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Transformation of polychlorinated biphenyls by a novel BphA variant through the *meta*-cleavage pathway

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Abstract

The transformation of 20 polychlorinated biphenyls (PCBs) through the *meta*-cleavage pathway by recombinant *Escherichia coli* cells expressing the *bphEFGBC* locus from *Burkholderia cepacia* LB400 and the *bphA* genes from different sources was compared. The analysis of PCB congeners for which hydroxylation was observed but no formation of the corresponding yellow *meta*-cleavage product demonstrated that only lightly chlorinated congeners including one tetrachlorobiphenyl (2,2′,4,4′-CB) were transformed into their corresponding yellow *meta*-cleavage products. Although many other tetrachlorobiphenyls (2,2′,5,5′-CB, 2,2′,3,5′-CB, 2,4,4′,5-CB, 2,3′,4′,5-CB, 2,3′,4,4′-CB) and one pentachlorobiphenyl (2,2′,4,5,5′-CB) tested were depleted from resting cell suspensions, no yellow *meta*-cleavage products were observed. For most of these congeners, dihydrodiol compounds accumulated as the endproducts, indicating that the *bphB*-encoded biphenyl-2,3-dihydrodiol-2,3-dehydrogenase is a key limiting step for further degradation of highly chlorinated congeners. These results suggest that engineering the biphenyl dioxygenase alone is insufficient for an improved removal of PCB. Rather, improved degradation of PCBs is more likely to be achieved with recombinant strains containing metabolic pathways not only specifically engineered for expanding the initial dioxygenation but also for the mineralization of PCBs.

Keywords: Polychlorinated biphenyl; Biphenyl dioxygenase; *Meta*-cleavage product

1. Introduction

Polychlorinated biphenyls (PCBs) are toxic pollutants widely distributed in the environment [1]. Despite their recalcitrant character, chlorinated biphenyls can undergo microbial transformation under both aerobic or anaerobic (reductive dechlorination) conditions by different metabolic pathways. Under aerobic conditions, PCBs are metabolized through the *meta*-cleavage pathway encoded by the *bph* operon described in many Gram-negative and to a lesser extend in Gram-positive bacteria [2–4]. Although this pathway has been well-described for many microorganisms, the extent of PCB metabolism (i.e. endproducts formed) has been analyzed only in a few cases and mainly for lightly chlorinated PCBs [5–7]. In many studies, the disappearance of specific PCB congeners in growing or resting cell suspensions is taken to assess microbial degradation.

Biphenyl dioxygenase (BDO), the first enzyme in-

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volved in aerobic degradation of PCBs, has been extensively studied. This enzyme introduces two oxygen atoms preferentially at the 2,3-position of the biphenyl ring. Depending on the chlorine substitution, dioxygenation can also occur at the 3,4-position as is the case for the BDO of *Burkholderia cepacia* LB400 [8]. Furthermore, for congeners with both *ortho*-positions substituted with chlorine (e.g. 2,2′-CB, 2,2′,5-CB), dioxygenation at one of the *ortho*-substituted carbons results in spontaneous dechlorination [9,10].

In contrast to these detailed studies on the substrate range of BDOs, only limited information is available on the recognition of dioxygenated PCBs by the enzymes acting downstream of BDO. Although the crystal structures of the *bphB*-encoded biphenyl-2,3-dihydrodiol-2,3-dehydrogenase [11] and the *bphC*-encoded 2,3-dihydroxybiphenyl-1,2-dioxygenase [12] offer insights into the catalytic mechanism and substrate binding, they allow only vague predictions regarding specificities for PCB congeners. Easily degradable, lightly chlorinated PCBs including several trichlorobiphenyls have been studied for the transformation into *meta*-cleavage products and chlorobenzoic acids, respectively [5,13]. However, data on metabolites accumulating from transformation of more recalcitrant PCBs are limited. In previous works [14,15], the metabolism of PCBs by *Alcaligenes* sp. Y42 and *Acinetobacter* sp. P6 was investigated for a variety of PCB congeners. However, the possible involvement of more than one metabolic pathway for the transformation of PCBs in these organisms did not allow for determination of the substrate range of any individual enzyme involved.

The substrate range of several BDOs has been modified by both rational [16,17] and directed evolution approaches [18,19]. Previously, we produced several chimeric biphenyl dioxygenases by DNA shuffling using the *bphA* genes from *B. cepacia* LB400 and *Pseudomonas pseudoalcaligenes* KF707 as templates [18]. One variant (S4.43) in particular exhibited an equal activity towards many *para*- and *ortho*-substituted PCBs as well as improved degradation capabilities for several tetra- and penta-chlorinated PCBs when compared with the parental enzymes. In addition, dioxygenated 4,4′-CB was recognized by the LB400 BphBC as seen by the formation of yellow *meta*-cleavage products, suggesting that the substrate range of BphBC may be far more relax than that of the biphenyl dioxygenase. To assess whether the extended capability for dioxygenation can result in further metabolism of PCBs, the metabolites accumulating from a broad range of PCBs were investigated by using *Escherichia coli* strains harboring gene cassettes containing the *bphEFGBC* locus of the LB400 strain and the *bphA* gene from either the LB400, KF707 or variant S4.43.

2. Materials and methods

2.1. Strains and plasmids

E. coli JM105 was used. Strain wt400 and wt707 contained the recombinant low copy number plasmid pAC31 harboring the *bphEFGBC* genes from *B. cepacia* LB400 and the *bphA* gene of the same strain or from *P. pseudoalcaligenes* KF707, respectively [18]. Strain S4.43 co-expressed a chimeric *bphA* gene previously obtained from shuffling of the *bphA* genes of the above-mentioned parental strains together with the *bphEFGBC* genes from the LB400 strain [18].

2.2. Resting cell assays

Depletion of PCB congeners was determined in resting cell assays. *E. coli* strains were grown in LB medium in the presence of 20 μg ml^{-1} kanamycin at 37°C and 300 rpm to an OD_{600} of 0.8, induced with 1 mM IPTG and grown for another 3 h. Cells were centrifuged at 5000 rpm for 5 min, washed once with 50 mM Tris-buffer, pH 7.5, then suspended in 50 mM Tris, pH 7.5, to give an OD_{600} of 1.0. 1 ml of the cell suspension was incubated with two different synthetic PCB mixes containing each congener at 1 ppm. Mix 1 was described previously [18]. Mix 2 contained the following congeners: 2-CB, 2,3-CB, 2,2′,5-CB, 2,2′,6,6′-CB, 2,4′,5-CB, 2,2′,4,6,6′-CB, 2,3′,4,4′,6-CB and 3,3′,4,4′-CB. The internal standard 2,2′,4,4′,6,6′-hexachlorobiphenyl was added to the synthetic PCB mix at 1.5 ppm. The reactions were carried out in sealed 4.5-ml glass vials on a rotary shaker at 150 rpm and 30°C for 24 h. PCBs were extracted twice with 0.5 ml hexane and combined extracts were analyzed on a Shimadzu gas

chromatograph/mass spectrometer QP-5000 (Shimadzu Corporation, Kyoto, Japan) as described [18].

2.3. Analysis of PCB metabolites and derivatization

E. coli cells were grown at 37°C and 300 rpm in 1 ml of supplemented M9 medium [18] in the presence of 20 μg ml^{-1} kanamycin until an OD_{600} of 0.8 was reached, induced with 1 mM IPTG and incubated for another 2 h. PCB solution in acetone was added to give a final concentration of 0.5 mM. The yellow *meta*-cleavage product formed from individual PCBs was determined spectrophotometrically in the cell-free supernatant of an overnight culture [16]. Hydroxylated PCBs were extracted with ethylacetate from a 1-ml overnight culture, derivatized with *n*-butyl boronoic acid (Sigma, St. Louis, MO, USA) as described [10] and analyzed by gas chromatography-mass spectrometry (GC-MS) using a 60-m capillary DB1 column (J&W Scientific, Folsom, CA, USA) with an inner diameter of 0.32 mm. The temperature program ran from 240 to 310°C using the following ramping conditions: 240°C for 1 min, 6°C min^{-1} to 310°C and 310°C for 15 min. Attempts to detect the molecular ions of TMS-derivatized dihydodiol compounds of higher chlorinated PCBs were not successful possibly due to degradation of the TMS-derivatized dihydrodiol compounds of higher chlorinated PCBs. In many cases, the fragment with the highest molecular mass may correspond to the derivatized monohydroxy compounds $[M\text{-}HOTMS]^+$.

3. Results and discussion

Depletion of PCB congeners was studied using resting cells of three recombinant *E. coli* strains (wt400, wt707 and S4.43). These recombinants carried the *bphEFGBC* genes from *B. cepacia* LB400 plus the *bphA* gene of either the same *B. cepacia* organism, *P. pseudoalcaligenes* KF707 or a chimeric *bphA* gene. The chimeric BDO of strain S4.43 was previously obtained by shuffling of LB400 and KF707 *bphA* sequences and exhibits an extended substrate range for PCBs [18]. As expected, strain S4.43 depleted nearly all congeners more efficiently from cell suspensions than either wt400 or wt707 (Fig. 1A). However, formation of the yellow *meta*-cleavage products was detected in the cell-free culture supernatant only for mono-, di- and tri-chlorinated PCBs and one tetrachlorobiphenyl (2,2′,4,4′-CB), an observation in line with previous works with *E. coli* cells expressing *bphAEFGBC* of the LB400 strain [5,13]. These results clearly indicated that improved dioxygenation by biphenyl dioxygenase alone is insufficient to provide improved degradation of PCBs.

To investigate which step along the degradation pathway was limiting, several congeners which were depleted but with no formation of the yellow *meta*-cleavage product (e.g. 2,2′,5,5′-CB, 2,2′,3,5′-CB, 2,4,4′,5-CB, 2,3′,4′,5-CB, 2,3′,4,4′-CB and 2,2′,4,5,5′-CB) were subjected to GC-MS analyses of derivatized metabolites. In all cases, only dihydrodiol compounds were found to accumulate in the supernatant, indicating that the LB400 2,3-dihydro-2.3-dihydroxybiphenyl dehydrogenase (BphB) limits further transformation of these dioxygenated PCBs. For 2,2′,4,5,5′-CB, it is possible that 3,4-dioxygenated PCBs were produced (Fig. 2), although it was difficult to differentiate between 2,3- or 3,4-dioxygenated compounds directly on GC-MS. If dioxygenation occurred at the 2,3-position, then, this would lead to a very unstable intermediate and elimination of hydrochloric acid would spontaneously occur as shown previously [10]. However, the mass spectrum indicates that it is an intact dihydrodiol compound with five chlorines which would suggest the formation of a 3,4-dioxygenated product. The inability of the LB400 BphB to catalyze the conversion of 3,4-dioxygenated PCBs has recently been reported [11]. In addition, the 2,3-dihydroxybiphenyl dioxygenase (BphC) of LB400 does not exhibit activity towards 3,4-dihydroxybiphenyl [20]. Therefore, further metabolism of these congeners would require enzymes capable to transform 3,4-dioxygenated PCBs. Congeners such as 2,3′,4,4′-CB or 2,4,4′,5-CB have likely undergone dioxygenation at an open *ortho-meta*-position, resulting in the accumulation of dihydrodiol products (Fig. 1).

Interestingly, strains wt707 and S4.43 were superior over the wt400 strain in transforming several double *para*-substituted congeners (4,4′-CB and 2,4,4′-CB), indicating that the LB400 BDO, but not the *bphB*- and *bphC*-encoded enzymes, is critical for the transformation of these congeners. It is noteworthy that absorbance maxima of the *meta*-cleavage

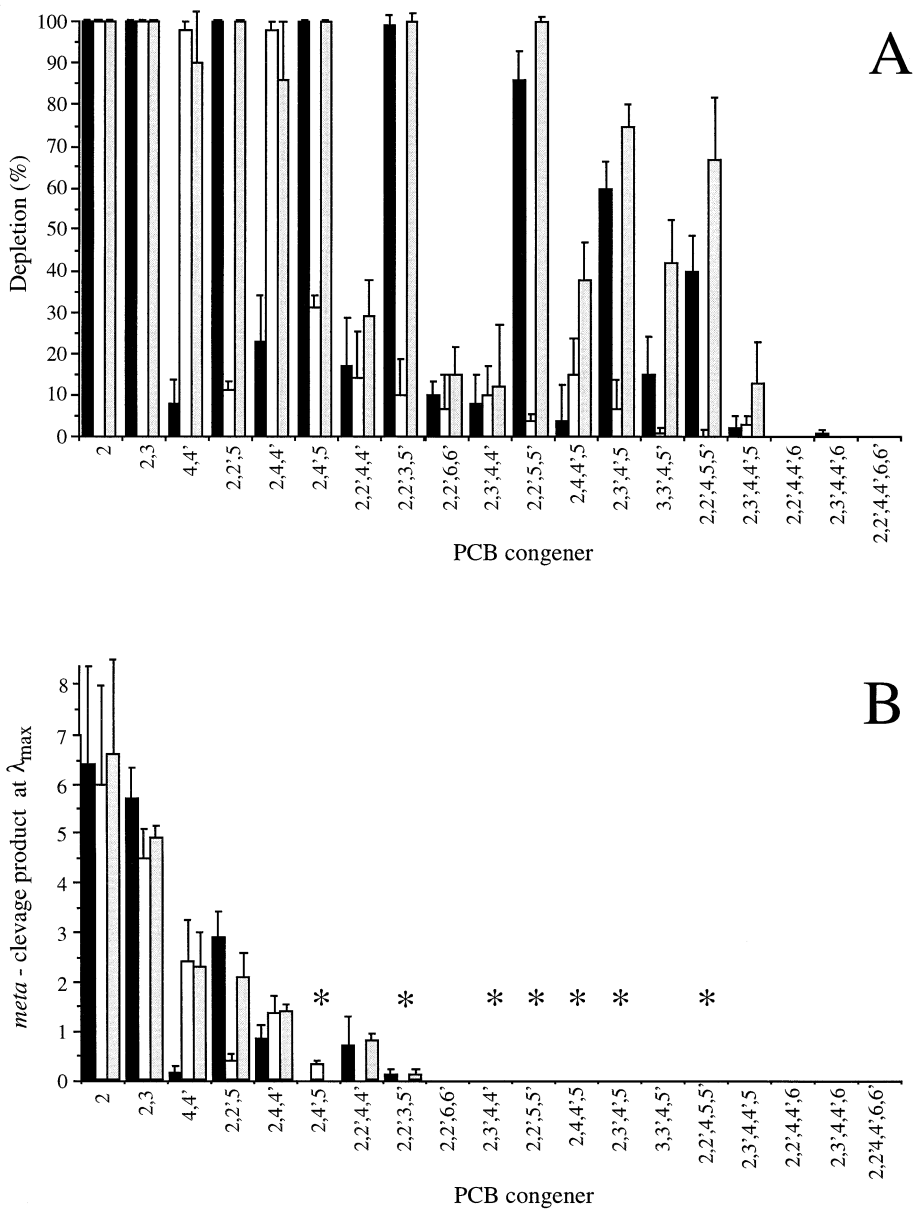

Fig. 1. (A) Depletion of PCB congeners by resting cells of recombinant *E. coli* strains expressing the *bphEFGBC* of *B. cepacia* LB400 and either *bphA* of the same strain (wt400, ■) or from *P. pseudoalcaligenes* KF707 (wt707, □) or a chimeric biphenyl dioxygenase (S4.43, ▨). Conditions for the assay were described in Section 2. The reactions were carried out in sealed 4.5-ml glass vials on a rotary shaker at 150 rpm and 30°C for 24 h. Values are the mean (±S.D.) of three independent experiments. (B) Formation of the yellow *meta*-cleavage product from individual PCBs by strain wt400 (■), wt707 (□) and S4.43 (▨). Yellow *meta*-cleavage product was determined spectrophotometrically by measuring the absorbance at 398 nm for *ortho*-substituted products and at 434 nm for *para*-substituted products. The asterisks indicate detection of the dihydrodiol compounds produced by strain S4.43. Values are the mean (±S.D.) of three independent experiments.

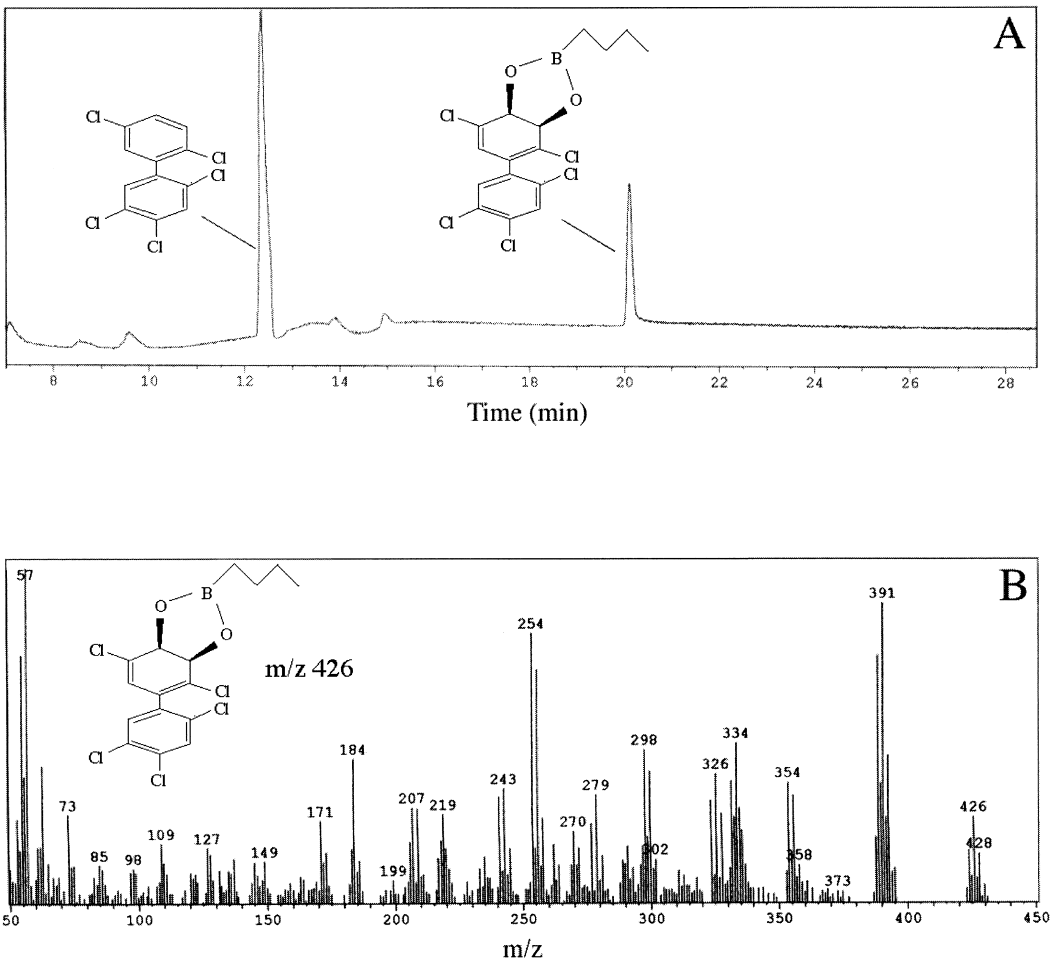

Fig. 2. GC-MS analysis of the dihydrodiol compound formed from 2,2′,4,5,5′-CB by strain S4.43. (A) Total ion chromatogram. (B) Mass spectrogram of the *n*-butyl boronate derivative of the dihydrodiol compound eluting after 20.1 min.

products from 2,4,4′-CB differed for strain wt400 (434 nm), wt707 (398 nm) and S4.43 (398 nm). It was shown previously that a chlorine substitution at the *ortho*-position of *meta*-cleavage products lowered their absorbance maxima [5]. This suggests that dioxygenation of 2,4,4′-CB occurred either at the 5,6-position (434 nm) or at the 2′3′-position (398 nm). Thus, depending on the congeners, the mode of dioxygenation of strain S4.43 mimics either that of LB400 (2,4′,5-CB) or KF707 (2,4,4′-CB).

The results obtained with three different recombinant *E. coli* strains show that only a limited number of the dioxygenated PCB congeners tested were further transformed by LB400 enzymes acting downstream of BDO. This is particularly true for highly chlorinated PCBs (≥ four chlorines). In contrast, lightly chlorinated PCBs, including trichlorobiphenyls, are transformed into their corresponding *meta*-cleavage products and chlorobenzoic acids [5,14]. There is a lack of information on the fate and impact of hydroxylated PCBs in the environment [21]. In many cases, analytical work targets for intact PCB congeners but neglects the identification of the corresponding metabolites. It remains to be elucidated to what extent enzymes other than those encoded by the *bph* operon are capable of further transforming hydroxylated PCBs. Interestingly, 3,4-dioxygenated biphenyl and 2,2′,5,5′-CB have recently been shown to be transformed into ring fission products by naphthalene-catabolizing enzymes [22]. Although the con-

ventional wisdom is to correlate the rate of initial dioxygenation with PCB degradation, our results clearly demonstrated that complete degradation of PCBs is more likely to be achieved with recombinant strains containing metabolic pathways not only specifically engineered for expanding the initial dioxygenation but for the mineralization of PCBs.

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