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Heterologous Expression of Biphenyl Dioxygenase-Encoding Genes from a Gram-Positive Broad-Spectrum Polychlorinated Biphenyl Degrader and Characterization of Chlorobiphenyl Oxidation by the Gene Products

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The *bphA1A2A3A4* gene cluster, encoding a biphenyl dioxygenase from *Rhodococcus globerulus* P6, a gram-positive microorganism able to degrade a wide spectrum of polychlorobiphenyls (PCBs), was expressed in *Pseudomonas putida*, thereby allowing characterization of chlorobiphenyl oxidation by this enzyme. While P6 biphenyl dioxygenase activity was observed in *P. putida* containing *bphA1A2A3A4*, no activity was detected in *Escherichia coli* cells containing the same gene cluster. In *E. coli*, transcription of genes *bphB* and *bphC1*, located downstream of *bphA1A2A3A4*, was shown to be driven solely by a vector promoter, which indicated that the lack of biphenyl dioxygenase activity was not due to a lack of mRNA synthesis. Radioactive labelling of *bph* gene products in *E. coli* implied inefficient translation of the *bphA* gene cluster or rapid degradation of the gene products. The biosynthesis of functional P6 biphenyl dioxygenase in *P. putida* cells containing the same plasmid construct that yielded no activity in *E. coli* emphasizes the importance of the host strain for heterologous expression and shows that synthesis, correct folding, and assembly of a *Rhodococcus* biphenyl dioxygenase can be achieved in a gram-negative organism. Dioxygenation of six mono- and dichlorinated PCB congeners by *P. putida* containing the P6 *bphA* gene cluster indicates the following ring substitution preference for this reaction (from most to least preferred): un-, *meta*-, *para*-, and *ortho*-substitution. No indications were found for dioxygenation of *meta*/*para* carbon pairs, or for hydroxylation of chlorinated carbons at any position of a monochlorinated ring, suggesting a strict specificity of this biphenyl dioxygenase for attack at nonhalogenated *ortho*/*meta* vicinal carbons. This contrasts the properties of an analogous enzyme from *Pseudomonas* sp. strain LB400, which can both dioxygenate at *meta* and *para* positions and dehalogenate substituted *ortho* carbons during *ortho* and *meta* dioxygenation.

Polychlorinated biphenyls (PCBs) are toxic, biologically recalcitrant compounds, composed of a biphenyl nucleus carrying from 1 to 10 chlorines. There are 209 theoretically possible PCB congeners. Commercially available PCB mixtures, such as Aroclors, Clophens, or Kanechlors, typically contain between 60 and 80 different congeners (8) differing in the number and position of chlorination. In the past, PCBs were produced in large quantities and used, typically as mixtures, in diverse substances such as plastics, carbon paper, cooling systems, hydraulic fluids, transformers, and fire retardants (8). Their widespread use, heterogeneity, persistence, toxicity, carcinogenic potential, and tendency to bioaccumulate as they ascend the food chain now make them major environmental pollutants (1, 21, 35).

A promising approach for dealing with PCB contamination is bioremediation, optimally through the combined action of anaerobic and aerobic microorganisms (1). Unfortunately, the aerobic PCB degraders studied so far, which belong mostly to gram-negative genera, are generally limited in the range of PCBs they are able to attack. An increase in the spectra of congeners which can be metabolized is essential if the effective potential of bioremediation of PCBs is to be realized.

One group of isolates which break down a wide range of PCBs, in part complementary to those attacked by the better gram-negative degraders, are gram-positive in nature, such as *Rhodococcus globerulus* P6. In this laboratory, *bph* genes of strain P6 have been isolated and characterized extensively (3, 4, 6). These genes were the first characterized genes that encode a biphenyl degradative pathway from a gram-positive organism. The *bph* genes of strain P6 encode a four-step catabolic pathway (Fig. 1) similar to that found in gram-negative organisms (8). Active enzymes responsible for the catabolism from biphenyl-2,3-dihydrodiol (BDHD) to benzoate and hydroxypentadienoate have been found in clones containing the respective genes (4, 6). However, attempts at heterologous expression of the genes encoding the ring-hydroxylating biphenyl dioxygenase (BDO) have so far been unsuccessful. This class IIB three-component dioxygenase (29) catalyzes the first step of the degradation pathway and is thought to be of major importance in determining its substrate spectrum (11, 20). Thus, heterologous expression of BDO-encoding genes is a key step in the development of novel recombinant PCB degraders (e.g., see reference 23). Additionally, it will enable the construction of recombinant strains for detailed analysis of the catabolic pathway (32, 33) and production of pathway inter-

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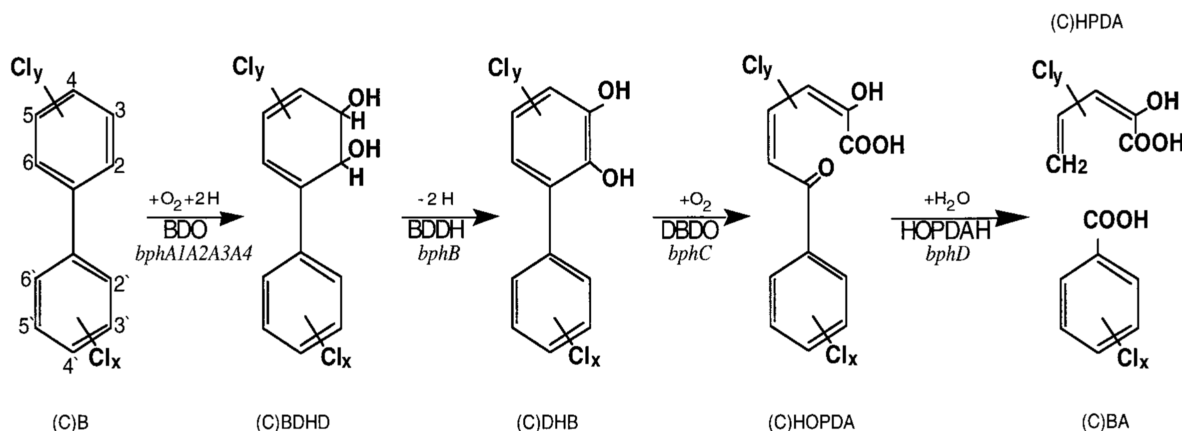

FIG. 1. The upper pathway of (polychloro)biphenyl degradation. The corresponding genes are listed below the abbreviated enzyme names (HOPDAH, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase). Pathway metabolites: (C)B, (chloro)biphenyl; (C)BDHD, (chloro)BDHD; (C)DHB, (chloro)2,3-dihydroxybiphenyl; (C)HOPDA, (chloro)HOPDA; (C)BA, (chloro)benzoic acid; (C)HPDA, (chloro)2-hydroxypenta-2,4-dienoic acid.

mediates for use as substrates in enzymological studies or as fine chemicals (15).

Here we report the functional expression of *Rhodococcus* BDO genes in a gram-negative host strain and its use in characterizing the dioxygenation of mono- and dichlorinated biphenyls by the encoded enzyme.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Pseudomonas putida* KT2442 used in this study is a rifampin-resistant and restriction-deficient derivative of *P. putida* mt-2 (12). *Escherichia coli* DH5α and BL21(DE3)[pLysS], the latter of which harbors the gene encoding T7 RNA polymerase, have been described previously (30, 36, 37). Strains were grown in Luria-Bertani medium unless otherwise indicated. Solid media contained 1.5% purified agar (Difco Laboratories, Detroit, Mich.). Antibiotics (and concentrations) used to select plasmids were chloramphenicol or ampicillin (100 μg/ml) for *E. coli* and chloramphenicol (500 μg/ml) for *P. putida*. Cultures were incubated at 30°C for *P. putida* and 37°C for *E. coli* unless otherwise indicated.

Construction of plasmids and recombinant strains. Plasmids were constructed and introduced into *E. coli* essentially by the standard methods described in reference 30 and introduced into *P. putida* by triparental matings using a filter technique (22). Constructions of plasmids containing *bph* genes from *R. globerulus* P6 (formerly *Acinetobacter* sp. strain P6 or *Corynebacterium* sp. strain MB1 [5]) are described in the Results section. Vectors used were pBluescript II SK and II KS (Stratagene, Heidelberg, Germany) and the broad-host-range vector pBBR1MCS (26). Plasmids pJA18 and pJA22, which are pUC18- and pBluescript II SK-derived subclones from an *R. globerulus* cosmid library (6), served as sources of the *bph* genes. pAIA743, which harbors *bphBCD* genes from *Pseudomonas* sp. strain LB400 downstream of a phage T7 promoter, was derived from pAIA74 (32) by digestion with *Pml*I and *Eco*RV and religation.

Radioactive labelling of insert-encoded proteins. [^{35}S]Met labelling of insert-encoded proteins and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using *E. coli* BL21(DE3)[pLysS] as the host strain were carried out as described previously (24).

Detection of enzymatic activities. The *bphC1* product, 2,3-dihydroxybiphenyl-1,2-dioxygenase (DBDO) was detected by the appearance of the yellow metabolite, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA), following addition to cells of a solution of 2,3-dihydroxybiphenyl (Wako Chemical Co., Tokyo, Japan) as previously described (14). The *bphB* product, biphenyl-2,3-dihydrodiol-2,3-dehydrogenase (BDDH), was detected in strains concomitantly expressing *bphC1* by the formation of HOPDA, following addition to cells of an aqueous solution containing approximately 1 mM BDHD. This compound was enzymatically prepared from biphenyl with a resting-cell culture of *E. coli* BL21 (DE3)[pLysS, pAIA11] that expresses genes *bphA1A2A3A4* of *Pseudomonas* strain LB400 (33). The product of the *bphA* gene cluster (BDO) was detected in strains concomitantly expressing *bphBC* by the formation of HOPDA, following addition to cells of a 7.5% solution containing biphenyl (dimethyl sulfoxide: ethanol, 1:1).

Resting-cell assays. Resting cell suspensions of *P. putida* KT2442[pDM10] were prepared by growing cells for 24 h on Luria-Bertani–chloramphenicol plates and suspending the cells in 50 mM sodium phosphate buffer (pH 7.5) to an optical density at 600 nm of 20 or 40. Suspensions (1 ml) were incubated with single PCB congeners at nominal concentrations between 0.25 and 2 mM (for details, see Table 2) for 18 h at 30°C on a gyratory shaker. When further conversion of initial metabolites to benzoates was required, supernatants of these incubations (0.5 ml) were added to equal volumes of resting-cell suspensions of *E. coli* BL21(DE3)[pLysS, pAIA743] at an optical density of 600 nm of 15, which were prepared as described previously (32). The cells were incubated for 24 h as described above. With 3,4′-chlorobiphenyl (3,4′-CB), conversion into chlorobenzoate (CBA) was carried out differently. This congener (nominal concentration, 125 μM) was incubated simultaneously with equal volumes of resting cells of *P. putida* KT2442[pDM10] and *E. coli* BL21(DE3)[pLysS, pAIA743]. After incubations, resting cells were pelleted and supernatants were filtered as previously described (32).

GC-MS analysis of metabolites. Extraction of resting-cell supernatants and drying and evaporation of solvents were carried out as previously described (33). To obtain butylboronate derivatives, dried extracts were dissolved in 80 μl of acetone, 20 μl of a 2-mg/ml solution of *n*-butylboronic acid in acetone was added, and the solution incubated at 50°C for 10 min (25). After derivatization, mixtures were evaporated to dryness under a stream of nitrogen and dissolved in 10 or 20 μl of *n*-octane. Samples (1 μl) were injected in the splitless mode into a gas chromatography-mass spectrometry (GC-MS) system: either a Hewlett-Packard 5890 series II gas chromatograph with an Rtx1 column (Restek, Bellefonte, Pa.) coupled to a Hewlett-Packard 5989A mass spectrometer, or a Shimadzu GC-17A gas chromatograph with an XTI-5 column (Restek, Bellefonte, Pa.) coupled to a Shimadzu QP-5000 mass spectrometer. Helium was used as the carrier gas. The mass spectrometers were operated in the electron ionization mode.

High-performance liquid chromatography-UV analysis of metabolites. This technique was carried out as previously described (32). CBAs were identified by comparison with authentic standards.

RESULTS

Cloning of *bph* genes from *R. globerulus* P6 in different expression vectors. To simplify detection of BDO activity encoded by genes *bphA1A2A3A4* from *R. globerulus* P6, the *bphA* gene cluster was cloned with genes *bphB* and *bphC1*, which encode BDDH and DBDO, respectively. Thus, in clones expressing all six genes, dioxygenation of biphenyl can be conveniently detected by conversion into yellow HOPDA (Fig. 1). Plasmids pDM3, pDM6, and pDM7 were constructed in a two-step fashion as shown in Fig. 2. Three different vectors which contain the same multiple-cloning site but differ in the orientation of vector promoters, RNA leader sequences, or host ranges were used. In this way, genes *bphA1A2A3A4BC1* were cloned downstream of (i) a phage T7 late promoter in pSK, yielding pDM6, (ii) the *lac* promoter in pKS, yielding pDM3, or (iii) the *lac* promoter in the broad-host-range vector pBBR1MCS, yielding pDM7. For experiments involving only BDO activity, a further plasmid, pDM10, containing only genes *bphA1A2A3A4*, was constructed in a fashion similar to that of the other plasmids (Fig. 2).

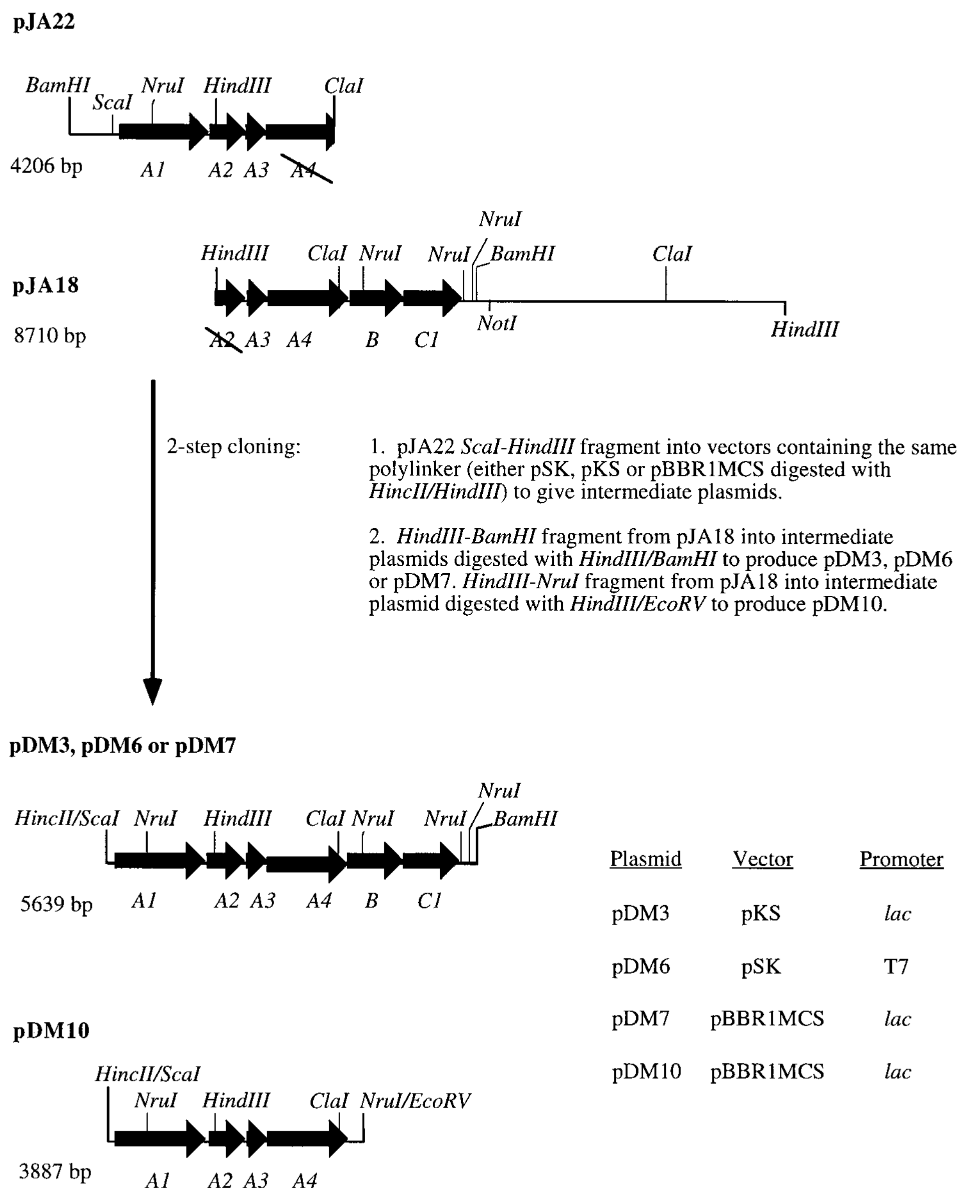

FIG. 2. Construction of plasmids for expression of genes *bphA1A2A3A4BC1* from *R. globerulus* P6. A stroke through a gene designation indicates truncation of the gene.

Expression of *bphA1A2A3A4* from *R. globerulus* P6 in *E. coli*. In the case of *E. coli* DH5α containing pDM3, both BDDH and DBDO activity was detected (Table 1), indicating expression of *bphB* and *bphC1*. In contrast, BDO activity, encoded by the *bphA* gene cluster, was observed neither by measuring O_2 consumption nor by monitoring HOPDA formation. This lack of detectable BDO activity could be due to the *bph* gene cluster being cloned downstream of the relatively weak *lac* promoter.

TABLE 1. Enzyme activities of various host-plasmid systems containing *bphA1A2A3A4BC1* from *R. globerulus* P6

Plasmid-host system component				Activity[a]		
Plasmid[b]	Vector	Promoter	Host	BDO	BDDH	DBDO
pDM3	pKS	*lac*	*E. coli* DH5α	−	+	+
pDM6	pSK	T7	*E. coli* DH5α	−	−	−
pDM6	pSK	T7	*E. coli* BL21(DE3)[pLysS]	−	+	+
pDM7	pBBR1MCS	*lac*	*E. coli* DH5α	−	+	+
pDM7	pBBR1MCS	*lac*	*P. putida* KT2442	+	+	+

[a] Activities were scored on the appearance (+) or absence (−) of HOPDA, as described in Materials and Methods.
[b] All plasmids contain the gene cluster *bphA1A2A3A4BC1*, which encodes the enzymes BDO, BDDH, and DBDO.

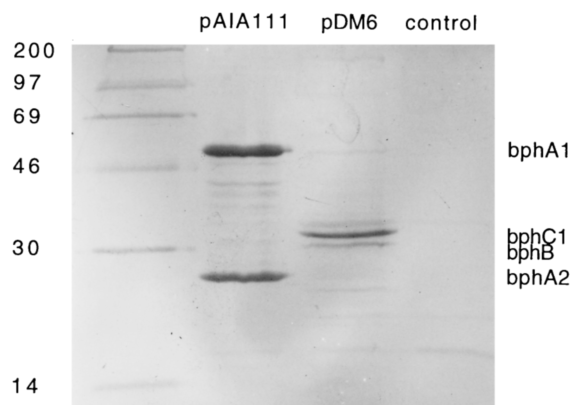

FIG. 3. SDS-PAGE of ^{35}S-labelled soluble proteins of *E. coli* BL21 (DE3)[pLysS] containing recombinant plasmids carrying *bph* genes. pAIA111 encodes *bphA1A2A3A4* from *Pseudomonas* sp. strain LB400. pDM6 encodes *bphA1A2A3A4BC1* from *R. globerulus* P6. The control lane shows the background of the host strain without a *bph*-containing plasmid. Molecular masses (in kilodaltons) of marker proteins (left lane) are indicated.

However, BDO activity was also undetectable when *bphA1A2A3A4BC* was cloned downstream of the strong T7 promoter (pDM6) and introduced into *E. coli* BL21(DE3) [pLysS], which contains the gene for T7 RNA polymerase.

The lack of BDO activity could originate from a number of factors, such as inefficient synthesis or efficient degradation of gene products, the synthesis of inactive proteins which may originate from improper assembly of polypeptides, or the requirement for an additional factor (or factors) not supplied by the host strain.

To investigate the cause of the lack of BDO activity, translation products of T7 promoter-initiated transcripts were radioactively labelled in vivo and visualized after SDS-PAGE (Fig. 3). Plasmid pAIA111, a *bphK* mutant of pAIA11 (33) that contains genes *bphA1A2A3A4* of *Pseudomonas* sp. strain LB400 under control of a T7 promoter, was used as positive control. While bands corresponding to the *bphA3* and *bphA4* products of this gene cluster are normally not detectable under these conditions (24), bands representing the *bphA1* and *bphA2* gene products (24) were readily visible. In the case of pDM6, bands corresponding in mobilities to *bphA* gene products did not stand out above background while the products of *bphB* and *bphC1* (apparent molecular masses, 30 and 32 kDa, respectively) were clearly visible. The lack of readily observable *bphA* gene products suggests inefficient transcription or translation of these genes or rapid degradation of their products.

To investigate whether or not the problem lies at the level of transcription of *bphA1A2A3A4* genes, strain DH5α[pDM6], which does not contain the gene for T7 RNA polymerase, was used to assay BDDH and DBDO activities. The lack of these activities (Table 1) indicates that the expression observed for *bphB* and *bphC1* is driven solely by the T7 promoter and is not due to transcription from a promoter upstream of *bphB* but downstream of *bphA1*. Consequently, detectable activities of the *bphB* and *bphC1* gene products in strain BL21 (DE3)[pLysS, pDM6] demonstrate transcription also of *bphA1A2A3A4*. This suggests that the problem of *bphA* expression is posttranscriptional.

Expression of *bphA1A2A3A4* from *R. globerulus* P6 in *P. putida*. The activity of foreign gene products may differ in different backgrounds. *P. putida* was therefore assessed as an alternative host. The gene cluster *bphA1A2A3A4BC1* was cloned into the broad-host-range vector pBBR1MCS to generate plasmid pDM7 (Fig. 2), and the corresponding enzyme activities encoded by these genes (BDO, BDDH, and DBDO) were assayed in both *E. coli* and *P. putida* (Table 1). All enzymes were detected in the *P. putida* KT2442 host, whereas only BDDH and DBDO activities were detected in the *E. coli* host. Interestingly, BDO activity was higher in *P. putida* grown on solid medium than in cells grown in liquid medium; this is currently being investigated further.

Characterization of dioxygenation of CBs by the *bph*-encoded BDO from *R. globerulus* P6. Biosynthesis of the *bph*-encoded BDO of *R. globerulus* P6 in a heterologous background enabled the characterization of the biodegradative potential of this enzyme. To investigate CB oxidation, resting cells of strain KT2442[pDM10] were incubated with each of three mono- and three dichlorinated CBs which were selected to deduce information about the ring preference and potential of the BDO to attack chlorinated carbons. The products of dioxygenation were characterized by GC-MS and, after conversion to CBAs, by high-performance liquid chromatography. Control experiments clearly showed that strain KT2442 is not able to oxidize CBs. The characterization of dioxygenation products is summarized in Table 2, giving some clear indications of the way in which this BDO oxidizes substituted biphenyls.

When 2-, 3-, and 4-CB were offered as substrates, only single, monochlorinated BDHDs were found as products. Coinjection of these metabolites with the previously characterized 2′,3′-dihydroxylated BDHDs formed from these same CBs with the *bph*-encoded BDO from strain LB400 (32, 33) in each case yielded only a single GC peak (data not shown), indicating the identity of the BDHDs formed by the two BDOs. This was corroborated by converting these products to benzoates with a resting-cell suspension of strain BL21(DE3)[pLysS, pAIA743], which contains the LB400 *bphBCD* genes. Complete disappearance of the BDHDs (analysis by GC-MS) indicated that they were substrates of the LB400 BDDH, which is specific for *ortho*- and *meta*-dihydroxylated BDHDs (20). The appearance of CBAs further supported this result, as attack at *meta* and *para* carbons cannot give rise to benzoates. Formation of only 2-, 3-, or 4-CBA indicated oxidation of the unchlorinated ring only (cf. Fig. 1).

When dichlorinated CBs were used as potential substrates, no oxidation product of 2,2′-CB was observed under the conditions employed, suggesting that *ortho*-chlorinated rings are poor substrates for this dioxygenase. In the case of 3,4′-CB, a single dichlorinated BDHD was observed, and conversion of this BDHD by resting cells of BL21(DE3)[pLysS, pAIA743] produced only 4-CBA, thus indicating that the DHD was produced by dioxygenation of the *meta*-substituted ring at carbons 5 and 6. However, the P6 BDO is also able to attack *para*-substituted rings, as evidenced by the dioxygenation of 4,4′-CB to a single dichlorinated BDHD. As this reaction did not involve a chlorinated carbon, which would have yielded a monochlorinated dihydroxybiphenyl, dioxygenation of this symmetrical congener must have taken place at *ortho* and *meta* positions.

In summary, the *bph*-encoded BDO of strain P6 dioxygenated only *ortho* and *meta* carbons with the CBs tested; there was no evidence of *meta* and *para* dioxygenation. The enzyme displayed the following chloro-substitution preference for ring dioxygenation (from most to least preferred): un-, *meta*-, *para*-, and *ortho*-substitution. It remains to be investigated to what extent, if any, the attack preference is influenced by the substitution pattern of the nonoxidized ring. There was no indication of oxidation of chlorinated carbons with concomitant

TABLE 2. Characterization of CB metabolites formed by the *bph*-encoded BDO of *R. globerulus* P6

Substrate	No. of possible products[a]	No. of observed products	T_r (min) of derivative[b]	Molecular mass of derivative[c]	Product no. of chlorines	Type of compound	Substrate for BDDH[d]	Conversion into CBA[e]	Assignment of oxidized carbons
2-CB[f]	6	1	21.3[i]	288	1	DHD	+	2-CBA	2′,3′
3-CB[g]	6	1	23.2[i]	288	1	DHD	+	3-CBA	2′,3′
4-CB[g]	4	1	23.5[i]	288	1	DHD	+	4-CBA	2′,3′
2,2′-CB[h]	4	0							
3,4′-CB[f]	6	1	25.1[j]	322	2	DHD	+	4-CBA	5,6
4,4′-CB[f]	2	1	25.5[j]	322	2	DHD	ND[k]	ND	2,3[l]

[a] Assuming dioxygenation of all vicinal carbons except for positions 1 and 1′.
[b] T_r, retention time, as determined by GC.
[c] As determined by MS.
[d] The indicated substrate was (+) or was not (−) a substrate for *bph*-encoded BDDH of *Pseudomonas* sp. strain LB400.
[e] By the respective *bph*-encoded enzymes of *Pseudomonas* sp. strain LB400.
[f] Nominal concentration, 2 mM.
[g] Nominal concentrations, 0.5 and 2 mM.
[h] Nominal concentrations, 0.25, 0.5, and 2 mM.
[i] Measured with the Shimadzu GC system.
[j] Measured with the Hewlett-Packard GC system.
[k] ND, not determined.
[l] Only one possibility without dechlorination.

elimination of HCl (20, 23, 33). Attack of *meta*- and *para*-chlorinated rings (3,4′- and 4,4′-CB) did not lead to dechlorination, and there is no conclusive evidence whether attack of the *ortho*-chlorinated ring could lead to dechlorination, since significant attack of 2,2′-CB was not observed.

DISCUSSION

In this paper we report on the expression of genes encoding a BDO from a gram-positive microorganism of the genus *Rhodococcus* in a gram-negative host strain and on the use of this heterologous expression system to analyze the type of attack and the chloro-substitution preference of this ring-activating enzyme.

Despite several attempts, we were not successful in obtaining recombinant *E. coli* cells expressing the *bphA1A2A3A4* gene cluster of strain P6. Difficulties in detecting activity of *Rhodococcus* BDOs in *E. coli* have also been reported by other groups working with similar systems. Masai et al. (28) cloned *bphA1A2A3A4* from *Rhodococcus* sp. strain RHA1 in pUC vectors and in expression vectors pKK223-3 and pTrc99A. BDO activity was not detected in vivo, although synthesis of the *bphA1*, *bphA2*, and *bphA4* gene products was observed by using pUC vectors in an in vitro transcription-translation system. Wang et al. (38) reported only very weak expression of closely related BDO-encoding genes from *Rhodococcus* sp. strain M5 in pUC18 and the T7 expression vector pT7-5. Failures to express other *Rhodococcus* genes in *E. coli* have occasionally been reported in the literature (10, 34). However, the causes of these problems have not been investigated. Our experiments clearly show that the deficit of BDO activity in *E. coli* is not due to a lack of transcription. A paucity of observable radioactively labelled *bph* translation products indicated either rapid degradation of products or inefficient translation of at least genes *bphA1* and *bphA2*.

Inefficient translation may be due to efficient degradation of the mRNA or to inefficient recognition of the (or a) ribosome binding site (RBS) which can originate from the formation of an interfering RNA secondary structure (9, 19, 27). Using the Wisconsin Package (version 8; Genetics Computer Group, Madison, Wis.), we analyzed one of our vector-insert fusions for potential secondary structure formation around the first RBS of the closely spaced *bphA* genes. Frequently, the first RBS of a polycistronic messenger is more crucial than the other downstream RBSs, because secondary structure formation at RBSs of closely spaced downstream genes is less likely to occur, as ribosomes translating an upstream gene tend to destabilize or prevent the formation of downstream secondary structures. Our analysis did not reveal the presence of obvious translationally hindering structures in the region of the first RBS.

Successful BDO biosynthesis in *P. putida*, using the same plasmid construct used for *E. coli*, clearly demonstrates that the host organism can play a crucial role in gene expression. Activity of the *Rhodococcus* BDO was much higher in *P. putida* cells harvested from plates than in those harvested from liquid cultures. Whether or not this effect is related to the lack of activity in *E. coli* remains to be elucidated.

Gene cloning enabled us to investigate CB dioxygenation specifically attributable to the *bph*-encoded BDO of *R. globerulus* P6. Several years ago, Furukawa and coworkers reported on CB degradation by the parental strain, then called *Acinetobacter* sp. strain P6 (13, 16–18). A comparison between the results is possible only to a limited extent. The generation of CBAs from 2-, 3-, 4-, and 4,4′-CB by strain P6 agrees with our findings. However, while Furukawa et al. found some conversion of 2,2′-CB into CBA, we did not observe significant attack of this congener. We also note that the Furukawa group detected the formation of dichlorodihydroxy compounds from 2,5,3′-, 2,5,4′-, 2,4,4′-, and 3,4,2′-CBs by strain P6, indicating dechlorination. In contrast, we did not observe that the *bph*-encoded BDO dechlorinated any of the dichlorinated CBs we tested. Although dioxygenation of trichlorinated congeners by the enzyme remains to be investigated, it seems unlikely that with four different congeners a higher degree of chlorination will result in dehalogenation since, usually, higher degrees of chlorination result in CBs that are less susceptible to attack. Rather, the dechlorination observed by Furukawa et al. may indicate the presence of additional dioxygenases or of other dehalogenating activity in this organism or in the culture used. We note that three different DBDO genes in this strain have already been reported (6) and that two microbes designated *Arthrobacter* sp. strain M5 and *Corynebacterium* sp. strain MB1 were later isolated from cultures of *Acinetobacter* sp. strain P6 (7).

R. globerulus P6 and *Pseudomonas* sp. strain LB400 are two

of the most effective PCB degraders known. They are able to transform a wide, yet partially complementary range of PCB congeners. It is therefore interesting to compare the substrate and product spectra of the BDOs from both enzymes. The P6 BDO more efficiently dioxygenates *meta*- and *para*-monochlorinated rings but less efficiently attacks *ortho*-monochlorinated rings. The converse is true for the LB400 enzyme, which is consistent with the proposal that the initial enzymes of PCB catabolic pathways are major determinants of substrate specificity. In contrast to the LB400 BDO, which attacks chlorinated *ortho*-carbons, thereby dehalogenating *ortho*-monochlorinated rings (20, 23, 33), the P6 BDO appears not to attack chlorinated *ortho*-, *meta*-, or *para*-carbons and therefore seems not to be capable of dehalogenation. There is no evidence that the P6 BDO catalyzes *meta* and *para* hydroxylation, as is the case with the LB400 BDO with some congeners possessing 3- and 2,5-chlorinated rings (20, 31), thereby producing dihydrodiols which do not serve as substrates for the next enzyme in the metabolic pathway.

Ahmad et al. (2) incubated *E. coli* cells containing the *bphAB* gene cluster of *P. testosteroni* B-356 with 2,4′- and 4,4′-CBs and observed partly dechlorinated dihydroxy-CBs by GC-MS analysis after trimethylsilylation. One hypothesis they advanced to explain this observation was that *ortho* and *meta* dioxygenation of the *para*-chlorinated ring resulted in an unstable dihydrodiol which spontaneously eliminates HCl. Our results indicate, however, that the 2,3-dihydro-2,3-dihydroxy-4-chlorophenyl ring is chemically stable since no elimination product was observed.

The functional expression of the P6 *bphA* gene cluster in *P. putida* now opens up the way to construct bacteria able to degrade a spectrum of PCB congeners wider than previously possible and to further investigate why some *Rhodococcus* genes are so difficult to express in gram-negative bacteria.

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