

# Tuning Biphenyl Dioxygenase for Extended Substrate Specificity

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Received 3 September 1998; accepted 15 November 1998

**Abstract:** Highly substituted polychlorinated biphenyls (PCBs) are known to be very resistant to aerobic biodegradation, particularly the initial attack by biphenyl dioxygenase. Functional evolution of the substrate specificity of biphenyl dioxygenase was demonstrated by DNA shuffling and staggered extension process (StEP) of the *bphA* gene coding for the large subunit of biphenyl dioxygenase. Several variants with an extended substrate range for PCBs were selected. In contrast to the parental biphenyl dioxygenases from *Burkholderia cepacia* LB400 and *Pseudomonas pseudoalcaligenes* KF707, which preferentially recognize either *ortho*- (LB400) or *para*- (KF707) substituted PCBs, several variants degraded both congeners to about the same extent. These variants also exhibited superior degradation capabilities toward several tetra- and pentachlorinated PCBs as well as commercial PCB mixtures, such as Aroclor 1242 or Aroclor 1254. Sequence analysis confirmed that most variants contained at least four to six template switches. All desired variants contained the Thr335Ala and Phe336Ile substitutions confirming the importance of this critical region in substrate specificity. These results suggest that the block-exchange nature of gene shuffling between a diverse class of dioxygenases may be the most useful approach for breeding novel dioxygenases for PCB degradation in the desired direction. © 1999 John Wiley & Sons, Inc. *Biotechnol Bioeng* 63: 544–551, 1999.

**Keywords:** random mutagenesis; DNA shuffling; staggered extension process; polychlorinated biphenyls (PCBs); biphenyl dioxygenase

## INTRODUCTION

PCBs are among the most widely identified organic contaminants found in environmental samples (Safe, 1992). Although PCBs are commonly considered as highly recalcitrant compounds, photochemical and microbial processes are known to contribute to the slow removal of PCBs from the environment. Microbial metabolism of PCBs occurs under both aerobic (dioxygenation) and anaerobic (dechlorination) conditions. Even though bacteria capable of degrading PCBs aerobically are known, these organisms generally have a narrow congener specificity and are able to degrade only lightly chlorinated PCBs (Minshull, 1995).

Dioxygenation of PCBs is the first enzymatic step in aerobic biodegradation. Biphenyl dioxygenase, being the first enzyme involved, plays a crucial role in the degradation of PCBs (Gibson et al., 1993). By catalyzing the incorporation of two hydroxyl groups into the aromatic ring, this enzyme increases the reactivity of PCBs, rendering them more susceptible to enzymatic ring fission reactions. Many highly chlorinated PCB congeners are resistant to aerobic biodegradation because of the inability of biphenyl dioxygenase to accept them as substrates.

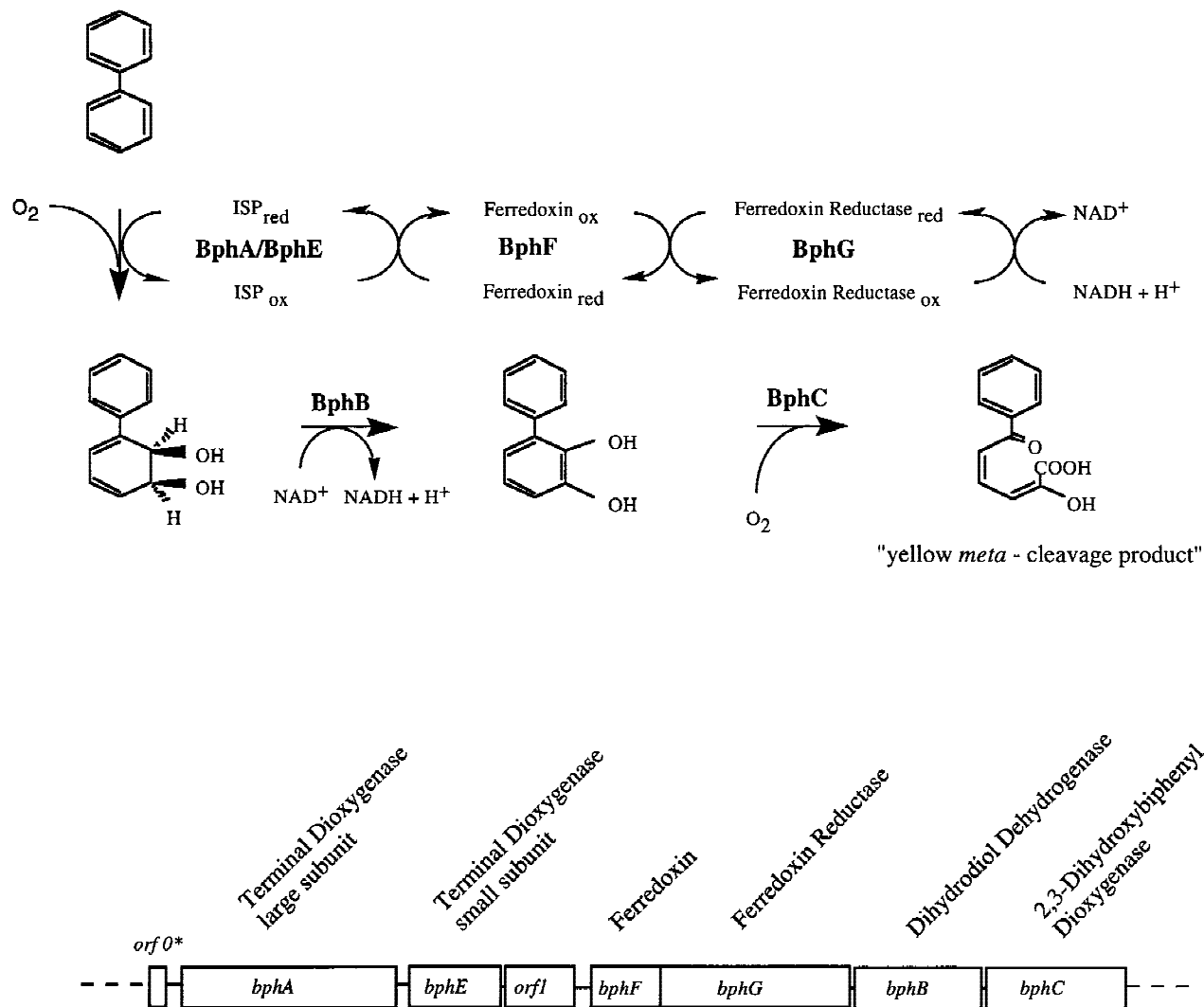
Biphenyl dioxygenase is a multicomponent enzyme consisting of a terminal dioxygenase (large and small subunit), ferredoxin, and ferredoxin reductase encoded by the *bph* operon (Erickson and Mondello, 1992; Haddock et al., 1993) (Fig. 1). However, only the large subunit (BphA) is primarily involved in substrate recognition (Erickson and Mondello, 1993; Furukawa et al., 1993; Hirose et al., 1994). Despite the high degree of similarity among the *bphA* genes, substrate specificity differs drastically. For example, the BphA protein for *Burkholderia cepacia* LB400 (LB400) and *Pseudomonas pseudoalcaligenes* KF707 (KF707) differ at only 20 positions, and yet the biphenyl dioxygenase from LB400 preferentially dioxygenates *ortho*-substituted PCBs whereas the biphenyl dioxygenase from KF707 preferentially attacks *para*-substituted PCBs (Erickson and Mondello, 1993; Gibson et al., 1993), indicating that the relatively few amino acid exchanges are responsible for the different substrate specificities.

Because very different substrate specificities have been observed for a relatively similar population of biphenyl dioxygenases, it is easy to envision that biphenyl dioxygenase can be modified *in vitro* for extended congener specificity. Site-directed mutagenesis has been used to generate recombinant biphenyl dioxygenases with extended substrate ranges (Erickson and Mondello, 1993; Kimura et al., 1997; Mondello et al., 1997). However, in the absence of detailed structural information, identifying all the amino acids responsible for congener specificity and those that might give rise to extended specificity remains an overwhelming challenge. *In vitro* directed evolution is perhaps the most useful way of evolving PCB substrate specificity, because microbial degradation is usually done by cometabolism and does not exert a selective pressure on bacteria.

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Contract grant sponsors: Office of Naval Research; National Science Foundation

Contract grant numbers: N00014-96-1-0599; BES-9700981



**Figure 1.** Top: Dioxygenation of biphenyl and ring fission mediated by enzymes of the *bph* operon (Kimura et al., 1997; Mondello et al., 1997). Only the reactions leading to the yellow *meta*-cleavage product are shown. ISP, iron sulfur protein of the large and small subunit of the terminal dioxygenase. The redox state of the different proteins is indicated (ox, oxidized; red, reduced). Bottom: Genes of the *bph* operon as utilized in vectors for the screening of *bphA* variants. Open reading frame *orf0\** is a truncated form of *orf0*. Boxes and solid lines indicate coding regions and noncoding regions, respectively. The function of *orf0* and *orf1* is unknown. The dotted line indicates plasmid DNA. For details see Materials and Methods.

“Irrational” design approaches such as DNA shuffling (Kumamaru et al., 1998) followed by screening have been demonstrated to be useful in identifying several chimeric biphenyl dioxygenases with altered activity. Surprisingly, no single variant was found to exhibit the combined capability of both parental clones toward the formation of *meta*-cleavage products from double *ortho*- and *para*-substituted PCBs, perhaps because of the arbitrary nature of their screening procedure. It is possible that, when combined with focused selection, DNA shuffling will be useful in guiding the extension of PCB substrate specificity in any desired direction. In this study, DNA shuffling (Stemmer, 1994) and the staggered extension process (StEP) (Zhao, 1998) were used to demonstrate the tuning of biphenyl dioxygenase with equal activity toward both *para*- and *ortho*-substituted PCBs.

## MATERIALS AND METHODS

### Strains and Plasmids

*Escherichia coli* JM105 was used in all experiments. *E. coli* strain FM4119 harboring the *bph* operon from *Burkholderia cepacia* LB400 in pUC18 was kindly provided by F. Mondello. Plasmid pKT18 containing *bphA1A2A3A4* of *Pseudomonas pseudoalcaligenes* KF707 was kindly provided by D. Focht. Plasmid pCAC11 contained the *bphAEFGBC* genes from LB400 on a *NsiI*–*EcoRI* fragment ligated into the *PstI* and *EcoRI* site of pUC119. Plasmid pCEC11 contained the *bphEFGBC* genes from LB400 on an *EcoRI*–*SacI* fragment, which was blunt end-ligated into the *SmaI* site of the pUC18 cloning vector (Yanisch-Perron et al., 1985) after treatment with the large Klenow fragment of DNA poly-

merase I. Plasmid pCEC31 is a derivative of the low copy number plasmid pK184 (Jobling and Holmes, 1990), harboring the *EcoRI*–*HindIII* insert of pCEC11 with the *bphE*–*FGBC* genes from the LB400 strain. Plasmid pPA400K was constructed by ligating an *EcoRI*–*KpnI* PCR fragment of the *bphA* gene from LB400 into pK184. Plasmid pPA707K is derived from pK184 carrying an *EcoRI*–*KpnI* PCR fragment of the *bphA* gene from KF707. Amplified *bphA* DNA from both strains was obtained by PCR with the following primers: 5′-GGGAATTCGGTTTGTGCGGTGACGCCA-3′ (introduced *EcoRI* site underlined) and 5′-GTTGGTACCTTCCGGTTGACAGATCTAA-3′ (introduced *KpnI* site underlined).

### Random Mutagenesis

If not otherwise stated, general procedures for the isolation, analysis, and manipulation of DNA fragments were carried out as described (Sambrook et al., 1989). Error-prone PCR in the presence of 0.5 mM manganese was performed using pPA400K as the DNA template as reported (Leung et al., 1989). Polymerase chain reactions were performed in an MJ Research (Watertown, MA) PTC-100 thermocycler. DNA shuffling was carried out as described elsewhere (Cramer et al., 1996). DNA fragments containing the *bphA* gene from LB400 and KF707 to be treated with DNase I were obtained by PCR. Plasmids pPA400K and pPA707K served as template DNA. Two primers (DE194: 5′-TGCGGGCCTCTTCGCTATTA-3′ and UH194: 5′-CCCCAGGCTTTACACTTAT-3′) were used, flanking the inserts by approximately 100 bp. Two micrograms of each amplified DNA was purified by phenol and chloroform extraction prior to digestion with 0.02 U of DNase I (Boehringer Mannheim) at 15°C for 2 min. The reaction was stopped by heating at 90°C for 10 min. DNA fragments of less than 100 bp were isolated from a 2% agarose gel with DEAE cellulose and subsequently purified by extraction with phenol and chloroform. Approximately 1 µg of DNA fragments of each *bphA* gene was mixed together and reassembled in 50 µL of a primerless PCR reaction using EasyStart (Molecular Bio-Products, San Diego, CA) and *Taq*-DNA polymerase (Perkin-Elmer Cetus, Emeryville, CA). A PCR program of 5 min 94°C, 45 cycles of 1 min 94°C, 1 min 45°C, and 1.5 min 72°C, followed by 10 min at 72°C, was used. After a 1:40 dilution of the primerless PCR products, DNA amplification was carried out in the presence of 0.4 µM M13/pUC sequencing primer and M13/pUC reverse sequencing primer using the following PCR program: 5 min 95°C, 35 cycles of 1 min 95°C, 1 min 51°C, and 2 min 72°C, followed by 10 min at 72°C. The second PCR resulted in a distinct fragment of 1.5 kb. Staggered extension process was carried out as described (Zhao et al., 1998), except that EasyStart PCR mixes (Molecular Bio-Products) were used in the following PCR program: 5 min 95°C, 80 cycles of 30 s 94°C, and 5 s 55°C (alternatively 4 s 55°C was also used). Plasmids pA400K and pA707K served as the template DNA using the aforementioned primers DE194 and UH194. The resulting 1.7-kb

DNA fragment was isolated from a 1% agarose gel using a GeneClean III kit (Bio 101 Inc., La Jolla, CA).

### Cloning and Selection

The PCR products obtained from DNA shuffling and staggered extension process were purified by gel isolation or by extraction with phenol and chloroform. The purified DNA fragments were digested with *EcoRI* and *KpnI* and ligated into pCEC31 previously cut with the same enzymes. Transformation of *E. coli* JM105 was carried out by heat shock of CaCl<sub>2</sub> competent cells. After transformation, cells were plated on M9 medium supplemented with 0.2% casamino acids, 0.2% tryptone, and 0.2% yeast extract. After an overnight incubation at 37°C, a piece of filter paper, soaked with 100 µL of a 0.1% solution of biphenyl (Sigma) or PCBs (AccuStandard, New Haven, CT) in ether, was inserted into the lid of the Petri dish with the transformants to be selected. After 20-h incubation at 37°C transformants were selected based on their ability to produce the yellow *meta*-cleavage product from PCBs. For quantitative analysis, cells were grown at 37°C in 1 mL of the supplemented M9 medium in the presence of 20 µg mL<sup>-1</sup> kanamycin until an OD<sub>600</sub> of 0.8 was reached. Cultures were then induced with 1 mM IPTG and incubated for another 2 h. Five microliters of 100 mM biphenyl or PCB solution in ether was then added to give a final concentration of 0.5 mM. Absorbance was measured after 1 h for biphenyl and after an overnight incubation for the various PCB congeners at 434 nm for 4,4′-CB or 395 nm for 2,2′,5-CB.

### DNA Sequencing

DNA sequencing was carried out by the dideoxynucleotide method using the fmol DNA sequencing system from Promega (Madison, WI). Alternatively, automated dye terminator DNA sequencing was performed on a Applied Biosystem 373A sequencer. Small-scale isolation of plasmid DNA for sequencing was carried out with the Wizard Mini-prep DNA purification system (Promega).

### Resting Cell Assays

*Escherichia coli* cells harboring the *bphA*–*AEFGBC* genes in pK184 were grown overnight on LB plates supplemented with 20 µg mL<sup>-1</sup> kanamycin at 37°C. Cells were scraped from agar plates and suspended in 50 mM Tris (pH 7.5). The cell suspension was centrifuged at 5000 rpm for 10 min and the cell pellet resuspended in fresh 50 mM Tris (pH 7.5) to give an OD<sub>600</sub> of 1.0. One half milliliter of the cell suspension was incubated with 20 ppm of Aroclor 1242 or Aroclor 1254 (AccuStandard) or a synthetic PCB mix (each congener at about 1 ppm). Incubations were carried out in sealed 4.5-mL glass vials on a rotary shaker at 150 rpm and 30°C for 2 days. PCBs were extracted twice with 0.3 mL of hexane. The combined hexane extracts were analyzed on a Shimadzu gas chromatograph/mass spectrometer QP-5000

(Shimadzu Corp., Kyoto, Japan) equipped with a splitter injector, operated at 260°C. A capillary column (RTX 5) of 30 m, with an inner diameter of 0.25 mm, was used (Restek, Bellefonte, PA). The temperature program ran from 100°C to 250°C using the following ramping conditions: 100°C for 2 min, 6°C min<sup>-1</sup> to 220°C; 220°C for 10 min, 20°C min<sup>-1</sup> to 250°C; 250°C for 5 min. The internal standard 2,2',4,4',6,6'-hexachlorobiphenyl was added to the Aroclors and the synthetic PCB mix at 1.5 ppm.

## RESULTS AND DISCUSSION

### Experimental Strategy for Evolving Biphenyl Dioxygenase

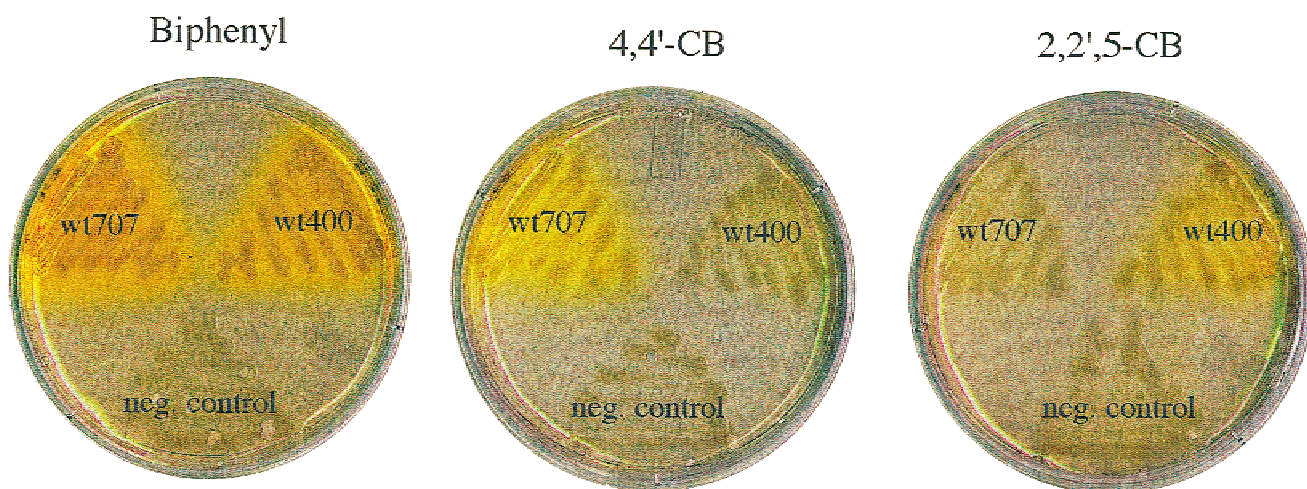
Molecular evolution requires the screening of a large number of variants because most mutations or recombinations are either neutral or deleterious. In this regard, colorimetric (visual) assays are convenient for rapid and sensitive screening. A plate assay based on the formation of the yellow *meta*-cleavage products from PCBs was developed for the selection of biphenyl dioxygenase with extended substrate specificity. Initially, *E. coli* cells harboring plasmid pCAC11, a pUC119 derivative carrying the *bphAEFGBC* genes from *Burkholderia cepacia* LB400 on an *Nsi*I–*Sac*I fragment, was shown to turn yellow on agar plates after exposure to biphenyl vapor. Subsequently, the formation of yellow *meta*-cleavage products was observed for PCBs (2-CB, 2,3-CB, and 2,2',5-CB) that are known to be highly degradable by the LB400 biphenyl dioxygenase. No color formation was observed for congeners that are degraded only very slowly and congeners that are not degradable at all by the LB400 dioxygenase. More importantly, simply by replacing the *bphA* fragment of LB400 with that from *Pseudomonas pseudoalcaligenes* KF707, yellow *meta*-cleavage products were detected with 4,4'-CB, a congener hardly degraded by LB400 (Fig. 2). A similar assay was also de-

veloped for growing cultures in supplemented M9 medium. The extent of PCB degradation was quantified by measuring the production of the yellow *meta*-cleavage products.

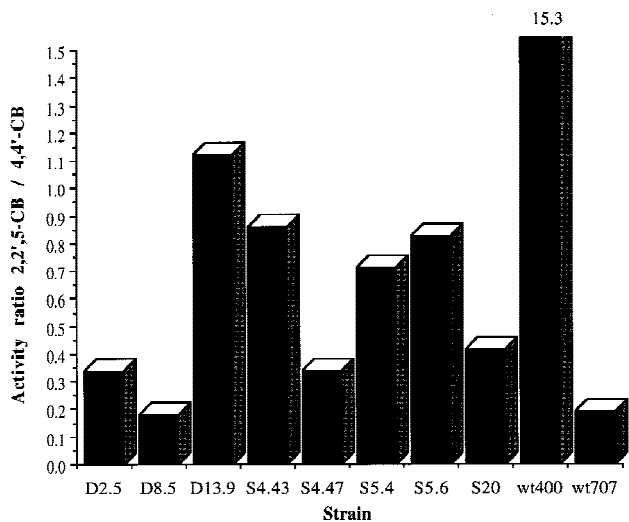
The biphenyl dioxygenase from the LB400 strain is capable of attacking a broad spectrum of PCB congeners, up to and including some hexachlorobiphenyls, but hardly degrades double-*para*-substituted congeners (Bedard et al., 1986; Bopp, 1986). Due to its broad substrate specificity, it was used as the starting template for generating variant enzymes that can act on an extended set of PCB congeners. Because substrate specificity is determined mainly by the *bphA* region, only this gene fragment was subjected to directed evolution. Initial attempts were to extend degradation toward both double-*ortho*- and *para*-substituted congeners. PCR-amplified *bphA* fragments were introduced into pCEC31, a low-copynumber construct based on pK184 (Jobling and Holmes, 1990) harboring the *bphEFGBC* genes from LB400. The pK184-based construct was chosen because it proved more stable in *E. coli* cells than did pUC-based constructs. Transformants were grown on supplemented M9 plates. Supplementation with yeast extract, tryptone, and casamino acids promoted growth and biphenyl-degrading activity of *E. coli* cells without increasing the background color, enabling easy identification of active clones. Plates were then exposed to vapors of 4,4'-CB or 2,2',5-CB at 37°C for about 15 h. Variants were selected based on their ability to generate the yellow *meta*-cleavage product from both 4,4'- and 2,2',5-substituted congeners. Positive clones were rescreened in liquid culture with 4,4'-CB and 2,2',5-CB, respectively. The formation of the yellow *meta*-cleavage product from these two PCB congeners was measured spectrophotometrically at 434 nm (4,4'-CB) and 395 nm (2,2',5-CB), respectively.

### Random Mutagenesis and Selection

Initially, error-prone PCR with mutational frequencies varying from 0.2% to 2% was attempted to create mutant librar-



**Figure 2.** Formation of the yellow *meta*-cleavage product from biphenyl, 4,4'-CB, and 2,2',5-CB by *E. coli* strain wt400 and wt707 grown on agar plates. In both strains, the *bphEFGBC* genes from *Burkholderia cepacia* LB400 are complemented either by the *bphA* gene from the same organism (wt400) or *Pseudomonas pseudoalcaligenes* KF707 (wt707). An *E. coli* strain expressing only the *bphEFGBC* genes from LB400 served as the negative control.



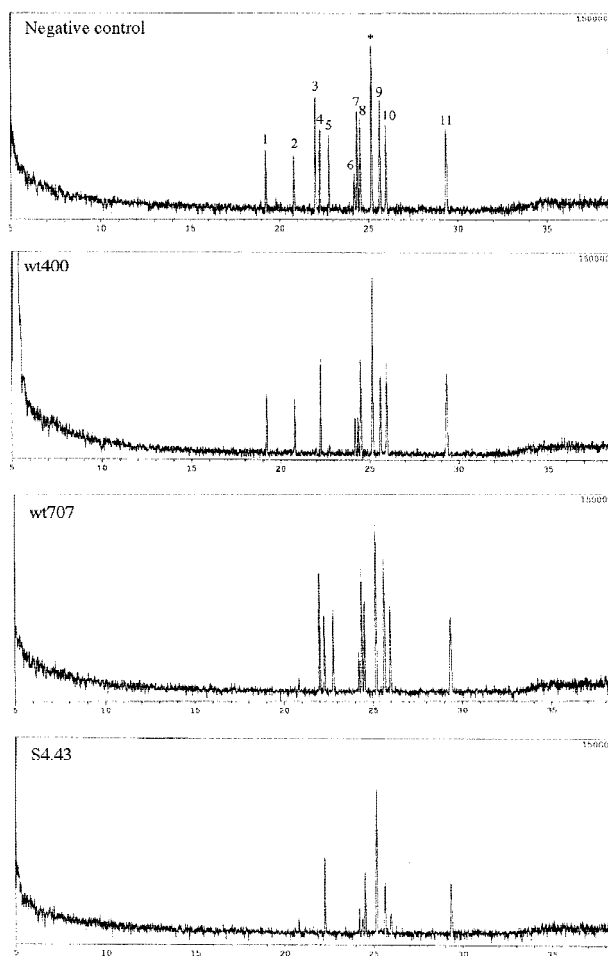
**Figure 3.** Cells of *E. coli* grown in liquid medium expressing chimeric forms of the *bphA* gene exhibit different activities for 2,2',5-CB and 4,4'-CB. The activity ratio for these two PCB congeners is shown for several mutants. Activity for 2,2',5-CB and 4,4'-CB was estimated by measuring absorbance of the corresponding yellow *meta*-cleavage in the cell-free culture supernatant. Strains with the prefixes "D" or "S" were derived from DNA shuffling and staggered extension process (StEP), respectively. Strain wt400 and wt707 express the corresponding wild-type forms of the *bphA* gene from *Burkholderia cepacia* LB400 and *Pseudomonas pseudoalcaligenes* KF707. Activities were calculated from three independent experiments.

ies of the *bphA* gene from LB400 using pCEC31 as the vector. However, screening of approximately 24,000 transformant did not result in the identification of clones with enhanced activity toward 4,4'-CB. In principle, a single point mutation (T-to-A transversion) at position 1006 would have changed a phenylalanine residue at position 336 into isoleucine, a mutation shown by site-directed mutagenesis to generate enhanced activity for 4,4'-CB (Mondello et al., 1997). However, it is possible that a strong bias for transition over transversion mutations (Cadwell and Joyce, 1992) lowered the probability of generating this particular mutation. DNA shuffling and staggered extension process (StEP) were subsequently used to generate chimeric *bphA* genes derived from the parental *bphA* gene from LB400 and KF707. Initially, all colonies that turned yellow when exposed to 4,4'-CB and 2,2',5-CB vapor were selected as potential positive variants and confirmed with degradation of 2,2',5- and 4,4'-CB in the liquid medium assay. Any transformant with an activity ratio (2,2',5-CB vs. 4,4'-CB) larger than 0.2 (wt707) but smaller than 15.3 (wt400) is indicative of a change in substrate specificity. Of these, several transformants exhibited elevated activities toward 4,4'-CB while maintaining high activity against 2,2',5-CB (Fig. 3). Five variants (D13.9, S4.43, S5.4, S5.6) in particular demonstrated a four- to fivefold improvement in their ability to attack both *para*- and *ortho*-substituted congeners, as indicated by an activity ratio close to one.

### Degradation of PCBs

We were interested in investigating whether any of the variants selected based on their ability to degrade both *para*-

and *ortho*-substituted congeners could also act on other new congeners, because an unexpected "gain in function" is not unusual during any in vitro evolution process. The degradation of Aroclor 1242 and Aroclor 1254 was, therefore, studied for several variants in resting cell assays. Several variants showed degradation capabilities superior to both the wild-type enzymes, suggesting that they may have acquired novel activities against other congeners during shuffling (data not shown). Because many of the peaks in the chromatograms have contributions from more than one PCB congener, the precise differences in substrate specificity among the different variants were somewhat difficult to determine. To address this shortcoming with the Aroclors, a synthetic mixture of PCBs similar to those proposed by Bedard et al. (1986) was used to differentiate more specifically between the degradation capabilities among the wild-types and the variants. A comparative degradation of the



**Figure 4.** Degradation of a synthetic mix of PCB congeners by resting cells of *E. coli* expressing the wild-type forms and the chimeric biphenyl dioxygenase of variant S4.43, respectively. A strain expressing only the *bphEFGBC* genes from *Burkholderia cepacia* LB400 served as the negative control. The internal standard (2,2',4,4',6,6'-CB) is marked with an asterisk. Peak numbers refer to the following PCB congeners: (1) 4,4'-CB; (2) 2,4,4'-CB; (3) 2,2',5,5'-CB; (4) 2,2',4,4'-CB; (5) 2,2',3,5'-CB; (6) 2,4,4',5-CB; (7) 2,3',4',5-CB; (8) 2,3',4,4'-CB; (9) 2,2',4,5,5'-CB; (10) 3,3',4,5'-CB; and (11) 2,3',4,4',5-CB.

**Table I.** Percentage degradation of PCB congeners in a synthetic mixture by different variants.

PCB	wt400	wt707	D13.9	S4.43	S4.47	S5.4	S5.6	S20
4,4'	15 (7)	95 (5)	10 (7)	100 (0)	100 (0)	93 (8)	100 (0)	97 (5)
2,4,4'	17 (12)	95 (5)	13 (15)	95 (7)	100 (0)	85 (10)	100 (0)	90 (9)
2,2',5,5'	96 (5)	3 (3)	28 (7)	99 (2)	100 (0)	56 (8)	59 (7)	23 (6)
2,2',4,4'	12 (8)	1 (3)	10 (7)	24 (11)	9 (9)	8 (10)	10 (7)	1 (2)
2,2',3,5'	97 (5)	6 (9)	16 (11)	99 (2)	92 (9)	61 (9)	65 (10)	33 (12)
2,4,4',5	4 (7)	5 (6)	16 (13)	33 (12)	36 (7)	19 (19)	27 (18)	7 (9)
2,3',4',5	68 (5)	0 (0)	27 (6)	91 (9)	79 (7)	56 (9)	60 (8)	15 (6)
2,3',4,4'	4 (5)	4 (6)	8 (9)	25 (16)	14 (4)	24 (5)	28 (9)	14 (7)
2,2',4,5,5'	37 (7)	5 (10)	5 (5)	61 (22)	50 (5)	26 (12)	30 (13)	5 (4)
3,3',4,5'	1 (1)	0 (0)	11 (16)	62 (22)	48 (1)	25 (10)	34 (12)	15 (10)
2,3',4,4',5	1 (2)	0 (0)	13 (12)	13 (12)	9 (9)	2 (3)	17 (5)	19 (11)

Standard deviations (in parentheses) were calculated from five different experiments. Resting cell assays were performed as described in Materials and Methods.

synthetic PCB mix by the wild-type and one of the variant biphenyl dioxygenases is shown in Figure 4. Most variants also exhibited enhanced activities against tetra- and pentachlorinated congeners. One variant (S4.43) in particular showed dramatically improved degradation toward these highly chlorinated PCBs. The percentage degradation of specific PCB congeners in the synthetic mix is shown in Table I.

### Sequence Analysis

To understand the molecular basis for the extended substrate range among the BphA variants, the *bphA* portion for several clones was sequenced. Of particular interest were region III (aa 335–341, numbers according to the LB400 sequence) and region IV (Asn377 in LB400), which has been suggested to be critical for substrate specificity (Kimura et al., 1997; Mondello et al., 1997). Even though the extent of template switching is somewhat difficult to define exactly because of the high degree of similarity between the two parental genes, most of the variants of interest contained between four and six crossovers. Although different crossovers occurred, as depicted in Figure 5, all variants with enhanced degradation capabilities contained a template crossover within region III of LB400 and KF707, confirming the importance of this region for substrate specificity. The sequence of variant S20 and S4.47 has region III identical to KF707 and region IV identical to LB400. This sequence combination has been shown by site-directed mutagenesis to result in a biphenyl dioxygenase which recognizes a 2,5-dichlorinated ring as well as a 4'-chlorinated ring (Kimura et al., 1997). More importantly, the substitutions from Thr335 and Phe336 into two smaller, more hydrophobic amino acids, Ala and Ile, respectively, are common to all variants of interest. The presence of only these two substitutions (as in the case for variant S4.43) seems sufficient to enhance the interaction of di-*para*-substituted congeners. This effect may be due to increased local hydrophobicity or by reducing steric hindrance, in line with the observations from site-directed mutagenesis of the LB400

biphenyl dioxygenase, where the Phe336Ile substitution alone dramatically increased activity toward both 4,4'- and 2,4,4'-CB (Mondello et al., 1997).

The three-dimensional structure of biphenyl dioxygenase is not known at present. However, the structure of a similar three-component naphthalene dioxygenase from *Pseudomonas* sp. NCIB 9816-4, which shares about 33% sequence identity at the protein level with the large subunit of the LB400 biphenyl dioxygenase, was recently determined (Kauppi et al., 1998). It is of interest to note that the two important residues of region III, Thr335 and Phe336, align structurally with Cys309 and Ser310 of naphthalene dioxygenase. This suggests that these two hydrophobic amino acid residues in region III may interact directly with PCB. The side chain of Phe336 is likely to be inside the active site and in close proximity to the substrate, whereas Thr335 is further away and close on the side of what it seems to be the entrance of the active site (Carredano and Ramaswamy, personal communication). Furthermore, Phe352 belonging to the narrowest part of the gorge of naphthalene dioxygenase, aligns with Phe378—a residue flanking region IV of LB400 biphenyl dioxygenase.

Variants S5.4, S5.6, and S4.43 degraded both *para*- and *ortho*-substituted congeners at high efficiency, while similar variants S20 and S4.47 with Thr338 and Thr341 had somewhat reduced activity toward 2,2',5-CB compared with the wt 400 strain. The presence of Ser325 and/or Val326 also appears to negatively affect activity against *ortho*-substituted congeners, suggesting that residues outside of the critical regions III and IV may have a supplementary or even an essential role in determining substrate specificity. This argument is further supported by comparing the *bphA* gene from *Comamonas testosteroni* B-356, which contains sequences in both regions III and IV very similar to KF707, yet it has 3,4-dioxygenase activity like LB400 and poorly degrades *para*-substituted congeners (Sylvestre et al., 1996). Because LB400 mutants with substitutions Ala4→Ser and Thr325→Ser have previously been generated with no noticeable alteration in specificity (Mondello et al., 1998), perhaps changes outside of the critical region

aa #	4	5	7	145	237	238	247	255	258	268	277	283	285
w1400	A	I	E	D	T	T	I	Q	I	A	Y	S	L
D2.5	-	-	-	-	-	-	-	-	-	-	-	M	M
D8.5	-	-	-	-	-	-	-	-	-	-	-	M	M
D13.9	-	V	G	N	-	-	-	-	-	-	-	M	M
S5.4	S	-	-	-	M	S	M	H	V	G	F	M	M
S5.6	S	-	-	-	M	S	M	H	V	G	F	M	M
S4.43	-	-	-	-	M	S	M	H	V	G	-	-	-
S4.47	-	-	-	-	-	-	-	-	-	-	-	-	-
S20	-	-	-	-	-	-	-	-	-	-	-	M	M
w1707	S	-	-	-	M	S	M	H	V	G	F	M	M

aa #	303	313	320	325	326	335	336	338	341	377	402	405
w1400	E	G	V	T	I	T	F	N	I	N	Y	K
D2.5	-	-	F	S	V	A	I	T	T	T	-	-
D8.5	D	DEL	F	S	V	A	I	T	T	T	H	R
D13.9	-	-	-	S	V	A	I	-	-	-	-	-
S5.4	D	DEL	-	-	-	A	I	-	-	-	-	-
S5.6	-	-	-	-	-	A	I	-	-	-	-	-
S4.43	-	-	-	-	-	A	I	-	-	-	-	-
S4.47	-	-	-	-	-	A	I	T	T	-	-	-
S20	D	DEL	F	S	V	A	I	T	T	T	-	-
w1707	D	DEL	F	S	V	A	I	T	T	T	-	-

**Figure 5.** Diversity of wild-type and chimeric biphenyl dioxygenases. Only amino acid residues different from the biphenyl dioxygenase sequence from *Burkholderia cepacia* LB400 are listed. Amino acid numbers (#aa) refer to the amino acid residues of the mature biphenyl dioxygenase from LB400. Roman numbers refer to amino acid residue regions as defined by Mondello et al. (1997). Code: -: Identical amino acids; ·: not sequenced; DEL: amino acid deletion.

must occur in concert with the appropriate changes within the critical regions in order to observe the complementary effects of these substitutions.

While this work was in preparation, several chimeric biphenyl dioxygenases have been generated by DNA shuffling with an extended substrate range for PCBs and related compounds (Kumamura et al., 1998). Two clones (pSHF2007 and pSHF2008) effectively dioxygenate 4,4'- and 2,5,2',5'-CB into 2,3-dioxygenated and 3,4-dioxygenated metabolites, respectively. However, virtually no formation of *meta*-cleavage products were detected from 2,2'-CB. Sequence analysis revealed the presence of Ser325, Val326, Thr338, and Thr341 on both of these clones, reaffirming the negative influence of these residues on dioxygenation of *ortho*-substituted congeners.

The different modes of degradation on *ortho*-substituted congeners between the two studies clearly reflect the differences in the screening procedure employed. By applying focused selection, we were able to generate variants not only with extended specificity, but also the combined activity of both parental clones. The best variants (S5.6, S5.4, S4.47, and S4.43) with substitutions of Thr335→Ala and Phe336→Ile effectively dioxygenate both 4,4'- and 2,2',5-CB into metabolites that can be further degraded into *meta*-cleavage products.

## CONCLUSION

DNA shuffling and staggered extension process were successfully applied in combination with a selective plate assay in tuning the substrate specificity of biphenyl dioxygenase. Several chimeric biphenyl dioxygenases derived from two related biphenyl dioxygenases with extended specificities toward both double *ortho*- and *para*-substituted PCB con-

geners were selected. The best variants acquired degradation capabilities for PCBs exceeding those of the two parental enzymes. It will be a challenging task to further extend the substrate range of these variants by crossing them with dioxygenases of more diverse sequences. Because dioxygenation initiates aerobic degradation of PCBs, engineering of biphenyl dioxygenases with an extended substrate range is a first step in evolving the entire *bph* operon for improved mineralization of highly chlorinated PCBs.

The authors thank Dr. Frank Mondello and Dr. Dennis Focht for providing the *E. coli* strain FM4119 and the plasmid pKT18, respectively. We appreciate the help of Pradeepa Selvakumar and Mona Zia during the early stage of this work. The support in DNA sequencing by Tammy Huynh and the critical reading of the manuscript by Dr. Rich Richins is also greatly appreciated. We also thank the reviewer for helpful suggestions.

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