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Conversion of Chlorobiphenyls into Phenylhexadienoates and Benzoates by the Enzymes of the Upper Pathway for Polychlorobiphenyl Degradation Encoded by the *bph* Locus of *Pseudomonas* sp. Strain LB400

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Metabolism of 21 chlorobiphenyls by the enzymes of the upper biphenyl catabolic pathway encoded by the *bph* locus of *Pseudomonas* sp. strain LB400 was investigated by using recombinant strains harboring gene cassettes containing *bphABC* or *bphABCD*. The enzymes of the upper pathway were generally able to metabolize mono- and dichlorinated biphenyls but only partially transform most trichlorinated congeners investigated: 14 of 15 mono- and dichlorinated and 2 of 6 trichlorinated congeners were converted into benzoates. All mono- and at least 8 of 12 dichlorinated congeners were attacked by the *bphA*-encoded biphenyl dioxygenase virtually exclusively at *ortho* and *meta* carbons. This enzyme exhibited a high degree of selectivity for the aromatic ring to be attacked, with the order of ring preference being non- > *ortho*- > *meta*- > *para*-substituted for mono- and dichlorinated congeners. The influence of the chlorine substitution pattern of the metabolized ring on benzoate formation resembled its influence on the reactivity of initial dioxygenation, suggesting that the rate of benzoate formation may frequently be determined by the rate of initial attack. The absorption spectra of phenylhexadienoates formed correlated with the presence or absence of a chlorine substituent at an *ortho* position.

Contamination of the biosphere with synthetic organic compounds that are persistent and toxic is of increasing concern (1, 3, 24). One such type of chemical that has been extensively used in past decades is polychlorinated biphenyl (PCB). There exist a total of 209 distinct PCB congeners which differ in number and position of the chlorines. A typical commercial PCB mixture, such as Arochlor, Clophen, or Kanechlor, contains between 60 and 80 different congeners (7).

A promising approach for PCB decontamination of soils and sediments is bioremediation involving the combined action of anaerobic and aerobic microorganisms. Anaerobic consortia that are able to partially dehalogenate highly chlorinated congeners have been described (1, 19), whereas a number of aerobic bacteria that are capable of oxidatively attacking the ring system of moderately chlorinated PCBs have been isolated (2, 6, 12). Although the spectra of PCBs that can be transformed by a number of aerobes have been characterized (4–6, 11, 17), little is known about the final metabolites formed from individual congeners. Moreover, in most cases observed transformations could not unequivocally be ascribed to specific enzymes, because the synthesis of isoenzymes by the original isolates cannot be excluded.

Gene cloning is a convenient approach to deal with such questions. Since genes encoding enzymes for any part of a pathway can be introduced into a recipient devoid of catalytic activities towards the substrates and incapable of further transformation of the products, characterization of individual enzymatic activities and identification of metabolites produced by them are considerably facilitated.

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DNA segments involved in PCB degradation have been isolated from a number of bacteria including *Pseudomonas* sp. strain LB400 (9, 17), a microorganism which is particularly noteworthy for its ability to oxidize a broad spectrum of congeners (4–6, 17). To date, 10 cistrons of the LB400 *bph* locus have been characterized and shown to be involved in the breakdown of biphenyls (10, 15, 16). Through this work, LB400 became the first strain for which complete sequence information at the DNA as well as at the enzyme level was available for the biphenyl upper pathway, i.e., the catabolism of biphenyls to benzoates and 2-hydroxypenta-2,4-dienoates which is catalyzed by four enzymes, biphenyl-2,3 dioxygenase (BphA), 2,3-dihydro-2,3-dihydroxybiphenyl-2,3 dehydrogenase (BphB), 2,3-dihydroxybiphenyl-1,2 dioxygenase (BphC), and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BphD) (Fig. 1).

We previously identified metabolites produced by the action of BphA and/or BphB and observed dioxygenase-mediated dechlorination of *ortho*-substituted congeners (21). Here we investigate which of a broad range of chlorinated biphenyls (CBs) are degraded to 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoates (HOPDAs or phenylhexadienoates) or chlorobenzoates (CBAs).

MATERIALS AND METHODS

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

Bacterial strains, plasmids, and culture conditions. The *Escherichia coli* strains used in this study were DH1 (14), DH5α (13), and BL21(DE3)[pLysS] (23). The recombinant plasmids pAIA50 and pAIA74 are described in Fig. 2. Bacteria were grown in Luria-Bertani medium (20) at 37°C. Where appropriate, chloramphenicol and/or ampicillin was used for selection at concentrations of 20 and 50 μg/ml, respectively.

Recombinant DNA techniques. Plasmids were constructed by standard recombinant DNA techniques similar to published protocols (20). Transformations of

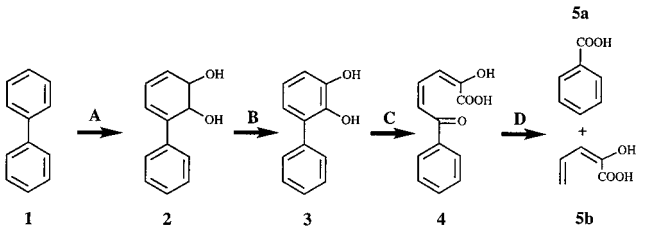

FIG. 1. Upper pathway for metabolism of biphenyls as encoded by the *bph* locus of *Pseudomonas* sp. strain LB400. Metabolites: 1, biphenyl; 2, 2,3-dihydro-2,3-dihydroxybiphenyl; 3, 2,3-dihydroxybiphenyl; 4, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid; 5a, benzoic acid; 5b, 2-hydroxypenta-2,4-dienoic acid. 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.

E. coli strains were carried out either by the Hanahan method (14) or by electroporation (8).

Resting cell assays. *E. coli* BL21(DE3)[pLysS] harboring either pAIA50 or pAIA74 was grown to an optical density of 0.6 at 600 nm. After further incubation in the presence of 0.4 mM isopropyl-β-D-galactopyranoside for 30 min at 30°C, cells were harvested, washed with 50 mM sodium phosphate buffer (pH 7.5), and resuspended in 1/10 volume of the same buffer. Cells were incubated with single PCB congeners (nominal concentration, 62 or 125 μM) for 20 or 24 h at 30°C on a gyratory shaker.

Monitoring of HOPDA and CBA formation. Formation of HOPDAs was monitored by visible spectral scanning of resting cell assay mixtures with a Beckman DU-70 spectrophotometer. Accumulation of CBAs was followed by high-performance liquid chromatography (HPLC). Cell-free supernatants of resting cell incubations (20 μl) were analyzed on a Beckman liquid chromatograph equipped with a diode array detector with a SC125/Lichrospher 5-μm column (Bischoff, Leonberg, Germany) and an aqueous solvent system containing 1 ml of 85% *ortho*-phosphoric acid and 640 ml of methanol per liter. CBAs were identified and quantitated by comparison with authentic standards.

RESULTS

Construction of recombinant strains and description of the assay system. Recombinant expression vectors harboring *bph* genes downstream of a phage T7 late promoter were described previously (16). Plasmid pAIA50 containing *bphABC* was generated from one of the earlier constructs, pAIA5, as shown in Fig. 2A. The construction of a *bphABCD*-containing expression vector comprised several steps. Firstly, *bphC* and *bphD* were separately amplified by PCR to introduce restriction sites at their ends and subsequently linked in such a way that 34 bp

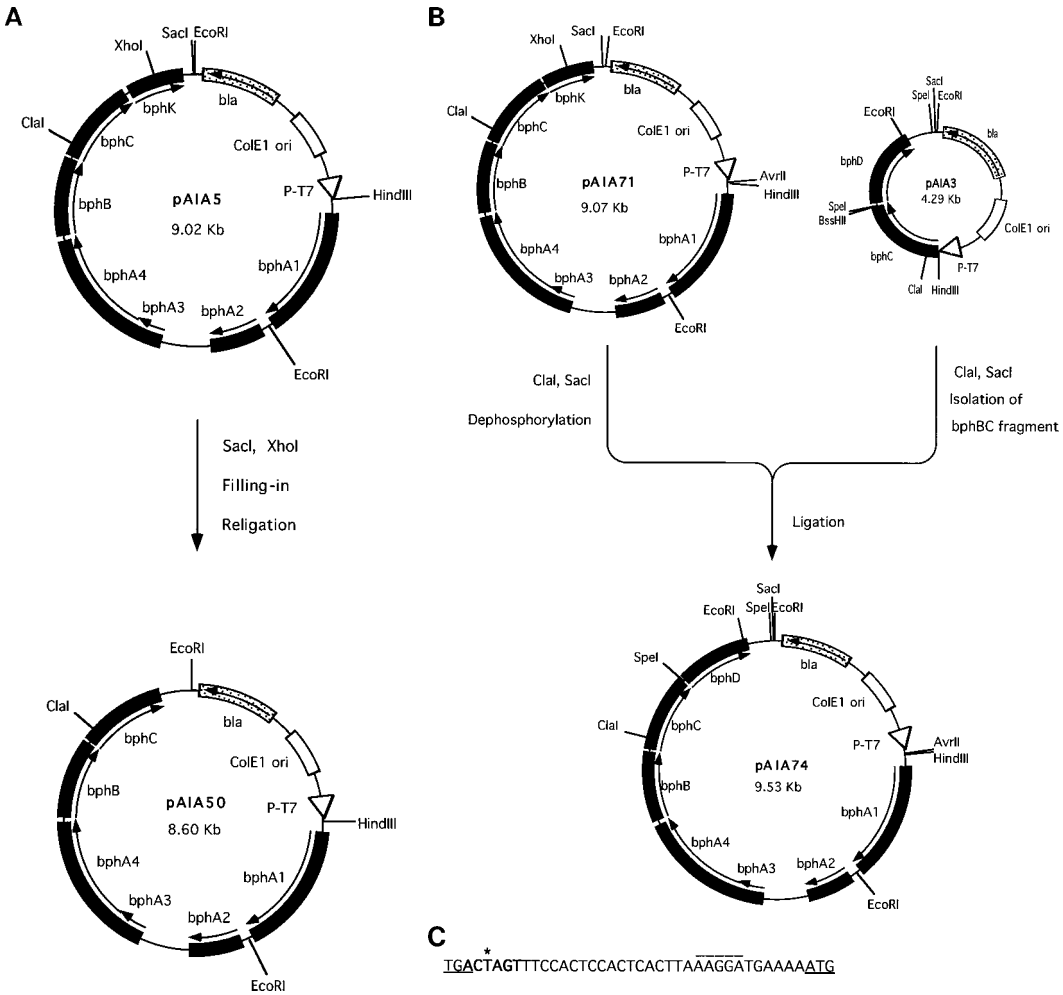

FIG. 2. Construction of recombinant plasmids. Directions of sense transcription of genes are indicated by arrows. P-T7, phage T7 gene 10 promoter initiating transcription in the direction indicated by the arrowhead. ori, origin of replication. bla, gene encoding β-lactamase which mediates ampicillin resistance. (A) Construction of pAIA50. A *Sac*I-*Xho*I fragment was deleted from pAIA5 to inactivate *bphK*. (B) Construction of pAIA74. For details, see text. (C) Sequence of the artificial *bphCD* junction. The stop codon of *bphC* and the start codon of *bphD* are underlined. The Shine-Dalgarno sequence of *bphD* is overlined. The newly introduced *Spe*I site is shown in boldface with the single base-pair exchange indicated by an asterisk.

TABLE 1. Formation of HOPDAs from various CBs by *E. coli* cells containing *bphABC*

CB	HOPDA	
	Absorption[a] (λ_{max} [nm])	Rate of formation[b]
2-[c]	393 ± 2	1
3-[c]	436 ± 2	1
4-[c]	437 ± 2	1
2,3-[d]	392 ± 2	1
2,4-[d]	392 ± 2	1
2,5-[d]	392 ± 2	1
2,6-[d]	ND	
3,4-[d]	440 ± 2	1
3,5-[c]	439 ± 2	1
2,2′-[c]	392 ± 2	1
2,3′-[d]	435 ± 2	1
2,4′-[d]	438 ± 2	1
3,3′-[d]	430 ± 5[e]	3
3,4′-[d]	432 ± 5	3
4,4′-[d]	430 ± 10	3
2,3,5-[d]	393 ± 2	1
2,3,3′-[d]	400 ± 5[f]	2
2,4,4′-[d]	437 ± 2	2
2,5,2′-[d]	392 ± 2	1
2,5,3′-[d]	ND	
2,5,4′-[d]	394 ± 2	2

[a] ND, not detected.
[b] See text for details.
[c] 20-h incubation; 62 μM CB.
[d] 24-h incubation; 125 μM CB.
[e] Measured after 2 h; shifted to 410 ± 5 nm during the incubation.
[f] Measured after 1 h; shifted to 370 ± 5 nm during the incubation.

of the *bphD* leader sequence (with a single exchange to create a *Spe*I site) was attached to the stop codon of *bphC* (Fig. 2C). The *bphD* start codon was thereby placed in almost the same position normally occupied by the *bphK* start codon (15). The resulting construct, pAIA3, is shown in Fig. 2B. An adapter containing several restriction sites was inserted into the *Hin*dIII site of pAIA5 (Fig. 2A) to yield pAIA71 (Fig. 2B). pAIA3 and pAIA71 were used to construct pAIA74 containing *bphABCD* as shown in Fig. 2B.

pAIA50 or pAIA74 was introduced into the *E. coli* host strain BL21(DE3)[pLysS], which permits induction of transcription of cloned genes from phage T7 late promoters (23). After induction, resting cells were incubated with single PCB congeners. Assay mixtures were analyzed directly, either by visible spectroscopy or by HPLC and UV spectroscopy.

Conversion of chlorinated biphenyls into phenylhexadienoates. Conversion of all mono- and dichlorinated and six trichlorinated biphenyls into their HOPDAs by *E. coli* cells expressing *bphABC* was assessed spectrophotometrically. All of the mono-, 11 of 12 di-, and 5 of 6 trichlorinated congeners were metabolized to HOPDAs. No HOPDAs were detected for the double *ortho*-substituted 2,6-diCB or for 2,5,3′-triCB (Table 1). While oxidation of 2,5,3′-triCB by biphenyl dioxygenase has been verified by gas chromatography-mass spectrometry (22), it is not clear at present whether 2,6-diCB is attacked by BphA, although oxidation by strain LB400 has been described previously (18).

The 19 congeners that were transformed to HOPDAs may be divided into three classes, according to the rate of conversion. The kinetics of conversion for one representative of each class is shown in Fig. 3. Class 3 congeners, which are substituted in both rings at the *meta* or *para* position (3,3′-, 3,4′-, and 4,4′-diCB), were converted slowly. Remarkably, their more

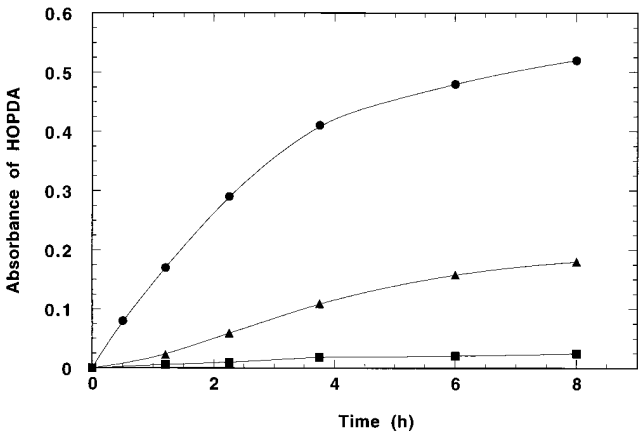

FIG. 3. Time course of conversion of CBs into HOPDAs by resting *E. coli* cells harboring *bphABC*. Absorptions were measured at the maxima of the individual HOPDAs (Table 1). HOPDAs were formed by degradation of 4-CB (circles), 3,3′-diCB (squares), and 2,3,3′-triCB (triangles).

highly chlorinated analogs containing an additional chlorine at an *ortho* position (2,3,3′-, 2,5,4′-, and 2,4,4′-triCB) were converted at moderate rates (class 2 congeners). The residual congeners (class 1) were quickly and probably quantitatively converted into HOPDAs. Although molar extinction coefficients of the HOPDAs are not known, completeness of conversion can be inferred in all but two cases from quantitative production of the corresponding benzoates (see Table 2, below).

Absorption maxima of the HOPDAs formed fell into two distinct wavelength regions lying either between 430 and 440 nm (as for the unsubstituted HOPDA) or between 390 and 400 nm (Table 1). We found a strict correlation between these values and the presence (low λ_{max}) or absence (high λ_{max}) of a chlorine substituent at an *ortho* position in the HOPDA, as deduced from identification of the corresponding CBAs (Table 2). We assume that the *ortho* chlorine prevents a coplanar conformation of the π electron systems of the aromatic ring and the aliphatic side chain, thereby leading to a hypsochromic shift of λ_{max}.

While λ_{max} values generally remained stable, significant shifts towards shorter wavelengths were observed during the incubations of 3,3′-diCB and 2,3,3′-triCB, suggesting a further reaction of the initially formed compounds.

Conversion of CBs into chlorinated benzoates. Conversion of CBs into CBAs by *bphABCD*-harboring *E. coli* cells was routinely analyzed after overnight incubations (20 or 24 h). Kinetics with some class 1 (Table 1) mono- and dichlorinated congeners had indicated that formation of benzoates reached completion within this time (data not shown). CBAs were identified by HPLC and UV spectrum by comparison with authentic standards. The results are summarized in Table 2.

With the exception of 4,4′-diCB, all of the mono- and dichlorinated congeners that were degraded to HOPDAs were also catabolized to benzoates. However, of the five trichlorinated CBs converted into HOPDAs, only two were metabolized into CBAs. Thus, 15 of 21 congeners tested were degraded to CBAs. In 11 cases, CBAs were obtained in virtually quantitative amounts.

Identification of the CBAs produced permitted us to trace back which of the biphenyl rings was attacked by the initial dioxygenase. All mono- and dichlorinated CBs which were converted into CBAs and were substituted in only one ring were found to be oxidized virtually exclusively in the nonhalo-

TABLE 2. Formation of CBAs from various CBs by *E. coli* cells containing *bphABCD*

CB (position[s] of chlorine[s])	CBA	
	Position(s) of chlorine(s)	Yield (%)[a]
2[b]	2	95–100
3[c]	3	95–100
4[b]	4	95–100
2,3[c]	2,3	95–100
2,4[c]	2,4	95–100
2,5[c]	2,5	95–100
2,6[c]		ND
3,4[c]	3,4	95–100
3,5[b]	3,5	95–100
2,2′[b]	2	95–100
2,3′[c]	3	95–100
2,4′[c]	4	95–100
3,3′[c]	3	20–25
3,4′[c]	4	25–30
4,4′[c]		ND
2,3,5[c]	2,3,5	40–45
2,3,3′[c]		ND
2,4,4′[c]		ND
2,5,2′[c]	2,5	35–40
2,5,3′[c]		ND
2,5,4′[c]		ND

[a] ND, not detected.
[b] 20-h incubation, 62 μM CB.
[c] 24-h incubation, 125 μM CB.

genated ring. In those cases of dichlorinated congeners possessing an *ortho*-monochlorinated ring, this ring was the sole target for oxidation. Also with 3,4′-, 2,3,5-, or 2,5,2′-substituted congeners, only one type of CBA was found (Table 2), indicating attack of the *meta*-, non-, or *ortho*-chlorinated ring. However, as turnover into CBAs was incomplete in these cases, additional oxidation of the other ring cannot be ruled out.

For three trichlorinated congeners, no CBAs were detected, but HOPDAs were formed. For these CBs, the observed correlation between λ_{max} and *ortho* chlorine substitution of HOPDAs permitted prediction as to which ring was oxidized. The λ_{max} values observed suggest that 2,3,3′- and 2,5,4′-triCB were oxidized in the monochlorinated rings yielding *ortho*-substituted HOPDAs, whereas 2,4,4′-triCB was oxidized in the dichlorinated ring yielding a *para*-chlorinated HOPDA. These conclusions are consistent with results obtained for the oxidation of 2,3,3′- and 2,4,4′-triCB by strain LB400 itself (4). Again, however, additional oxidation of the other ring not leading to HOPDA formation cannot be ruled out.

DISCUSSION

The results obtained indicate the location of the initial oxidative attack and a correlation between chlorine substitution pattern and degradability of CB congeners specified by the *bph* gene cluster of *Pseudomonas* sp. strain LB400. The *bphA*-encoded biphenyl dioxygenase of strain LB400 is able to oxidize not only the *ortho* and *meta* carbons but also the *meta* and *para* carbons (18, 22). Given the enzymatic repertoire encoded by the cloned *bph* genes, the recombinant strain can produce benzoate only if the initial oxidation is directed towards the *ortho* and *meta* positions. Thus, the percentage of benzoate formation gives a lower limit value for *ortho-meta* oxidation by biphenyl dioxygenase. Our results demonstrate that all mono- and at least 8 of 12 dichlorinated congeners are attacked virtually exclusively in the *ortho-meta* mode. 2,3,5- and 2,5,2′-triCB are at least partly oxidized at these positions. Interestingly, attack at *ortho* and *meta* positions is not necessarily directed towards unchlorinated carbons. With 2,2′- and 2,4′-diCB, virtually quantitative dioxygenation at positions 2 and 3, leading to elimination of hydrochloric acid, was observed (21).

If CBAs are obtained from an asymmetric congener in quantitative yield, their percentage of formation reflects the ratio of oxidation of the two rings by biphenyl dioxygenase. The virtually quantitative formation of only one CBA from asymmetrical congeners demonstrates a high degree of preference of the enzyme for one of the two aromatic rings. The types of CBAs produced indicate that the reactivity of the biphenyl rings towards BphA-catalyzed oxidation decreases according to the following order: non- > *ortho*- > *meta*-substituted. Furthermore, the exclusive formation of 4-CBA from 3,4′-diCB suggests that a *meta*-chlorinated ring is more easily attacked than a *para*-chlorinated one. This is consistent with the observation of poorer HOPDA formation from 4,4′-diCB than from 3,3′-diCB.

For mono- and dichlorinated congeners, the percentages of benzoate formation observed correlate with the chlorine substitution pattern of the metabolized ring. Congeners oxidized in the non- or *ortho*-chlorinated ring yielded benzoates in virtually quantitative amounts. CBs attacked in the *meta*-chlorinated ring were incompletely converted into CBAs. If the *para*-chlorinated ring was dioxygenated, no formation of benzoate was observed. This suggests that the chlorine substitution pattern of the metabolized ring decreases the efficiency of degradation through the entire upper pathway in the order non- ≈ *ortho*- > *meta*- > *para*-substituted. The trichlorinated congeners assayed yielded similar results. Only trichlorinated CBs containing a non- or mono-*ortho*-chlorinated ring were converted into benzoates. The influence of the chlorine substitution pattern of the attacked ring on benzoate formation resembles its influence on reactivity towards BphA-catalyzed oxidation (compare above). This suggests that the rate of CBA formation may frequently be determined by the rate of initial attack.

Data on CB metabolites produced by the parental strain, *Pseudomonas* sp. strain LB400, are rather limited. Comparison of such data (4) with the results obtained with the *bphABCD*-containing *E. coli* strain indicates that they are similar. In both cases, 3- or 4-CBA, but no or only trace amounts of 2-CBA, was detected as metabolites of 2,3′- or 2,4′-diCB. The monochlorinated ring of 2,3,3′-, but the dichlorinated ring of 2,4,4′-triCB, was found to be (predominantly) attacked. However, Bedard and Haberl (4) reported formation of 2,3-diCBA (no quantitation given) or 4-CBA (13%), respectively, from these two congeners, whereas we detected only the HOPDAs. This might be due to an additional HOPDA hydrolase produced by strain LB400, but other explanations such as a higher hydrolase-to-substrate ratio in the experiments of these authors cannot be ruled out. Neither recombinant *E. coli* nor strain LB400 was able to convert 2,5,3′-triCB into an HOPDA. The similarity between the data obtained with the cloned *bph* genes and those with the parental strain is consistent with the view that the isolated *bph* genes specify the catabolic capability of the strain for the conversion of chlorobiphenyls into CBAs.

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