β subunit), bphF (FER<sub>BP</sub>BP), and bphG (RED<sub>BP</sub>BP). BPDO can oxygenate several polychlorinated biphenyl (PCB) congeners. The application of BPDO for efficient PCB degrading processes will require an expansion of the range of PCB substrates it can oxygenate. The catalytic oxygenation of biphenyl occurs normally on carbons 2 and 3 to generate the cis-2,3-dihydro-2,3-dihydroxybiphenyl, which is dehydrogenated by the cis-2,3-dihydro-2,3-dihydroxybiphenyl 2,3-dehydrogenase encoded by bphC in strain LB400. The resulting catechol 2,3-dihydroxybiphenyl is then cleaved by the 2,3-dihydroxybiphenyl 1,2-dioxigenase encoded by bphC in strain LB400. 2-Hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid (HOPDA) is then hydrolyzed by the HOPDA hydrolase (BphD) to yield benzoate and 2-hydroxypentanoic acid (Fig. 1).

Based on site-directed mutagenesis and family shuffling of genes, variant BPDOs with extended PCB degrading potency were obtained (6–9). Several persistent congeners that are of concern for health are not degraded, even by the most performing BPDO variants described to date. Amino acid residues of the C-terminal portion of the ISP<sub>BP</sub>BP α subunit (BphA) were found to influence the substrate selectivity of the enzyme (6–9). A stretch of seven amino acid residues termed region III was of particular interest (6, 9). The sequence of amino acids for region III of *B. xenovorans* LB400 BPDO, which is the wild-type enzyme exhibiting the highest PCB degrading potency, is Thr335-Phe336-Asn337-Asp338-Leu339-Arg340-Ile341 (4). The sequence pattern of corresponding region is Ala335-Ile336-Asn337-Thr338-Ile339-Arg340-Thr341 in *Pseudomonas pseudoalcaligenes*KF707 BphA (10) and Gly335-Ile336-Asn337-Thr338-Ile339-Arg340-Thr341 in *Comamonas testosteroni* strain B-356 BphA (11). KF707 BphA BPDO shares more than 95% homology with LB400 BphA (9). KF707 BPDO and B-356 BPDO catalyze the oxygenation of a much narrower range of PCB congeners than LB400 BPDO. Nevertheless, replacement of region III of LB400 BphA by that of KF707 or of B-356 created enzymes that oxygenated a much...
broader range of PCB congeners (6, 8, 9, 12). This shows that region III amino acids are not the only ones influencing the catalytic activity toward chlorobiphenyl, but it also shows that the sequence pattern of region III of LB400 BphA is not optimal for catalytic activity toward chlorobiphenyl.

In addition to their influence on the range of chlorobiphenyls used as substrate by the enzyme, amino acids of region III also appear to be implicated in regiospecificity toward 2,2'-CB. Thus Suenaga et al. (12) found that replacing Ile336 of KP707 BphA with Phe as in LB400 BphA changed the regiospecificity toward 2,2'-dichlorobiphenyl (2,2'-CB) to favor production of 2,3-dihydroxy-2'-chlorobiphenyl.

The ortho-substituted PCB congeners such as 2,2', 2,6-, and 2,6,2',6'-CB are among the most resistant to microbial attack. LB400 BPDO is among the few BPDOs of natural occurrence that can oxygenate 2,2'-CB efficiently (9). In previous work we found that evolved BPDOs selected for their ability to oxygenate 2,2'-CB at a higher rate than the parental LB400 BPDO were able to catalyze a broader range of PCB congeners than LB400 BPDO (6). Although the amino acid residues of region III BphA influence greatly the range of PCB congeners oxygenated by the enzyme, based on the alignment of sequences of BphAs that are found in data bases, the diversity of sequence patterns of this stretch of amino acids is rather limited (6). In this work, we have used an approach that combines a PCR-based method of random mutagenesis at targeted multi-sites plus DNA shuffling to create a library of BPDO variants that were randomly mutated simultaneously on four residues Thr335-Phe336-Asn338-Ile341 of region III of LB400 BphA. The purpose of the work was to evaluate the extent of variation that can occur in region III of BphA of LB400 with no loss of activity toward 2,2'-CB and the influence of new sequence patterns on catalytic activity toward this congener and other chlorobiphenyls. One of the most potent variants of the library (variant p4) exhibited a higher turnover rate of oxygenation of 2,2'-CB, but its regiospecificity toward this congener was altered. The rates of metabolism and the metabolite profiles of this variant toward a range of chlorobiphenyl congeners were compared with those obtained when LB400 BPDO catalyzed the reaction. The data suggest that despite the fact that residues 335 and 336 exert a strong influence on the regiospecificity toward 2,2'-CB and on the turnover rate toward several congeners, the pattern of chlorine substitution on the PCB substrate imposes its orientation toward the catalytic active center.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and General Protocols—**Escherichia coli DH11S (13) was used in this study. Plasmid pQE31[LB-400-bphAE], in which bphA was mutated to introduce an AvrII site at position 1354, was described previously (14); it encodes for a fused His-tagged ISPH (14, 15) carrying the His tag on BphA. Plasmid pYH31[LB400-bphF-GBC] was described previously (16). This plasmid encodes for the ferredoxin (BphF) and the ferredoxin reductase (BphG) components of LB400 BPDO and for 2,3-dihydro-2,3-dihydroxybiphenyl-2,3-dehydrogenase (BphB) and 2,3-dihydroxybiphenyl 2,3-dioxygenase (BphC) from *B. xenovorans* LB400, which are the second and third enzymes of the biphenyl catabolic pathway. The coupled reaction involving BPDO plus BphB and BphC transforms biphenyl into its yellow HOPDA.

To construct pDB31[LB400-bphFG], bphFG was amplified as a 1555-bp BamHI/KpnI fragment from LB400 DNA using antisense primers 5'-GGGCGGATCCATAGAATTTACGAGATTGTTG-3' and 5'-CTGG-TACCCTTCAAATTTTGTTCA-3' and then cloned into the BamHI/KpnI-digested pQEE1. DNA general protocols were done according to Sambrook et al. (17). DNA was sequenced by DNA Landmark (St-Jean, Canada).

**Preparation of DNA and PCRs to Evolve BPDO and Screening Protocol—**Seven degenerated primers, each of which contained degenerated nucleotides at positions to be mutated, were used with appropriate antisense primers to amplify fragments of LB400-bphAE that overlapped over the stretch of DNA that encode for region III (Fig. 2). Primers 1 and 2 were used in conjunction with the reverse primer 3 to amplify the 1065-bp fragments A and B that corresponded to the right end portion of bphA plus bphE. Primer 1 and 2 were degenerated at bp positions 1021, 1022, and 1023 to introduce a mutation to replace Ile431 of the protein by any other amino acid. In addition, primer 1 was degenerated at position 1008, and primer 2 was degenerated at bp position 1007. The base pairs at positions 1007 and 1008 belong to the codon encoding amino acid 336 (Phe) of the protein. Fragment A and B extended from the beginning of the DNA stretch that encoded for region III of BphA sequence Thr335-Phe336-Asn338-Ile339 to the end of the gene. The five other primers, primers 4, 5, 6, 7, and 8, containing degenerated nucleotides were used in conjunction with primer 9 to amplify a 1023-bp fragment that corresponded to the left end portion of bphA. These five sets of primers amplified DNA fragments A, C, D, E, F, and G that overlapped from the beginning of A to bp position 1023, which corresponded to the right end of region III of BphA (Fig. 2). Therefore fragments C, D, E, F, and G overlapped with fragment A and B inside the DNA stretch that encoded region III. Primer 4 was degenerated at bp positions 1006, 1007, and 1008 to mutate Phe336 of the protein; primer 5 was degenerated at bp positions 1003, 1004, and 1005 to mutate Thr335 of the protein; primer 6 was degenerated at bp positions 1012, 1013, and 1014 to mutate Asn338 of the protein; primer 7 was degenerated at bp positions 1003, 1005, 1006, and 1008 to mutate Thr335 and Phe336 together; and primer 8 was degenerated at bp positions 1006, 1008, 1011, and 1014 to mutate Phe336 and Asn338 together.

The seven fragments that overlapped inside region III were shuffled to generate a library of 1238-bp fragments (Fig. 3). The DNA fragments were treated with DNAse I to prepare the 50–100-bp fragments for DNA shuffling (18). Taq DNA polymerase was used for the primerless PCR step. The conditions were set as recommended by Roche Applied Science except that the program was as follows for 60 cycles: 94 °C for 45 s, 42 °C for 45 s, and 72 °C for 1.5 min. The primerless PCR product was PCR-amplified using the following antisense oligonucleotides 10′-ACGCGCTGGCGCTCAGCATAC-3′ and 11′-CTGGGCTCTGGCGG-TAGTGAACAGTC-3′. Taq DNA polymerase was used, setting conditions as recommended by Roche Applied Science. The resulting library of 1238-bp fragments was digested with MluI and AvrII, and the resulting 864-bp MluI/AvrII fragments were ligated to pQEE1[Lb400-bphAE] previously deleted from its MluI/AvrII fragment. The library of plasmids was transformed into *E. coli* DH11S YHI31[LB400-bphF-GBC] cells that expressed the entire set of enzymes required to convert chlorobiphenyls into corresponding chloro-HOPDAs. Transformants were inoculated on nylon membranes on the surface...
of LB agar plates (17). After 18 h of incubation at 37 °C, the membranes were transferred to fresh LB plates containing 1 mM isopropyl β-D-thiogalactoside (IPTG), the plates were incubated for 3 h at 37 °C, and then crystals of 4-CB or of 2,2'-CB were placed into the lid of the Petri dishes. 4-CB is a chlorobiphenyl congener that is normally oxidized at rate similar or higher than biphenyl by most BPDOs of natural occurrence, including LB400 BPDO (8). It was therefore used to determine the proportion of the clones of the library expressing an enzyme for which the mutations that occurred in region III did not alter their capacity to oxygenate this easily oxidizable chlorobiphenyl. The plates were incubated at 37 °C, and colonies exposed to 2,2'-CB were periodically inspected visually over a period of 48 h to search for those that developed a stronger yellow color than a control expressing LB400 BphA.

Whole Cell Assays to Evaluate the PCB Degrading Potency—E. coli DH11S[pYH31[LB400-bphFGBC]] + pQE31[bphAE]] cells expressing variant BphAAs were tested for their ability to degrade a synthetic mix of 18 PCBs using a protocol published previously (6). The synthetic mix comprised 1 μM each of the following congeners: 2,6-CB, 2,4,3'-CB, 2,4,4'-CB, 2,3,4'-CB, 2,6,2'-CB, 2,5,5'-CB, 2,2',3'-CB, 2,4,2'-CB, 2,4,3',4'-CB, 3,4,3',5'-CB, 3,4,2',5'-CB, 2,4,5,2',5'-CB, 2,4,5,2',4',5'-CB, 2,4,5',2',5',5'-CB, and 5 μM of 3,3'-CB and 4,4'-CB. The internal standard 2,3,4,5,6,3',5',6'-CB, presumed to be not degraded, was present at a concentration of 1 μM. The chlorobiphenyls were obtained from ULTRAScientific (Kingstown, RI). Each experiment included control cultures of E. coli [pYH31[bpFGBC]] + [pQE31] that were run under conditions identical to the experimental cultures. All of the values reported in the present study are averages from triplicate experiments.

Assays to Identify the Metabolites and Quantify the Catalytic Activity—Metabolites were analyzed by suspensions of IPTG-induced whole cells of E. coli [pDB31[bpFG]] + [pQE31[bphAE]]. In this case, log phase cells grown in LB broth were induced for 2 h with 0.5 mM IPTG and then washed and suspended to an optical density at 600 nm of 2.0 in M9 medium (17) containing 0.5 mM IPTG. The cell suspension was distributed by portions of 2 ml in 7-ml glass tubes covered with Teflon-lined screw caps. Each tube received 10 μl of a 50 mM acetone solution of the appropriate chlorobiphenyl substrate. They were incubated for 18 h at 37 °C with shaking. Cell suspensions were extracted at neutral pH with ethyl acetate. The metabolites were identified by gas chromatography-mass spectrometry (GC-MS) analyses of their butylboronate or trimethylsilyl derivatives (6, 19). In some cases, the dihydro-dihydropyridine metabolites were oxidized by IPTG-induced cell suspensions of E. coli [pQE31 bphB] (19) following a protocol similar to the one described above.

In some experiments, the metabolites were analyzed from catalytic oxygenation using reconstituted BPDO prepared from His-tagged purified enzymes components that were obtained following protocols published previously (2). Catalytic activities were determined from measurement of substrate depletion recorded by GC-MS analysis (15).

RESULTS

Screening for BPDO Variants Able to Oxygenate 2,2'-CB—To randomly mutate targeted residues of region III simultaneously, we used the approach described under "Experimental Procedures" and summarized in Fig. 3. A library of E. coli transformants carrying pQE31[bphAE] plus pYH31[bphFGBC] was generated. Transformants were grown on nitrocellulose membranes and exposed to the vapors of a chlorobiphenyl. The screening assay was based on the production of yellow chloro-HOPDA by these transformants. In a first set of experiments, we found that 20% of the transformants were able to oxygenate the easily oxidizable 4-CB; 50% of them were unable to transform 2,2'-CB into the yellow chloro-HOPDA. Therefore approximately half of the variants that remained active toward 4-CB contained (a) mutation(s) that caused a loss of activity toward 2,2'-CB.

70,000 colonies of the library were exposed to 2,2'-CB vapors. Among the ~7,000 yellow-colored colonies, only seven exhibited a clearly more intense color than control colonies expressing parental LB400 BPDO. Based on the assumption that variant exhibiting a higher turnover rate of reaction toward 2,2'-CB would produce larger amount of chloro-HOPDA from 2,2'-CB, we retained these colonies for further examination.

We also noticed that a fraction of the 70,000 transformants examined exhibited a pinkish gray color rather than the expected yellow color when exposed to 2,2'-CB. The latter were presumed to generate a metabolite that BphC was unable to cleave. Therefore, these variants were expected to have changed their regiospecificity toward 2,2'-CB because it has been established that catalytic oxygenation of this congener by LB400 BPDO produces as major metabolite 2,3-dihydroxy-2'-chlorobiphenyl (20, 21) that can be cleaved by BphC. These pinkish gray variants were also characterized.

All of the variants retained were examined for their capacity to degrade a mixture of 18 PCB congeners. Based on previous reports (20, 22–24), with the exception of 2,5,2',5'-CB and 2,3,2',3'-CB, all chlorobiphenyls of the mix are metabolized at a low rate by purified preparations of LB400 BPDO or by E. coli
clones expressing this enzyme. For this reason, under our experimental conditions the depletion levels of these congeners by IPTG-induced E. coli cells expressing LB400 BPDO was lower than 20% after 18 h of incubation. These levels of depletion were too low to report statistically significant variations and were therefore not reported precisely (Table I). The mix of 18 congeners did not contain 2,2'-CB because it was not discriminated from 2,6-CB by our GC-ECD chromatography setting, but its degradation was demonstrated in other experiments (see below).

**Characterization of Yellow-colored Variants**—Seven yellow transformants were confirmed to metabolize 2,2'-CB at a higher rate than LB400 BPDO. Suspensions of IPTG-induced cells of these E. coli clones metabolized 2,2'-CB at rates ranging from 3.0 to 3.5 nmol/h compared with 1.5 nmol/h for E. coli cells expressing LB400 BPDO. Sequence analysis of these clones indicated that most of them were single or double mutants for which the amino acid sequence patterns of region III were identical or very similar to the patterns of variants obtained by Mondello et al. (9). Three variants (y7, y8, and y9) exhibited the sequence pattern Ala<sup>335</sup>-Phe<sup>336</sup>-Asn<sup>338</sup>-Ile<sup>341</sup>, two variants (y1 and y5) exhibited the sequence pattern Gly<sup>335</sup>-Phe<sup>336</sup>-Asn<sup>338</sup>-Ile<sup>341</sup>, and two variants (y10 and y11) exhibited the sequence pattern Ala<sup>335</sup>-Leu<sup>336</sup>-Asn<sup>338</sup>-Ile<sup>341</sup>. With respect to the range of PCB substrates degraded, the data confirmed those of Mondello et al. (9) where the replacement of Thr<sup>335</sup> by a small hydrophobic amino acid (Ala or Gly) did not extend the range of PCB congeners used as substrate (not shown). Replacement of Thr<sup>335</sup>-Phe<sup>336</sup> simultaneously by Ala-Leu created variant y10, which was able to oxygenate more efficiently doubly para-substituted congeners such as 4,4'-CB and 2,2',4,4'-CB, confirming previous report (9). In addition, the y10 variant was able to oxygenate efficiently the doubly meta-substituted congeners such as 3,3'-CB that LB400 BPDO degraded poorly (Table I).

**Characterization of the Pinkish Gray-colored Variants**—Approximately 50 of the 70,000 clones examined for their ability to convert 2,2'-CB into chloro-HOPDA exhibited a pinkish gray
coloration. Seven were sequenced. Variant $p1$ was a triple mutant in which Thr$^{335}$-Phe$^{336}$-Ile$^{341}$ was replaced by Ala$^{335}$-Leu$^{336}$-Val$^{341}$. Variant $p8$ was a double mutant where Thr$^{335}$-Phe$^{336}$ was replaced by Ala$^{335}$-Ile$^{336}$. It is noteworthy that of the seven variants, five ($p2$, $p3$, $p4$, $p5$, and $p7$) were double mutants that shared an identical amino acid sequence pattern where Thr$^{335}$ was replaced by Ala and Phe$^{336}$ was replaced by Met.

Induced *E. coli* cell suspension expressing $p4$ BPDO metabolized 2,2'-CB at a rate of 1.5 nmol/h, which was similar to that of cells expressing LB400 BPDO. The rate of depletion of 2,2'-CB by cells expressing $p8$ BPDO was, however, much lower (0.38 nmol/h). Assays measuring the rate of depletion of 2,2'-CB by His-tagged purified preparations of $p4$ variant (Ala$^{335}$-Met$^{336}$) revealed that its specific activity toward 2,2'-CB was in the range of 70–90 nmol 2,2'-CB oxidized per min per mg enzyme compared with values of 35–55 nmol/min/mg LB400 BPDO. The specific activity of purified preparations of $p4$ BPDO was significantly higher than that of LB400 when biphenyl was the substrate. Values of 350–400 nmol biphenyl converted per min per mg $p4$ BPDO were obtained, compared with 120–150 nmol/min/mg LB400 BPDO. Therefore, the turnover rate of oxidation of biphenyl had increased significantly for this enzyme. It was also the case for several other PCB congeners that were oxygenated more efficiently by *E. coli* cells expressing $p4$ BPDO than those expressing LB400 BPDO (Table I). On the other hand, cells expressing $p8$ BPDO oxygenated 3,3'-CB more efficiently than those expressing LB400 BPDO, but overall, the range of chlorobiphenyls degraded was much smaller than for variant $p1$ and $p4$.

**Metabolism of 2,2'-CB by BPDO Variants—**Haddock et al. (20) reported that two metabolites were generated from oxygenation of 2,2'-CB by LB400 BPDO. The major one, representing about 90% of the product, was identified as 2,3-dihydroxy-2',2'-chlorobiphenyl. In a recent report (29) we identified the less abundant metabolite as cis-3,4-dihydroxy-3,4-dihydroxy-2,2'-dichlorobiphenyl.

When the pinkish gray variant $p4$ was used to catalyze the reaction, the ratio of the metabolites produced from 2,2'-CB differed significantly from the ratio obtained with LB400 BPDO. *E. coli* cells expressing $p4$ BPDO produced 30–40% of 2,3-dihydroxy-2',2'-chlorobiphenyl and 60–70% of cis-3,4-dihydroxy-2,2',dichlorobiphenyl from 2,2'-CB (Table II). In addition, there was a small amount (1–5%) of a dihydroxy-dichlorobiphenyl, which was confirmed to be 3,4-dihydroxy-2,2',dichlorobiphenyl (29).

The ratio of 2,3-dihydroxy-2'-chlorobiphenyl to cis-3,4-dihydroxy-3,4-dihydroxy-2,2'-dichlorobiphenyl was determined for all of the pinkish gray and yellow variants obtained in this study. The metabolite profile of all yellow variants was similar to that of LB400 BPDO (Table II). However, it had changed for all of the pinkish gray variants that generated a larger amount of cis-3,4-dihydroxy-2,2'-dichlorobiphenyl than LB400 BPDO. No other metabolites than 2,3-dihydroxy-2'-chlorobiphenyl, cis-3,4-dihydroxy-3,4-dihydroxy-2,2'-chlorobiphenyl, and 3,4-dihydroxy-2,2'-dichlorobiphenyl were produced from 2,2'-CB by any of these variants.

**Metabolism of Other PCB Congeners by Variant p4 BPDO—**Table III and Fig. 4 show the metabolite profile obtained from selected chlorobiphenyls when LB400 and $p4$ BPDOs were used to catalyze the oxygenation. The metabolites were identified by GC-MS analysis in comparison with the available data reported in the literature. The table also shows the relative rate of oxygenation of these congeners by $p4$ BPDO compared with that of LB400 BPDO.

LB400 BPDO oxygenates 3,3'-CB very poorly (20, 23). There are two possible sites of attack of 3,3'-CB (carbons 5 and 6 or carbons 4 and 5). In previous report (24), based on the analysis of metabolites generated from 3,3'-CB by coupled reactions involving the four enzymes of the biphenyl catabolic pathway, 5,6-dihydro-5,6-dihydroxy-3,3'-dichlorobiphenyl was identified as the major metabolite, and 4,5-dihydro-4,5-dihydroxy-3,3'-dichlorobiphenyl was identified as the minor one. This was confirmed using *C. testosteroni* B-356 BPDO. A purified preparation of B-356 BPDO produced a single metabolite from 3,3'-CB whose GC retention time and mass spectral features were identical to the major metabolite produced from LB400 BPDO (not shown), and this metabolite is converted to 3-chlorobenzoate by *C. testosteroni* B-356 cells (25). Variant $p4$ produced the same two metabolites as LB400 BPDO in much larger amounts. When 1 nmol of enzyme was used to catalyze the reaction, the area under the GC-MS peaks of metabolites generated by $p4$ BPDO after 2 min of incubation was 1000 times higher than corresponding peaks of metabolites produced by 1 nmol of LB400 BPDO during the same time.

Similarly, the mutation that occurred in $p4$ BPDO did not change the regiospecificity toward 2,4,3'-CB, 2,3,4'-CB, 2,4',4'-CB, and 2,4,2',4'-CB, but the rate of conversion of these congeners was faster when $p4$ was used to catalyze the reaction (Table III). Only one metabolite was produced from 2,4,2',4'-CB by both enzymes. Based on previous report (24) it was identified as 2,3-dihydroxy-4,4'-trichlorobiphenyl. Two metabolites were produced from 2,4,4'-CB. Its trichlorinated dihydro-dihydroxy derivative could not be identified precisely. However, based on the previous report (24) its dichlorinated derivative was identified as 2,3-dihydroxy-4,4'-dichlorobiphenyl, showing that a portion of the substrate was oxygenated on the ortho-meta carbons of the 2,4-chlorinated ring. Unlike 2,4,4'-CB neither LB400 nor $p4$ BPDO oxygenated 2,4,3'-CB on carbons 2 and 3 of the 2,4-chlorinated ring. Both enzymes generated two trichlorinated dihydro-dihydroxy metabolites that could not be identified precisely from available data. However, the metabolite profiles were identical whether the oxygenation was catalyzed by LB400 or $p4$ BPDO. Two trichlorinated dihydro-dihydroxy metabolites were also obtained from 2,3,4'-CB. Their precise identity was not determined, but an identical metabolite profile was obtained for both enzymes.

The metabolite profile and rate of metabolism of the ortho-
TABLE I

| Sequence patterns (variant designations) | Degradation of indicated congenera |
|-----------------------------------------|-----------------------------------|
|                                         | 2,6-CB | 3,4-CB | 4,4-CB | 2,4,3'-CB | 2,4,4'-CB | 2,5,2',5'-CB | 2,4,2',4' CB | 2,4,3',5'-CB | 2,3,4',5'-CB | 2,3,4',5'-CB | 2,3,4',5'-CB | 2,3,4',5'-CB | 2,3,4',5'-CB | 2,3,4',5'-CB |
| Thr\(^{335}\)-Phe\(^{336}\)-Asn\(^{337}\), Asn\(^{338}\)-Ile\(^{339}\)-Arg\(^{340}\)-Ile\(^{341}\) (LB400) | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| Ala\(^{335}\)-Leu\(^{336}\)-Asn\(^{337}\), Asn\(^{338}\)-Ile\(^{339}\)-Arg\(^{400}\)-Ile\(^{411}\) (y10 and y11) | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| Ala\(^{335}\)-Leu\(^{336}\)-Asn\(^{337}\), Asn\(^{338}\)-Ile\(^{339}\)-Arg\(^{410}\)-Val\(^{411}\) (p1) | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |
| Ala\(^{335}\)-Leu\(^{336}\)-Asn\(^{337}\), Asn\(^{338}\)-Ile\(^{339}\)-Arg\(^{410}\)-Ile\(^{411}\) (p2, p3, p4, p5, and p7) | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 20 |

a The values are the percentages of depletion of each PCB congener of a mix of 18 added to a culture of E. coli DH118/pYH31 [LB400-bphFGBC]/pQE31[bbphAE] expressing either one of the BphA variants. The protocol is described under “Experimental Procedures.” The values are the averages of results from three separate experiments done in triplicate. The variance was less than 10% of the measured value in all cases. The variance for depletion values 20% was too high to report statistically significant values. 2,6-CB, 2,4,3',5'-CB, 2,4,5,3'-CB were also present in the PCB mixture but not shown on the table because no significant depletion was recorded, and metabolites were not detected. 2,3,4,5,6,2',3',5',6'-CB was the internal standard and is also not shown in the table. Production of metabolites not confirmed.

b Production of metabolites not confirmed.
tural factors that influence the regiospecificity toward 2,2′-CB are not the same as those that determine the regiospecificity toward the homologous congener 2,6-CB.

**DISCUSSION**

In this work we have used the in vitro molecular evolution approach to examine the contribution to regiospecificity and substrate turnover of amino acid residues of BPDO a subunit region III. Screening was set up to select for variants exhibiting increased turnover rate or modified regiospecificity toward 2,2′-CB. When compared with LB400 BPDO, several of the selected variants had increased their rates of metabolism of several PCB congeners. In vitro molecular evolution is a very powerful approach to identify structural features associated to selected phenotypes. Many variants selected on the basis of the intensity of the yellow coloration produced from 2,2′-CB were similar or identical to those obtained previously (9) by exchanging residues of LB400 BphA by those of KF707 BphA1. The data suggest that the number of amino acid combinations of region III leading to the increased rate of oxygenation of 2,2′-CB accompanied by no change of regiospecificity are rather limited. The diversity of sequence patterns of region III of variants exhibiting a change in regiospecificity toward 2,2′-CB was also low, but two of the three sequence patterns analyzed conferred an increase of the turnover rate of oxidation toward a range of chlorophenyls.

Engineering BPDOs to catalyze the oxygenation of PCBs is a formidable task. The enzyme has to learn to catalyze the oxygenation of an array of structurally distinct compounds, which is determined by the position of their chlorine substitutes. Thus some congeners can take a co-planar configuration; others, the ones that are doubly ortho-substituted on the same ring, can never be co-planar. Regiospecificity toward each chlorobiphenyl is directed by its chlorine substitution pattern, through interaction between the chlorine atoms and specific amino acid residues of the protein. The identity of these amino acid residues and their interactions with various chlorobiphenyl congeners is still unknown. However, several amino acids of the region III of BphA and especially Thr335-Phe336 as being critical for enzyme turnover rate and regiospecificity toward 2,2′-dichlorobiphenyl; diCldiol, cis-3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl.

| BPDO   | Sequence pattern                  | Ratio of monoCldiolOH to diCldiol |
|--------|----------------------------------|----------------------------------|
| LB400  | Thr335-Phe336-Asn337,Asn338-Ile339,Arg340-Ile341 | 90/10                            |
| y1 and y5 | Gly335-Phe336-Asn337,Asn338-Ile339,Arg340-Ile341 | 80/20                            |
| y7, y8, and y9 | Ala335,Phe336-Asn337,Asn338-Ile339,Arg340-Ile341 | 85/15                            |
| y10 and y11 | Ala335,Leu336-Asn337,Asn338,Ile339,Arg340-Ile341 | 85/15                            |
| p1     | Ala335,Leu336-Asn337,Asn338,Ile339,Arg340-Val341 | 40/60                            |
| p2, p3, p4, p5, and p7 | Ala335,Met336-Asn337,Asn338,Ile339,Arg340-Ile341 | 40/60                            |
| p8     | Ala335,Ile336,Asn337,Asn338,Ile339,Arg340-Ile341 | 40/60                            |

*The ratios were determined from the relative amounts each metabolite produced in the culture media of *E. coli* cells expressing the indicated BPDO and are based on the area under the peak of each metabolite obtained from GC-MS analysis. monoCldiolOH, 2,3-dihydroxy-2′-chlorobiphenyl; diCldiol, cis-3,4-dihydro-3,4-dihydroxy-2,2′-dichlorobiphenyl.

In this work we have used the in vitro molecular evolution approach to examine the contribution to regiospecificity and substrate turnover of amino acid residues of BPDO a subunit region III. Screening was set up to select for variants exhibiting increased turnover rate or modified regiospecificity toward 2,2′-CB. When compared with LB400 BPDO, several of the selected variants had increased their rates of metabolism of several PCB congeners. In vitro molecular evolution is a very powerful approach to identify structural features associated to selected phenotypes. Many variants selected on the basis of the intensity of the yellow coloration produced from 2,2′-CB were similar or identical to those obtained previously (9) by exchanging residues of LB400 BphA by those of KF707 BphA1. The data suggest that the number of amino acid combinations of region III leading to the increased rate of oxygenation of 2,2′-CB accompanied by no change of regiospecificity are rather limited. The diversity of sequence patterns of region III of variants exhibiting a change in regiospecificity toward 2,2′-CB was also low, but two of the three sequence patterns analyzed conferred an increase of the turnover rate of oxidation toward a range of chlorophenyls.

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| Congener | Activity of p4 relative to LB400 BPDO<sup>a</sup> | Metabolite ratio<sup>b</sup> | Metabolite features | Comments |
|----------|-----------------------------------------------|-----------------------------|---------------------|----------|
| 3,3'-CB  | 1000                                         | 90                          | 5,6-dihydro-5,6-dihydroxy-3,3'-dichlorobiphenyl | 322, 287, 265, 222 Based on Ref. 24 |
| 2,3,4'-CB| 100                                          | 95                          | 4,5-dihydro-4,5-dihydroxy-3,3'-dichlorobiphenyl | 322, 287, 265, 222 Based on Ref. 24 |
| 2,4,3'-CB| 100                                          | 80                          | dihydro-dihydroxy-trichlorobiphenyl           | 356, 321, 299, 256 OH position unknown |
| 2,4,4'-CB| 10                                           | 70                          | dihydro-dihydroxy-trichlorobiphenyl           | 356, 321, 299, 256 OH position unknown |
| 2,4,2',4'-CB| 100                                         | 10                          | 2,3-dihydroxy-4,4'-dichlorobiphenyl           | 320, 264 Based on Ref. 24 |
| 2,5,2',5'-CB| 1                                           | 100                         | 2,3-dihydroxy-2,3-dichlorobiphenyl            | 354, 298 Based on Ref. 24 |
| 2,3,4',2',5'-CB| 1                                         | 100                         | 3,4-dihydro-3,4-dihydroxy-2,5,2',5'-tetrachlorobiphenyl | 390, 355, 333, 290 Based on Refs. 20 and 22 |
| 2,3,3',3'-CB| 1                                         | 90                          | 4,5-dihydro-4,5-dihydroxy-2,3,2',3'-tetrachlorobiphenyl | 424, 389, 367, 324 Assuming an hydroxylation on carbons 3 and 4 |
| 2,4,3',4'-CB<sup>d</sup> | ND<sup>e</sup> | ND                          | 5,6-dihydro-5,6-dihydroxy-2,3,2',3'-tetrachlorobiphenyl | 390, 355, 333, 290 Based on NMR analysis (29) |
| 2,6-CB<sup>f</sup> | ND<sup>e</sup> | ND                          | 2,3-dihydroxy-6-chlorobiphenyl                | 286, 230 Assuming dehalogenation during attack on carbons 2 and 3 |

<sup>a</sup> The activity of p4 BPDO relative to LB400 BPDO was calculated from the ratio of the sum of area under the peaks of all metabolites produced from each congener after 2 min of incubation with 1 nmol of enzyme.

<sup>b</sup> Based on GC-MS analyses of butylboronate-derived metabolites generated by His-tagged purified LB400 or p4 BPDOs. ND, no metabolite detected.

<sup>c</sup> Mass spectral features of the butylboronate-derived metabolite.

<sup>d</sup> Metabolites were obtained with IPTG-induced E. coli cells expressing LB400 or p4 BPDO.

<sup>e</sup> ND, not determined because no metabolite produced with LB400 BPDO.

<sup>f</sup> Spectral features of the trimethylsilyl-derived metabolite.
ferred site of attack of 2,2′-CB by p4 BPDO, the single metabolite obtained from 2,4,2′,4′-CB was the same as the one obtained when LB400 BPDO catalyzed the reaction, and it was identified as a trichlorinated dihydroxybiphenyl. On the other hand, 2,3,2′,3′-CB was oxygenated principally on the meta-meta carbons by LB400 BPDO. The mutation that occurred in p4 did not alter the regiospecificity of the enzyme toward this substrate. It is noteworthy that the two symmetrical ortho-meta-substituted congeners 2,5,2′,5′-CB and 2,3,2′,3′-CB are both oxygenated principally on meta-para carbons by both LB400 and p4 BPDO. Altogether, data suggest that despite the fact that residues 335 and 336 exert a strong influence on the regiospecificity toward 2,2′-CB and on the turnover rate toward several congeners, the pattern of chlorine substitution on the PCB substrate imposes its orientation toward the catalytic active center. We will need to wait for the crystal structure of the enzyme to understand how the residues of region III interact with individual congeners to influence their rate of oxygenation and, for some of them, their site of oxygenation. However, by analogy with cytochrome P450cam, it is likely that hydrophobic interactions or hydrogen bonding between the chlorine substitutes on the biphenyl skeleton and specific amino acid residues of the protein influence the orientation of the biphenyl ring inside the catalytic pocket. The identification of the amino acid residues involved in these interactions are mandatory to design strategies to broaden the range of PCB congeners that the enzyme can oxygenate efficiently.

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Evolution of the Biphenyl Dioxygenase BphA from *Burkholderia xenovorans* LB400 by Random Mutagenesis of Multiple Sites in Region III

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*J. Biol. Chem. 2004, 279:47480-47488.*

doi: 10.1074/jbc.M406805200 originally published online August 31, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406805200

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