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1 **Generation of a Hybrid Dioxygenase Showing Improved Oxidation of**
2 **Polychlorobiphenyls by a Widely Applicable Approach**

3
4 RUNNING TITLE: Improved oxidation of PCBs by a hybrid dioxygenase

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SUMMARY

1
2 Recently, a sequence-based approach has been developed for the fast isolation and
3 characterization of class II aryl-hydroxylating dioxygenase activities (S. Kahl and B. Hofer,
4 Microbiology **149**:1475-1481, 2003). It comprises the PCR-amplification of segments of
5 alpha subunit genes of unknown sequence that encode the catalytic centre, and their fusion
6 with sequences of the *bphA* gene cluster of *Burkholderia xenovorans* LB400. One of the
7 resulting chimaeric enzymes, harbouring the core segment of a dioxygenase from
8 *Pseudomonas* sp. strain B4-Magdeburg, has now been characterized with respect to the
9 oxidation of chlorobiphenyls (CBs). Its substrate and product specificities differed favourably
10 from those of the parental dioxygenase of strain LB400. The hybrid possessed a higher
11 regiospecificity and yielded less unproductive dioxygenations at *meta* and *para* carbons. It
12 attacked *ortho*-, *meta*- and *para*-chlorinated rings with comparable efficiencies. It gave
13 significantly higher yields in *ortho,meta*-dioxygenation of recalcitrant congeners containing
14 a doubly *ortho*-chlorinated ring. Whilst the parental enzyme yielded mainly unproductive
15 *meta,para*-dioxygenation of 2,5,4'-CB, the hybrid predominantly converted this congener
16 into an *ortho,meta*-dioxygenated product. The subsequent enzymes of the LB400 catabolic
17 pathway were able to transform most of the metabolites formed by the novel dioxygenase,
18 indicating that the substrate ranges of these biocatalysts are not adapted to that of their initial
19 pathway enzyme. Some of the catabolites, however, were identified as problematic for further
20 degradation. Our results demonstrate that the outlined approach can successfully be applied
21 to obtain novel the dioxygenase specificities that favourably complement or supplement
22 known ones.

INTRODUCTION

1
2 Industrial mixtures of polychlorobiphenyls (PCBs) constitute an important class of
3 persistent and potentially carcinogenic pollutants. Certain aerobic bacteria are able to oxidize
4 some of the more lightly substituted PCB congeners through pathways that are basically
5 identical in the different organisms (Fig. 1) (e.g., 1, 8, 11, 13, 26). However, commercial
6 PCB mixtures pose a huge problem to catabolic pathways, as they typically consist of dozens
7 of different congeners. Even if broad in substrate range, no single pathway is able to
8 metabolize all PCBs in such mixtures. Moreover, the characterized pathways convert a
9 fraction of the PCBs into dead-end metabolites (18). Thus, enzymes with novel specificities
10 that are useful to replace or supplement known ones are of particular interest.

11 The initial pathway enzyme, biphenyl dioxygenase (BphA), is of crucial importance for
12 the successful breakdown of PCBs. Firstly, as shown in previous publications (15, 42) as well
13 as in the present work, its substrate range frequently is narrower than that of subsequent
14 pathway enzymes. Secondly, its regioselectivity of dioxygenation is a critical parameter, as it
15 determines the (potential) site(s) of attack by the subsequent enzymes of the metabolic route
16 (cf. Fig. 1). In this way it controls whether and how further enzymatic degradation of a given
17 congener may take place.

18 Two approaches appear particularly promising to obtain “missing” enzymatic activities.
19 One is the generation of altered enzymes through protein engineering and strategies of
20 artificial evolution (14, 42). The other is the detection and isolation of larger numbers of
21 naturally occurring enzymatic activities. The developments of high throughput formats
22 enable the screening of large clone libraries generated by these methods (16). Using the first
23 approach, we recently were able to generate BphAs with improved dioxygenation of PCBs by
24 segmental random mutagenesis (42). For the second approach, we developed a sequence-
25 based strategy for the fast isolation and characterization of BphA and other class II aryl-

1 hydroxylating dioxygenase activities (20). According to the latter method, the part of the
2 alpha subunit gene encoding the catalytic centre is amplified by PCR and is fused with
3 sequences of a *bphA* gene cluster that is efficiently expressed in an appropriate host (Fig. 2).
4 This method was initially tested using the DNA of cultivated micro-organisms as template for
5 PCR amplification. However, it can likewise be applied as a metagenomic approach (C.
6 Standfuß-Gabisch, D. Al-Halbouni, and B. Hofer, in preparation), thereby circumventing the
7 cultivation of organisms (23, 31). Depletion assays with different aromatic compounds had
8 indicated that the substrate spectra of the generated hybrid dioxygenases were largely
9 determined by their BphA1 core segment. However, a characterization of their catabolic
10 potential with respect to chlorobiphenyls (CBs) and its comparison to that of the parental
11 enzyme had not been carried out. Now the properties of one of the resulting chimaeric
12 enzymes have been investigated with a selection of CBs as potential substrates. This hybrid
13 (BphA-B4h) harbours the core segment of a dioxygenase from *Pseudomonas* sp. strain B4-
14 Magdeburg, a bacterium isolated from a polluted sediment of the Elbe river near Magdeburg,
15 Germany (7, 10). The other sequences were provided by the BphA of *Burkholderia*
16 *xenovorans* (17; formerly *Burkholderia* sp.) LB400 (BphA-LB400), a metabolically very
17 well characterized dioxygenase (2, 18, 24, 33, 35, 36, 40, 41). The present investigations
18 showed in detail how substrate and product ranges of the hybrid enzyme differed from those
19 of its parental BphA. They revealed that, with several CBs, the newly generated dioxygenase
20 showed complementing or improved degradative properties.

MATERIALS AND METHODS

Chemicals. CB congeners (99% purity) were obtained from Lancaster Synthesis (White Lund, Morecombe, England), Promochem (Wesel, Germany), or Restek (Sulzbach, Germany). Chlorobenzoates (CBAs) (98% purity) were purchased from Fluka AG (Buchs, Switzerland) or Lancaster Synthesis.

Bacterial strains, plasmids and culture conditions. The *E. coli* strain used in this study was BL21(DE3)[pLysS] (38) harbouring either pAIA111, pAIA6100, pAIA1104, pAIA6104 or pAIA51. These plasmids are based on the phage T7 expression vector pT7-6. pAIA111 (27) carries *bphA1A2A3A4* (collectively referred to as *bphA*), and pAIA6100 (20) harbours *bphABC* of *B. xenovorans* LB400. pAIA6104 contains a *bphA1* gene that is a fusion of the *bphA1* genes of *B. xenovorans* LB400 and *Pseudomonas* sp. B4-Magdeburg (20) and genes *bphA2A3A4BC* of *B. xenovorans* LB400. pAIA1104 was obtained from pAIA6104 by cleavage with *Ppu*MI and recircularization. This deleted most of genes *bphBC*. pAIA51 (42) carries the *bphD* gene of strain LB400. Bacteria were grown in Luria-Bertani medium (30) at 37 °C. Chloramphenicol and ampicillin at concentrations of 20 and 50 mg/ml, respectively, were used for selection.

Preparation of resting cells. Preparation of resting cells was carried out as previously described (34) with some modifications. Cells of *E. coli* BL21(DE3)[pLysS] harbouring the respective *bph*-containing plasmid were grown in LB medium at 30 °C. At an optical density at 600 nm (OD_{600}) of 0.6 - 1.0, 0.4 mM IPTG was added and the incubation was continued for another 30 - 60 min. Cells were harvested, washed with one vol. of 50 mM sodium phosphate buffer (pH = 7.5) and resuspended in the same buffer to give the concentrations specified below.

Biotransformations. Substrates were dissolved in hexane or cyclohexane. They were dispensed into teflon-sealed Erlenmeyer flasks. After evaporation of the solvent, reactions

1 were started by addition of typically 10 ml of resting cells. Individual details of the different
2 transformation experiments are given below.

3 **Transformations of CBs by BphA-B4h or BphA-LB400 and analysis of products.**

4 Resting cell suspensions ($OD_{600} = 18$) of *E. coli* BL21(DE3)[pLysS] harbouring either
5 pAIA111 or pAIA1104 were incubated with a CB congener at a nominal concentration of 1
6 mM on a gyratory shaker for 6 h at 30 °C. Reaction mixtures were extracted with ethyl
7 acetate, and the dried extracts were derivatized with *n*-butylboronic acid as described before
8 (36). After derivatization, mixtures were evaporated to dryness and dissolved in 20 μ l of
9 hexane. Samples (1 μ l) were injected in the splitless mode into a gas chromatography-mass
10 spectrometry (GC-MS) system consisting of a Autosystem XL gas chromatograph (Perkin-
11 Elmer, Boston, MA, USA) with a MDN-1 column (Supelco, Bellefonte, PA, USA), coupled
12 to a Perkin-Elmer Turbo Mass mass spectrometer. Helium served as carrier gas. The mass
13 spectrometer was operated in the electron impact ionization mode at 70 eV.

14 **Transformations of CBs by BphA-B4h and BphBC-LB400 or by BphABC-LB400**

15 **and analysis of products.** Resting cell suspensions ($OD_{600} = 2.0$) of *E. coli*
16 BL21(DE3)[pLysS] harbouring either pAIA6104 or pAIA6100 were supplemented with
17 glucose to 0.5 % and were incubated with a CB at a nominal concentration of 125 μ M, on a
18 rotary shaker at 30 °C. The formation of chlorinated 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-
19 dienoates (HOPDAs) was monitored at intervals up to 24 h by spectral scanning of the
20 supernatants with a UV-2401 PC spectrophotometer (Shimadzu, Kyoto, Japan).

21 **Transformations of CBs by BphA-B4h and BphBCD-LB400 or by BphABCD-**

22 **LB400 and analysis of products.** Equal volumes of resting cell suspensions ($OD_{600} = 2.0$)
23 of *E. coli* strains BL21(DE3)[pLysS](pAIA6104) and BL21(DE3)[pLysS](pAIA51) or
24 BL21(DE3)[pLysS](pAIA6100) and BL21(DE3)[pLysS](pAIA51), respectively, were
25 supplemented with glucose to 0.5 % and were incubated as above with a CB at a nominal

1 concentration of 125 μ M for 16 h. Cell-free supernatants were analysed by high performance
2 liquid chromatography (HPLC) as described previously (34). CBAs were identified and
3 quantitated by comparison with authentic standards.

RESULTS

1
2 It had been shown that *Pseudomonas* sp. strain B4-Magdeburg is able to grow with
3 biphenyl as sole source of carbon and energy (7, 10). However, its abilities to metabolize
4 CBs had so far not been investigated. The construction of a hybrid *bphA1* gene carrying
5 sequence from strain B4-Magdeburg in its core region (Fig. 2) has been described (20). The
6 respective plasmid, pAIA6104, also harboured genes *bphBC* from strain LB400. In order to
7 avoid further catabolism of the products formed through catalysis by the initial pathway
8 dioxygenase, major parts of the latter genes were deleted, as described in the experimental
9 section. This yielded pAIA1104. *E. coli* strains harbouring either pAIA1104 (encoding BphA-
10 B4h) or pAIA111 (encoding BphA-LB400) were incubated with a selection of CBs. As a
11 first group, the three symmetrical di-CBs were chosen to compare the attack at mono-
12 substituted rings chlorinated at all possible positions. The dioxygenation products were
13 analyzed by GC-MS (Table 1).

14 **The *ortho*-monochlorinated ring.** The dioxygenation of 2,2'-CB by the two enzymes
15 was targeted to completely different positions. The parental BphA-LB400 yielded two
16 metabolites, a monochlorinated dihydroxybiphenyl (DHB) as major and a dichlorinated
17 biphenyldihydrodiol (BDHD) as minor product, in accordance with previous findings (18). It
18 has been shown before that the DHB is hydroxylated at carbons 2 and 3 (18, 35). For the
19 BDHD, hydroxylation at carbons 5 and 6 has been proposed (18) which, however, is in
20 conflict with our results (see below). BphA-B4h formed a different BDHD as sole product.

21 In order to monitor the further degradation of the dioxygenation products and to obtain
22 additional evidences for the assignment of dioxygenation sites, congeners were incubated
23 with cells also harbouring the subsequent pathway enzymes, BphB, BphC and BphD, of
24 strain LB400 (cf. Fig. 1).

25 The results on the formation of HOPDAs are shown in Table 2. It should be noted that

1 BphC of strain LB400 does not cleave 3,4-DHB (12). Thus, the formation of ring-cleavage
2 products suggests dioxygenation at *ortho* and *meta* carbons.

3 Both, the wild-type and hybrid pathways converted 2,2'-CB into HOPDAs. This
4 indicated that also BphA-B4h generated an *ortho,meta*-dioxygenated metabolite. Thus, the
5 BDHD produced by BphA-B4h must be 5,6-dioxygenated. It follows that the BDHD formed
6 by BphA-LB400 was *meta,para*-dioxygenated. This is in agreement with the results of
7 Barriault et al. (4), who assigned, on the basis of its NMR spectrum, hydroxylation at carbons
8 3 and 4 to a metabolite, produced by a BphA variant, that showed the same retention time as
9 the BDHD formed by BphA-LB400.

10 The results of the conversion of congeners into CBAs in the presence of recombinant
11 cells synthesizing the hydrolase BphD of *B. xenovorans* LB400 are shown in Table 3.

12 Extradiol fission between carbons other than 1 and 2 or 1 and 6, respectively,
13 (equivalent to 1' and 2' or 1' and 6', respectively) would not lead to benzoates. Thus, the
14 formation of 2-chlorobenzoate (CBA) from the different dioxygenation products of 2,2'-CB
15 generated by the two BphAs provided additional evidence for their attacks at *ortho* and *meta*
16 carbons. It furthermore demonstrated that the subsequent pathway enzymes were able to
17 convert metabolites that were not formed via their cognate dioxygenase.

18 **The *meta*-monochlorinated ring.** The major site of attack of 3,3'-CB was identical for
19 both enzymes and yielded a BDHD. Studies with BphA-LB400 had shown that this
20 metabolite is dioxygenated at carbons 5 and 6 (36). This agrees with a decreasing effect of
21 *ortho* substituents on the retention time (29), as shown in Table 1, and with the finding that
22 both, the BphA-LB400- and -B4h-initiated pathways converted 3,3'-CB into significant
23 amounts of HOPDA and 3-CBA (Table 3). The observed instability of the extradiol fission
24 product agrees with the slow spontaneous hydrolytic dehalogenation, followed by a
25 tautomeric shift to the 2-oxo form, that has been described for HOPDA chlorinated at carbon

1 4 (32). BphA-B4h additionally only formed trace amounts of a second metabolite, identified
2 as a monochlorinated DHB. Based on the high preference of the hybrid for *ortho,meta*-
3 dioxygenation, we tentatively assign oxidation at carbons 2 and 3. The LB400 enzyme
4 catalyzed formation of a second BDHD to which we assign dioxygenation at positions 4 and
5 5, in agreement with its larger retention time and with previous results (18, 36).

6 **The *para*-monochlorinated ring.** Both enzymes showed identical regioselectivity in
7 the dioxygenation of 4,4'-CB. They formed the same BDHD as the only metabolite, which
8 has previously been identified to be dioxygenated at carbons 2 and 3 (36). However, yields
9 were approximately 5-fold higher for the hybrid enzyme. A low level of HOPDA formation
10 from 4,4'-CB, but no conversion of this HOPDA into 4-CBA was detected, in agreement
11 with previous results (34).

12 Three CBs that were not efficiently or not productively dioxygenated by BphA-LB400
13 were selected for further comparison of the two enzymes. Not productively is used in the
14 sense that congeners were mainly converted into dead-end metabolites (18).

15 **CBs possessing a doubly *ortho*-chlorinated ring.** With 2,6-CB, no DHB was detected
16 as metabolite, ruling out a dechlorinating attack at an *ortho* carbon. Both enzymes yielded
17 two BDHDs of which the major one was common to both dioxygenases. However, yields
18 with the hybrid enzyme were about eight-fold higher.

19 Conversion of 2,6-CB into a HOPDA was not detected after dioxygenation by BphA-
20 LB400. However, it was observed after hydroxylation by the hybrid enzyme. This suggests
21 that the BDHD exclusively formed by BphA-B4h was dioxygenated at positions 2' and 3'.
22 Moreover, it is in keeping with the observation that this BDHD showed the smallest retention
23 time of the three dioxygenation products. The formation of a small quantity of a metabolite
24 with a UV spectrum and retention time consistent with that of 2,6-CBA agrees with 2',3'-
25 dioxygenation by BphA-B4h. The major product formed by BphA-B4h could be either 3,4-

1 or 3',4'-dioxygenated. We favour the latter possibility, because in this case the two attacks
2 observed with this enzyme would not require two completely different orientations of the
3 substrate at the active site. One of the best ways to position a substrate for catalysis is
4 hydrogen bonding. Chloroaromatics, however, can at best act as very weak hydrogen bond
5 acceptors. Thus, a certain oscillation between similar orientations of such a less tightly bound
6 substrate is not unexpected. In the case of BphA-B4h, a position of the activated dioxygen
7 close to C_{3'} of 2,6-CB in conjunction with some motion around the axis perpendicular to the
8 plane of the oxidized ring could lead to the involvement of either C_{4'} or C_{2'} as the second site
9 of attack. The probably best characterized BphA in terms of regiospecificity with CBs is the
10 enzyme from strain LB400. A survey of available data indicates that about 50 % of the
11 reported cases of relaxed regiospecificity could be explained by the described rationale.

12 When incubated with BphA-LB400, the structurally related 2,6,4'-CB yielded a
13 trichlorinated BDHD and very small amounts of a dichlorinated DHB, resulting from a partly
14 dechlorinating attack at carbons 2 and 3 (42). The former metabolite had not been detected
15 after overnight incubations (42), presumably due to the known tendency of some DHDs to
16 readily re-aromatize by elimination of water. In contrast to the parental enzyme, the hybrid
17 formed only a single metabolite, a BDHD, that was different from the LB400 product. The
18 yield of dioxygenation was approximately 40-fold higher than with BphA-LB400. As only
19 two BDHDs are theoretically possible (Table 1), the retention times suggest that BphA-B4h
20 formed the 2',3'-dioxygenated isomer, whereas BphA-LB400 attacked carbons 3 and 4.
21 However, the former metabolite was not converted into a HOPDA. In contrast, the 2,3-
22 dioxygenated DHB formed by BphA-LB400 was further transformed into 4-CBA by the
23 LB400 pathway (Table 3).

24 **The *para*-monochlorinated ring in combination with the 2,5-dichlorinated ring.**

25 There are several indications that both chlorination patterns are problematic for the

1 catabolism through the LB400 pathway. As shown above, dioxygenation at the *para*-
2 chlorinated ring is productive, but only gives minor yields. Hydroxylation of the 2,5-
3 disubstituted ring is usually efficient (2, 9, 18), but typically leads to dead-end metabolites.

4 The two BphAs behaved complementary towards 2,5,4'-CB. They yielded the same two
5 BDHDs as the only products, however in roughly inverse amounts. As only two BDHDs are
6 theoretically possible (Table 1), this indicates dioxygenation at positions 3 and 4 or 2' and 3',
7 respectively. Their retention times suggest that the major metabolite of BphA-LB400 is
8 dioxygenated at carbons 3 and 4. This is in accordance with the reported preference of this
9 enzyme for the 2,5-dichlorinated ring.

10 Significantly higher yields of a HOPDA with an absorption maximum at 397 nm were
11 observed after dioxygenation of 2,5,4'-CB by BphA-B4h. Moreover, in the presence of
12 BphD, a low concentration of a product with a UV spectrum and retention time consistent
13 with that of 2,5-CBA was found. These results confirm the above assignment of 2',3'-
14 dioxygenation.

15 When HOPDA formation via dioxygenation by BphA-LB400 was monitored early in the
16 reaction, an absorption maximum at 413 nm was initially observed. During the first three to
17 four hours, it was shifted to and then stably remained at 397 nm, the value of the HOPDA
18 also generated via dioxygenation by BphA-B4h (Table 2). This suggests, in the case of
19 BphA-LB400, the transient appearance of an unstable second extradiol fission product,
20 derived from the 3,4-hydroxylated BDHD, which was the only other product formed by this
21 enzyme. Low activities of the BphB of *Comamonas testosteroni* B-356 and of strain LB400
22 against the 3,4-DHD derived from 2,5,2',5'-CB have been described (6). Moreover, extradiol
23 cleavage of the resulting catechol by DoxG from *Pseudomonas* sp. strain C18 has been
24 reported (5). Oxygenolytic ring fission on either side of a 2,5-dichlorinated 3,4-diol would
25 yield a reactive acylchloride. Nucleophilic attack either by its own hydroxy group at carbon 2

1 (21) or by water (25) would lead to conversion into colourless metabolites, a lactone or a
2 dimuconate, respectively, consistent with the observed disappearance of the long wavelength
3 absorption maximum.

4 **Time course of HOPDA formation.** When the time course of HOPDA formation was
5 monitored, generally two types of curves were obtained, depending on both, the CB and the
6 BphA involved. If 4,4'- or 2,5,4'-CB were initially dioxygenated by BphA-B4h, the HOPDA
7 concentration showed an essentially linear increase up to the last sample taken (Fig. 3). In all
8 other cases, the increases of the absorptions levelled off after one or a few hours. As an
9 example, the degradation of 3,3'-CB via dioxygenation by BphA-B4h, is shown in Fig. 3.
10 These apparent decreases in the rate of HOPDA formation cannot be explained by substrate
11 depletion. They are likely due to chemical instability of some ring-cleavage products (32)
12 and to enzyme inhibition along the pathway.

DISCUSSION

The dioxygenation of various CBs by the two enzymes showed that substrate acceptance and regiospecificity of dioxygenation by the hybrid BphA investigated in this study differed fundamentally from that of the parental enzyme. This is illustrated in Fig. 4.

BphA-LB400 showed major differences in the amounts of productive *ortho,meta*-dioxygenation of the *ortho*-, *meta*- and *para*-chlorinated ring. Such behaviour is typical for BphAs (3, 9, 19, 27). In contrast, BphA-B4h attacked all three rings with comparable efficiencies.

Congeners with a doubly *ortho*-chlorinated ring are known to generally be recalcitrant to attack by BphAs (9, 24, 39). Thus, the LB400 enzyme showed no *ortho,meta*-dioxygenation of 2,6-CB, although this congener possesses an unchlorinated ring, which normally is easily attacked. Similarly, the enzyme yielded only small amounts of *ortho,meta*-dioxygenation products of 2,6,4'-CB. In contrast, BphA-B4h was able to catalyze significant 2',3'-dioxygenation of both congeners.

Available data suggest that typically the 2,5-dichlorinated ring is less recalcitrant to CB dioxygenation than the 2,6-dichlorinated ring, and that for some BphAs, including the enzyme of strain LB400, the 2,5-dichlorinated ring even is a preferred target (2, 9, 18). Remarkably, the only site of attack are carbons 3 and 4. Typically, 3,4-BDHDs are dead end metabolites (12, 18), although a low turnover of the 3,4-DHD derived from 2,5,2',5'-CB by BphB of *Comamonas testosteroni* B-356 and by strain LB400 has been reported (6), and our results suggest a low level of dehydrogenation and subsequent extradiol cleavage of the 3,4-DHD formed from 2,5,4'-CB. Moreover, transformation of chlorinated *meta,para*-BDHDs by BphB and BphC may form acylchlorides, which may inactivate enzymes by attack of side chains in the active site. In contrast, ring cleavage of a chlorinated *ortho,meta*-DHB between carbons 1 and 2 or 1 and 6, respectively, can never yield an acylchloride. Contrary to the

1 LB400 enzyme, BphA-B4h converted 2,5,4'-CB into only marginal amounts of the 3,4-
2 dioxygenated metabolite, but into almost 100-fold higher amounts of the BDHD resulting
3 from the more favourable *ortho,meta*-dioxygenation of the other ring.

4 In summary, BphA-B4h showed a higher regioselectivity of dioxygenation. In only one
5 case the main product represented less than 95 % of the dioxygenated CB (Table 1). BphA-
6 B4h also possessed a greater preference for *ortho,meta*-dioxygenation. Thus, BphA-LB400
7 formed *meta,para*-dioxygenated products from five of the six substrates (Fig. 4), with an
8 average contribution of 61 % (Table 1), whilst BphA-B4h yielded such metabolites only
9 from three CBs, with a major contribution only in the case of 2,6-CB (Table 1).

10 Interestingly, the subsequent enzymes of the *bph*-encoded metabolic pathway of strain
11 LB400 were able to transform most of those metabolites, produced by the hybrid BphA, with
12 which they normally are not confronted. Thus, 2',3'-dioxygenated 2,6-CB was converted at
13 least into the respective HOPDA, and 5,6-dioxygenated 2,2'-CB was transformed to 2-CBA.
14 These results show that subsequent metabolic enzymes can possess a broader substrate
15 spectrum than the initial pathway enzyme. This is particularly remarkable for the hydrolase
16 BphD, which has been reported to constitute a major bottleneck (14, 15, 32).

17 On the other hand, it is directly apparent from a correlation of Tables 1 and 3 that the
18 products of 2,3-dioxygenation of 4,4'-CB, and of 2',3'-dioxygenation of 2,5,4'-, 2,6- and
19 2,6,4'-CB were not or only marginally converted into the respective CBAs by the subsequent
20 pathway enzymes. When 4,4'- and 2,5,4'-CB were dioxygenated by BphA-B4h, significant
21 amounts of the *meta*-fission products were found. This indicates that these HOPDAs were
22 not or very slowly converted by the hydrolase BphD (Fig. 5). Only low or no detectable
23 quantities of HOPDAs were formed from the BDHDs generated by 2',3'-dioxygenation of
24 2,6- and 2,6,4'-CB. This indicates a problem with the dehydrogenase BphB and/or the
25 extradiol dioxygenase BphC (Fig. 5). A comparison of the structures of the respective

1 *ortho,meta*-dioxygenated BDHDs suggests that it is the double *ortho* substitution of the non-
2 oxidized ring which prevents efficient turnover by one or both of these enzymes.

3 We note that Seah et al. (32) found that HOPDA chlorinated at position 3 was
4 hydrolysed by BphD-LB400 at a 500-fold lower rate than the unchlorinated metabolite. This
5 is consistent with no or only marginal conversions of the 3,10-dichlorinated or 3,8,11-
6 trichlorinated HOPDA, resulting from 4,4'- or 2,5,4'-CB, respectively, into 4-CBA (Fig. 5).
7 The same authors also reported that HOPDA chlorinated only at position 4 was
8 hydrolytically cleaved at a 10000-fold lower rate than the unsubstituted compound (32).
9 Thus, the observed conversion of 3,3'-CB into a 4,9-dichlorinated HOPDA that was further
10 metabolized to 3-CBA suggests that, unexpectedly, the additional chlorine substituent at the
11 non-oxidized ring significantly enhances this rate. A positive electronic effect appears
12 unlikely, as recently Speare et al. (37) has shown that electron-withdrawing substituents at
13 the non-oxidized ring decrease the rate of HOPDA hydrolysis by BphD-LB400.

14 The exchange of the BphA1 core segment resulted in 24 AA differences between the
15 LB400 and the hybrid sequence (see supplemental material). Therefore, alterations in
16 catalytic behaviour cannot directly be ascribed to single or a few AA substitutions. However,
17 several of the replaced residues have previously been exchanged, either singly or in groups of
18 two and three (22, 28, 41, 42). In most cases, this did not result in significant changes of the
19 examined properties. An exception was the region comprising AAs 335 to 341 (LB400
20 numbering), where four of the present substitutions are located. However, more than these
21 exchanges are likely to play a role, because seemingly "unimportant" residues may exert
22 significant effects, when replaced in concert with additional AAs. Such a context-dependence
23 of substitutions has repeatedly been reported (e. g., 22, 28).

24 The fundamental differences in substrate and product spectra between the parent and
25 hybrid enzyme demonstrate that BphAs with novel catabolic potential can be obtained

1 through a rapid approach involving PCR amplification of partial genes encoding the large
2 subunit of aryl-hydroxylating dioxygenases and their fusion with cloned “helper” genes and
3 gene segments to reconstitute a complete aryl-hydroxylating dioxygenase system. The speed
4 of this approach and, in addition, the possibility to carry out PCR amplifications directly with
5 metagenomic DNA samples may permit a rapid access to a much larger part of natural BpHA
6 diversity than was previously possible. Such an approach could thus favourably complement
7 or supplement methods of artificial evolution (14) for the acquisition of novel dioxygenase
8 activities.

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FIGURE LEGENDS

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FIG. 1. Upper pathway for catabolism of biphenyls encoded by the *bph* locus of *B. xenovorans* LB400. Compounds: 1, biphenyl; 2, biphenyl-2,3-dihydro-2,3-diol (BDHD); 3, 2,3-dihydroxybiphenyl (DHB); 4, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA); 5a, 2-hydroxypenta-2,4-dienoic acid; 5b, benzoic acid (BA). Enzymes: BphA, biphenyl 2,3-dioxygenase; BphB, 2,3-dihydro-2,3-dihydroxybiphenyl 2,3-dehydrogenase; BphC, 2,3-dihydroxybiphenyl 1,2-dioxygenase; BphD, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase.

FIG. 2. Generation of the fusion dioxygenase. The top line shows a representation of the *bphA* gene cluster of *B. xenovorans* LB400, encoding alpha and beta subunits (*bphA1/bphA2*), ferredoxin (*bphA3*) and ferredoxin reductase (*bphA4*). The hatched *bphA1* segment between the restriction sites was exchanged with a PCR amplicon. The bottom line shows a representation of the alpha subunit. Catalytic and Rieske domains are shown in grey or hatched, respectively. Horizontally connected vertical bars indicate sites encoding amino acid ligands of the Rieske iron-sulphur cluster (“[2Fe-2S]”) and of the active site mononuclear iron (“mono-Fe”). The part encoded by the amplicon and the flanking amino acid positions of the recipient subunit are indicated.

FIG. 3. Time course of HOPDA formation. Absorbance (milli-units) at absorption maxima are shown. Substrates: 3,3'-CB (circles) and 2,5,4'-CB (squares). Both substrates were initially oxidized by BphA-B4h.

FIG. 4. Overview of the regiospecificity of CB dioxygenation by BphA-B4h and -LB400.

1 Regiospecificities of attack are symbolized by O₂ molecules with arrows. Relative quantities
2 are indicated as follows. Black arrows, > 33 % of total dioxygenation; grey arrows, 10-33 %
3 of total dioxygenation; light grey arrows, < 10 % of total dioxygenation. For experimental
4 evidences of site assignments and further details, see text and Table 1. A lack of
5 experimental evidence for the assignment of an oxidation site is indicated by “S?”, a lack of
6 experimental evidence for the assignment of the relative quantity of oxidation is indicated by
7 “Q?”.

8
9 FIG. 5. Bottlenecks in the metabolism of specific *ortho,meta*-dioxygenated chlorinated
10 BDHDs by enzymes BphB, BphC and BphD of the pathway from *B. xenovorans* LB400.
11 Thin arrows symbolize low transformation, crossed-out thin arrows symbolize no detectable
12 transformation.

TABLE 1. Characterization of CB metabolites formed by dioxygenation catalyzed by BphA-B4h or BphA-LB400.

Substrate	No. theoretically possible ^a		Meta- bolite No.	t _r (GC) [min] of derivative	m (MS) of derivative	No. of Cl	Type of com- pound	Products				Assignment of oxidized carbons ^c
	BDHDs	DHBs						Apparent absolute yield ^b [area units]		Apparent relative yield ^b [%]		
								BphA-B4h	BphA-LB400	BphA-B4h	BphA-LB400	
2,2'-CB	3	1	1	21.3	286	1	DHB	no ^d	80.8 ± 8.5	no	93	2,3
			2	22.1	322	2	BDHD	25.6 ± 5.7	no	100	no	5,6
			3	22.6	322	2	BDHD	no	6.1 ± 1.0	no	7	3,4
3,3'-CB	2	2	1	22.5	286	1	DHB	0.34 ± 0.12	no	4	no	2,3
			2	23.7	322	2	BDHD	9.5 ± 3.6	6.7 ± 0.3	96	68	5,6
			3	24.1	322	2	BDHD	no	3.1 ± 0.1	no	32	4,5
4,4'-CB	1	1	1	24.1	322	2	BDHD	20.0 ± 4.6	4.4 ± 1.6	100	100	2,3
2,6-CB	3	1	1	22.4	322	2	BDHD	4.46 ± 0.47	no	25	no	2',3'
			2	22.6	322	2	BDHD	no	0.51 ± 0.31	no	23	3,4 or 3',4'
			3	23.0	322	2	BDHD	13.6 ± 4.3	1.71 ± 1.00	75	77	3,4 or 3',4'
2,6,4'-CB	2	2	1	23.4	320	2	DHB	no	0.05 ± 0.03	no	16	2,3
			2	24.1	356	3	BDHD	11.6 ± 4.2	no	100	no	2',3'
			3	24.4	356	3	BDHD	no	0.26 ± 0.14	no	84	3,4
2,5,4'-CB	2	4	1	24.4	356	3	BDHD	72.5 ± 38.5	14.4 ± 0.46	99	16	2',3'
			2	25.0	356	3	BDHD	0.97 ± 0.68	72.9 ± 16.3	1	84	3,4

^a Not taking into account dioxygenations involving C₁ or C_{1'}, respectively. These would not yield cyclic boronates and thus would not be detected in the analysis. To our knowledge, dioxygenations at these carbons have so far not been observed.

^b Deduced from total ion chromatogram peak areas.

^c For details see text.

^d no, not observed.

TABLE 2. Chlorinated HOPDAs formed from CBs by BphA-B4h or BphA-LB400, respectively, and BphBC-LB400.

Substrate	BphA	λ_{\max} [nm] of HOPDA
2,2'-CB	B4h	395 ± 2
	LB400	393 ± 2
3,3'-CB	B4h	420 ± 3^a
	LB400	425 ± 3^a
4,4'-CB	B4h	434 ± 2
	LB400	432 ± 2
2,6-CB	B4h	392 ± 2
	LB400	no ^b
2,6,4'-CB	B4h	no
	LB400	403 ± 5
2,5,4'-CB	B4h	397 ± 3
	LB400	413 ± 3^c
		397 ± 3^c

^a Shifted to 395 nm during the incubation.

^b no, not observed.

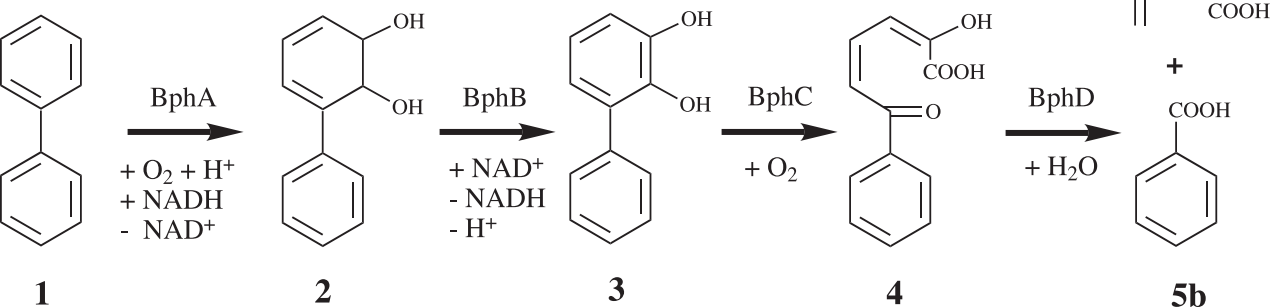
^c For details see text.

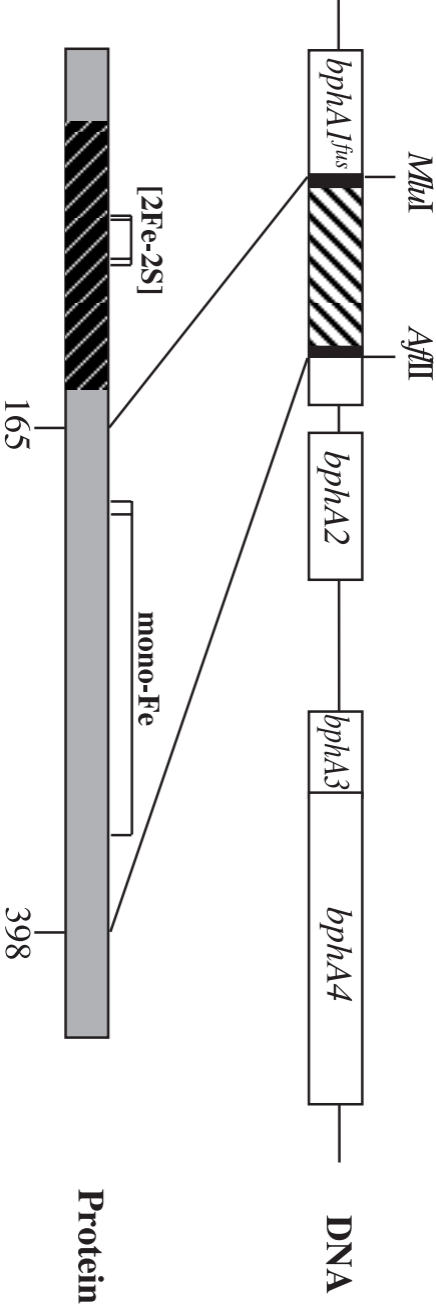
TABLE 3. CBAs formed from CBs by BphA-B4h or BphA-LB400, respectively, and BphBCD-LB400.

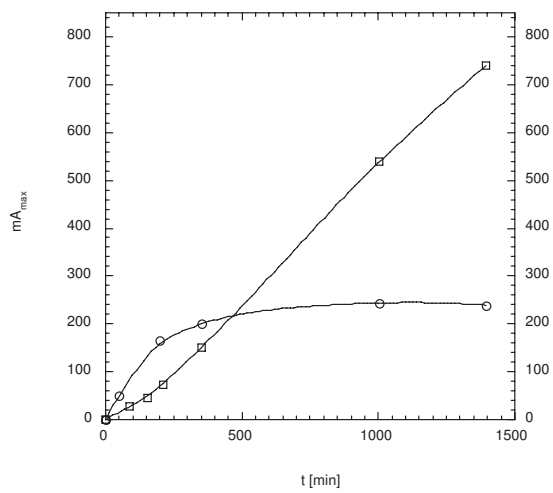
Substrate	BphA	CBA			
		t_r [min]	λ_{\max} [nm]	Identification as	Concentration [μM]
2,2'-CB	B4h	3.40 ± 0.01	204 ± 2	2-CBA	44.8 ± 15.4
	LB400	3.38 ± 0.01	204 ± 2	2-CBA	121.0 ± 13.0
3,3'-CB	B4h	5.80 ± 0.05	231 ± 2	3-CBA	25.4 ± 4.6
	LB400	5.75 ± 0.04	231 ± 2	3-CBA	9.9 ± 2.7
4,4'-CB	B4h			no ^a	
	LB400			no	
2,6-CB	B4h	2.98 ± 0.05	206 ± 5	2,6-CBA ^b	2.9 ± 1.4
	LB400			no	
2,6,4'-CB	B4h			no	
	LB400	5.58 ± 0.04	240 ± 2	4-CBA	0.5 ± 0.3
2,5,4'-CB	B4h	5.84 ± 0.05	200 ± 5	2,5-CBA ^b	0.5 ± 0.3
	LB400			no	

^a no, not observed.

^b Identification based mainly on t_r due to uncharacteristic spectrum of the respective CBA.

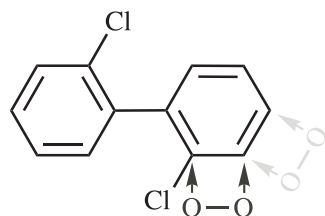
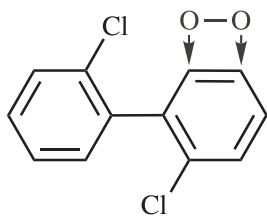




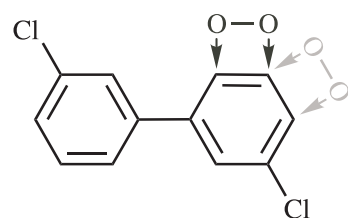
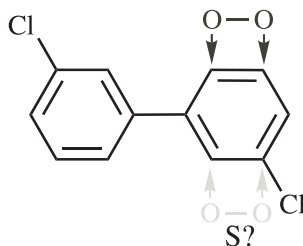


BDO-B4h**BDO-LB400**

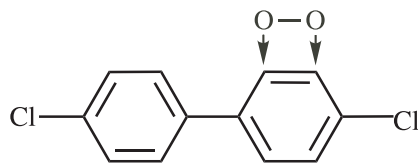
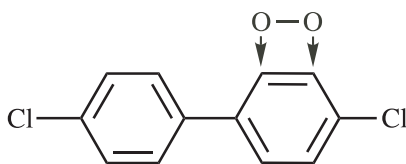
2,2'-CB



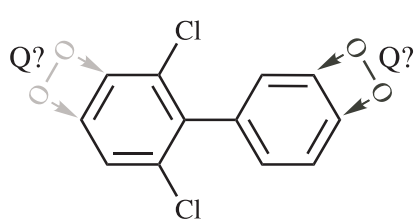
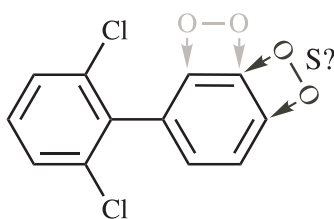
3,3'-CB



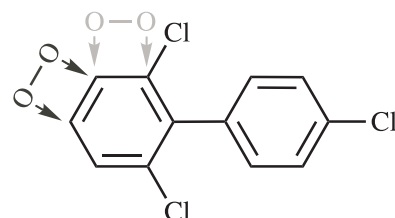
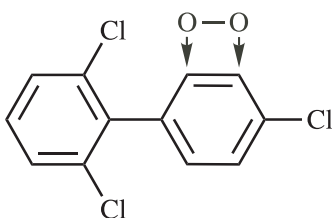
4,4'-CB



2,6-CB



2,6,4'-CB



2,5,4'-CB

