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BIOTECHNOLOGY and BIOENGINEERING

Biphenyl and Ethylbenzene Dioxygenases of *Rhodococcus jostii* RHA1 Transform PBDEs

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ABSTRACT: Polybrominated diphenyl ethers (PBDEs) are a class of flame retardants that have been widely used in consumer products, but that are problematic because of their environmental persistence and endocrine-disrupting properties. To date, very little is known about PBDE degradation by aerobic microorganisms and the enzymes involved in PBDE transformation. Resting cells of the polychlorinated biphenyl-degrading actinomycete, *Rhodococcus jostii* RHA1, depleted nine mono- through penta-BDEs in separate assays. Extensive depletion of PBDEs occurred with cells grown on biphenyl, ethylbenzene, propane, or styrene, whereas very limited depletion occurred with cells grown on pyruvate or benzoate. In RHA1, expression of *bphAa* encoding biphenyl dioxygenase (BPDO) and *etbAa1* and *etbAc* encoding ethylbenzene dioxygenase (EBDO) was induced 30- to 3,000-fold during growth on the substrates that supported PBDE depletion. The BPDO and EBDO enzymes had gene expression profiles that matched the PBDE-depletion profiles exhibited by RHA1 grown on different substrates. Using the non-PBDE-degrading bacterium *Rhodococcus erythropolis* as a host, two recombinant strains were developed by inserting the eth and bph genes of RHA1, respectively. The resultant EBDO extensively depleted mono- through penta-BDEs, while the BPDO depleted only mono-, di-, and one tetra-BDE. A dihydroxylated-BDE was detected as the primary metabolite of 4-bromodiphenyl ether in both recombinant strains. These results indicate that although both dioxygenases are capable of transforming PBDEs, EBDO more potently transforms the highly brominated congeners. The availability of substrates or inducing compounds can markedly affect total PBDE removal as well as patterns of removal of individual congeners.

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Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of flame retardants that have been used for three decades in many manufactured materials such as foams, textiles, and plastics at up to 20% by weight (de Wit 2002). As of 2006, the penta- and octa-BDE technical mixtures were banned and removed from production in Europe and California due to their toxicity (California Assembly 2003; European Parliament 2002). Production of the remaining technical mixture deca-BDE will voluntarily cease in 2013 and in 2009, tetra- through hepta-BDEs were added to the list of persistent organic chemicals of the Stockholm Convention (EPA 2009; Stockholm Convention, 2009a,b). As a consequence of their extensive long-term use in consumer products, PBDEs have been detected in a wide variety of environmental samples, ranging from water, soil, and sediment, to biota (North 2004; Oros et al., 2005; She et al., 2007; Xia et al., 2008). The main congeners in the industrial penta-BDE formulation, notably tetra- and penta-BDEs, are those most frequently found in environmental samples and in humans (Elliott et al., 2005; Lindberg et al., 2004; North 2004; Oros et al., 2005).

To date, very little is known about aerobic microbial transformation of PBDEs and in particular about the substrates and enzymes necessary to achieve PBDEs biodegradation. A few *Sphingomonas* strains have been isolated that are capable of growth on diphenyl ether and transforming mono- through tri-BDE congeners (Kim et al.,

2007; Schmidt et al., 1992, 1993). In a previous study, we showed that PBDEs with up to six bromines, including the major congeners in the industrial penta-BDE formulation, can be aerobically transformed individually and in the combination present in the DE-71 industrial penta-BDE mixture by microorganisms capable of degrading polychlorinated biphenyls (PCBs) (Robrock et al., 2009). One bacterium in particular, *Rhodococcus jostii* RHA1, depletes mono- through penta-BDEs when grown on biphenyl, and releases stoichiometric quantities of bromide when transforming mono- and tetra-BDEs. However, the range of PBDEs transformed by RHA1 depends dramatically on the organic growth substrate, suggesting that the enzymes responsible for degradation of higher-congener PBDEs are neither constitutively expressed nor induced by PBDEs, but are upregulated in response to other substrates. Similar results were observed with the *Sphingomonas* isolates. Although one *Sphingomonas* strain was able to grow on 4-bromodiphenyl ether, diphenyl ether was required as a substrate to achieve transformation of all other congeners. The dioxygenases responsible for degradation of diphenyl ether and less brominated PBDEs are diverse. Typically, 2,3-dioxygenases are involved in the initial dihydroxylation of diphenyl ether as shown with *Sphingomonas* sp. PH-07 and *Burkholderia cepacia* Et4, although a 1,2-dioxygenase from *Sphingomonas* sp. SS3 has also been implicated (Kim et al., 2007; Pfeifer et al., 1993; Schmidt et al., 1992). Complete mineralization of diphenyl ether was achieved with strain PH-07, while phenol and 2-pyrone-6-carboxylate were end products produced by strain Et4. Identification of the enzymes involved in transformation of the more highly brominated PBDEs by RHA1 will improve our understanding of the conditions required to promote degradation of the environmentally prevalent congeners and will facilitate the development of PBDE bioremedation strategies. *R. jostii* RHA1 has a 9.7-Mbp genome encoding substantial catabolic diversity and is predicted to have 203 oxygenases, of which 30 are hypothesized to degrade aromatic compounds (McLeod et al., 2006). In particular, the biphenyl and ethylbenzene 2,3-dioxygenases (biphenyl dioxygenase—BPDO and ethylbenzene dioxygenase—EBDO) are implicated in initiating PCB degradation (McLeod et al., 2006). Each enzyme catalyzes the 2,3-dihydroxylation of the aromatic ring (Fig. 1). There is substantial overlap in the respective substrate specificities of BPDO and EBDO, a factor that likely contributes to RHA1's potent PCB-degrading properties (Iwasaki et al., 2007; Masai et al., 1997). BDPO and EBDO each comprise four subunits, encoded by the *bph* and *etb* genes, respectively (Fig. 1), and their catalytic components (BphAa/BphAb and EtbAa/EtbAb) share 37% amino acid sequence identity. Furthermore, there are two copies of the *etbAa* and *etbAb* genes, which differ by a single nucleotide substitution (Patrauchan et al., 2008). While BPDO and EBDO each transform various aromatic compounds, including benzene, biphenyl, ethylbenzene, and naphthalene, EBDO prefers larger substrates, such as naphthalene, consistent with its phylogeny, while BPDO prefers substrates containing a single aromatic nucleus (Iwasaki et al., 2007; Masai et al., 1997). The *bph* and *etb* genes are regulated by a two-component regulatory system, BphST, that responds to numerous aromatic compounds (Takeda et al., 2004).

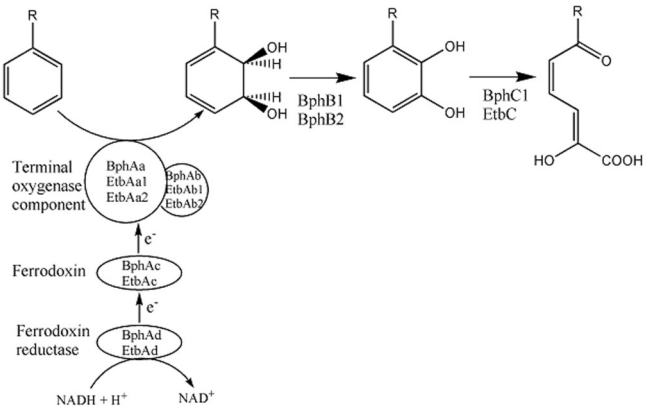

Figure 1. The first three steps in the degradation of biphenyl and similar aromatic compounds. R equals H (to form benzene) CH_2–CH_3 (to form ethylbenzene), CH=CH_2 (to form styrene), or an aromatic group (to form biphenyl). The enzymes involved in each step are listed adjacent to the arrows. BphA and EtbA are a biphenyl 2,3-dioxygenase and an ethylbenzene 2,3-dioxygenase, respectively, with four subunits. The first two subunits of the ethylbenzene dioxyganse are present in two duplicate copies in the genome. BphB1 and BphB2 are a *cis*-2,3-dihydrobiphenyl-2,3-diol dehydrogenase and a *cis*-3-phenylcyclohexa-3,5-diene-1,2-diol dehydrogenase, respectively. BphC1 and EtbC are both 2,3-dihydroxybiphenyl 1,2-dioxygenases.

Iwasaki et al. (2006, 2007) used gene disruption mutants and recombinant strains to demonstrate that both BPDO and EBDO are involved in PCB degradation. Given the structural similarity between PCBs and PBDEs and evidence of 2,3-dioxygenase involvement in PBDE transformation in a *Sphingomonas* strain (Kim et al., 2007), the goal of this study was investigate whether these enzymes are also involved in PBDE transformation by RHA1 and what conditions are necessary to induce these enzymes and achieve PBDE transformation. Using reverse transcription quantitative PCR (RT-qPCR) we quantified the expression of these genes when RHA1 was grown on different substrates. Using a related species, *Rhodococcus erythropolis* IAM1399, which does not transform PBDEs or PCBs, as a host strain and plasmids constructed by Iwasaki et al. (2007) containing the full complement genes encoding BPDO and EBDO, we created recombinant strains to assess the involvement of BPDO and EBDO in PBDE transformation. This study is the first to demonstrate that 2,3-dioxgenases are capable of transforming PBDEs with up to five bromines and to assess their relative substrate specificities.

Materials and Methods

Bacterial Cultures

R. erythropolis IAM1399 (ATCC 15963) was kindly provided by Dr. Masao Fukuda at the Nagaoka University of

Technology, Nagaoka, Japan. IAM1399 was grown in Luria Bertani (LB) broth. RHA1 (McLeod et al., 2006) was grown on LB agar plates and then transferred to minimal medium (Sharp et al., 2005). The following growth substrates were used for RHA1: 20 mM pyruvate, 10 mM benzoate, 10 mM biphenyl, or 16 mmol of propane added as 40 mL of gas into the headspace of the bottle. Cultures were grown in 160 mL bottles sealed with teflon-lined Mininert caps (VICI Valco Instruments, Houston, TX), containing 50 mL of medium. RHA1 was alternatively grown on ethylbenzene or styrene vapors by suspending a separate tube with 2 mL of either ethylbenzene or styrene liquid inside a 1-L Erlenmeyer flask with 200 mL of bacterial culture. For PBDE transformation assays, IAM1399 and derivative strains were grown in a 1-L Erlenmeyer flask with 200 mL LB and a suspended tube of ethylbenzene to induce gene expression. All PBDE congeners were purchased from Accustandard, Inc. (New Haven, CT) as 50 μg/mL standard dissolved in nonane. 4-chlorobiphenyl was purchased from Sigma–Aldrich (St. Louis, MO) and dissolved in nonane at 50 μg/mL.

Analytical Techniques

One milliliter of isooctane extract was transferred into a 2 mL crimp cap vial with a disposable glass pipette and analyzed using a gas chromatograph equipped with an electron capture detector (GC-ECD) 3800 from Varian (Walnut Creek, CA). A 30-m DB-1 column with 0.25 mm ID and 0.25 μm film thickness was used. The GC temperature program was as follows: 110°C for 2 min, ramp at 30°C/min to 200°C, and then ramp at 1.5°C/min until 300°C. The injector and detector temperatures were 250 and 325°C, respectively. Concentrations for the individual congeners were determined using a three-point calibration curve, ranging from 0.5 to 50 ng/mL. The instrument detection limits for PBDE congeners were 0.1–1.0 pmol, corresponding to low nM concentrations in the samples analyzed. Extraction efficiencies in the experiments for the di- through hexa-BDEs ranged from 73% to 118%. The extraction efficiency for mono-BDE was much less, around 54%, probably due to its volatilization during evaporation of the nonane. The extraction efficiency for the internal standard averaged 93%.

Plasmids

The plasmids, pKPEB4–2, pK4TBA214, and pFST1, were kindly donated by Dr. Masao Fukuda (Iwasaki et al., 2007). The pKPEB4–2 plasmid contains the *etbAa2Ab2AcAd* genes (formerly *edbA1A2A3etbA4*) plus a kanamycin resistance gene. The pK4TBA14 plasmid contains the *bphAaAbAcAd* genes (formerly *bphA1A2A3A4*) plus kanamycin and thiostrepton resistance genes. The pFST1 plasmid contains the *bphST* genes, which encode an ethylbenzene-responsive two-component signal transducer necessary for transcription of the above *etb* and *bph* genes, plus genes specifying resistance against ampicillin and chloramphenicol. The plasmids were transformed into chemically competent *E. coli* TOP10 cells, and the propagated plasmid was purified using standard procedures (Invitrogen, Carlsbad, CA). Plasmids were transformed into *R. erythropolis* IAM1399 as previously described (Rodrigues et al., 2001) with minor modifications (see Supporting Information for details of the electroporation protocol).

PBDE Degradation Assay

Bacteria were exposed to nine different congeners individually, listed in Table I. One microliter of a stock solution containing 50 μg/mL of a PBDE congener dissolved in nonane was added to an 8-mL culture vial. Vials were left in a laminar hood for 5 min to evaporate to dryness. RHA1 cells were initially grown on LB agar plates. Colonies were then transferred to 250 mL mini-inert bottles or 1 L Erlenmeyer flasks with minimal medium containing the appropriate growth substrate. Three milliliters of cells at mid log-phase growth (OD_{600} of 0.26–0.27) were added to the culture vials sealed with Teflon-lined screw caps. Any undissolved biphenyl in the culture was allowed to settle out before transfer of the bacteria to avoid diminishing PBDEs extraction efficiency. For assays performed with IAM1399 and its recombinant strains, cells were grown overnight in LB broth to a final OD_{600} of 1.1, then exposed for 8 h to ethylbenzene vapors, before being transferred to the PBDE-containing vials. Recombinant strains were also exposed to 4-chlorobiphenyl dissolved in nonane as a positive control. Final concentrations for all PBDE congeners and 4-chlorobiphenyl was 17 ng/mL. Negative controls were constructed using autoclaved cells. Samples were shaken at 200 rpm for 72 h at 30°C. All samples and controls were performed in triplicate. For analysis, the entire vial contents were extracted with 3 mL of isooctane using 0.6 ng of 2,2′,4,5′-tetrabromobiphenyl dissolved in methanol as an internal standard. The vials were briefly vortexed and then extracted by shaking for 16 h. Samples were analyzed by GC-ECD as previously described (Robrock et al., 2009).

Gene Expression Analysis

RT-qPCR analyses were performed essentially as previously described (Gonçalves et al., 2006). Briefly, RHA1 cells from

Table I. List of the tested PBDE congeners and their bromine substitution patterns.

Congener name	Bromine substitution pattern
Mono-BDE 3	4
Di-BDE 4	2,2'
Di-BDE 7	2,4
Tri-BDE 17	2,2',4
Tri-BDE 28	2,4,4'
Tetra-BDE 47	2,2',4,4'
Tetra-BDE 49	2,2',4,5'
Penta-BDE 99	2,2',4,4',5
Penta-BDE 100	2,2',4,4',6

1.5 mL of mid-log phase culture (OD_{600} of 0.26–0.27) were disrupted via bead-beating and extracted using the Qiagen RNeasy kit (Qiagen, Valencia, CA). The extracted RNA was treated six times with DNase using the DNA-free kit by Ambion (Austin, TX). The RNA was reverse transcribed to cDNA using Superscript (Invitrogen) and the appropriate primers (Table SI-1). Quantitative PCR was performed using a SYBR green probe (Applied Biosystems, Foster City, CA) and the appropriate primers. Four replicate cultures were analyzed for each condition. The expression of the target genes was normalized using the housekeeping gene DNA polymerase IV and compared to the expression of DNA polymerase IV in pyruvate-grown cells, which was set to be equal to 1.0 (Table II).

Results

Dependence of PBDE Depletion on RHA1 Growth Substrate

RHA1 was grown separately on each of six different substrates followed by incubation separately with each of nine PBDE congeners to assess the inducibility of the strain's PBDE-transformation activity (Fig. 2). Biphenyl, ethylbenzene, styrene, and propane were chosen because they were known to induce EBDO or BPDO, and benzoate was chosen because it is catabolized by a different enzyme system—the Ben-Cat and Pca pathways (Patrauchan et al., 2008). Pyruvate was used for representative baseline expression for RHA1. No transformation was observed in autoclaved controls. The percent transformation was determined by normalizing the amount of PBDEs remaining in the live samples to the concentration in the controls. RHA1 grown on biphenyl, ethylbenzene, styrene, or propane extensively depleted PBDEs. PBDE-depletion profiles of biphenyl-, ethylbenzene-, and propane-grown cells, respectively, were strikingly similar, with 100% removal of mono- and di-BDEs, and decreasing removal of more highly brominated congeners. Styrene-grown cells transformed only the mono- through tetra-BDE congeners. Pyruvate- and benzoate-grown cells depleted PBDEs much less effectively and removed none of the more highly brominated congeners. Surprisingly, the mono-BDE was not transformed by benzoate-grown cells.

RHA1 cells were also exposed to different PBDEs concentrations. Experiments were conducted with 10- and 100-fold higher concentrations (170 and 1,700 ng/mL) tetra-BDE 47, although with longer incubation times. Similar degradation was observed, with 94% degradation of 170 ng/mL over 5 days and 88% degradation of 1,700 ng/mL over 4 days. At 1,000-fold higher concentrations, a decrease in transformation ability was noted. RHA1 was exposed to 17 μg/mL mono-BDE 4 and only achieved 50% transformation over the course of 3 days.

Under growth conditions with pyruvate, RHA1 can deplete mono-BDE, but not tetra-BDE congeners. To determine whether the presence of easily transformable PBDEs can help achieve transformation of more recalcitrant PBDEs, cells growing on 20 mM pyruvate were exposed to 17 ng/mL tetra-BDE 47 or tetra-BDE 49 in the presence of 17 ng/mL mono-BDE 3. No depletion of either tetra-BDE occurred over the course of 3 days while between 84% and 86% of the mono-BDE was depleted. Although 17 ng/mL mono-BDE may be an insufficiently low concentration, this result suggests that the presence of certain congeners does not determine PBDE transformation ability in RHA1.

Gene Expression Results

Expression of genes encoding BPDO and EBDO on each growth substrate was investigated using RT-qPCR. As *etbAa1* and *etbAa2*, paralogous genes encoding the catalytic subunit of EBDO, cannot be distinguished by RT-qPCR, we also quantified the transcription of *etbAc*, which is unique to one of the two *etb* operons. *etbAc* is located one gene (601 bp) downstream from *etbAa2* and 11 ORFs (8,593 bp) downstream from *etbAa1*. The results show that when grown on biphenyl, ethylbenzene, styrene, and propane, the *bph* and *etb* genes were highly upregulated (Fig. 3). The *etb* genes were most highly expressed on ethylbenzene, whereas *bphAa* was most highly expressed on styrene. The *bph* and *etb* genes were expressed at essentially the same low levels on pyruvate and benzoate.

Depletion of PBDEs by Recombinant Strains

In order to conclusively demonstrate the role of BPDO and EPDO in PBDE transformation by RHA1 and to distinguish

Table II. Relative fold difference and total error of *bphAa*, *etbAa1*, and *etbAc* expression measured by RT-qPCR.

	bphAa		etbAa1		etbAc	
Growth substrate	Fold difference	Total Error	Fold difference	Total Error	Fold difference	Total Error
Pyruvate	1	1.1	1	2.1	1	1.1
Benzoate	1.2	1.3	1.0	2.3	1.2	1.2
Biphenyl	32	1.5	290	2.8	104	1.4
Ethylbenzene	66	1.5	2,758	2.5	624	1.3
Propane	38	1.5	450	2.3	184	1.2
Styrene	144	1.3	251	2.4	87	1.2

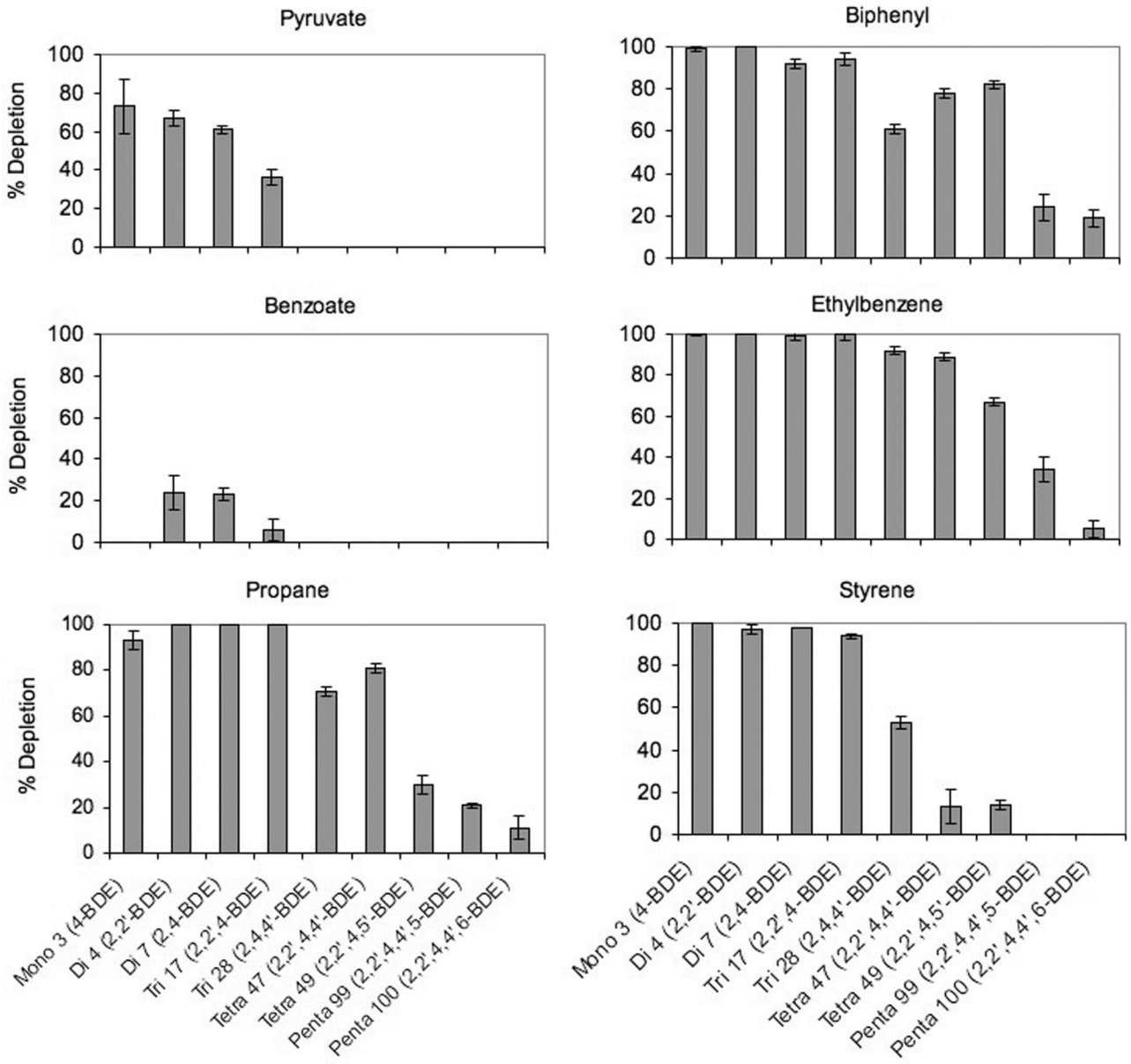

Figure 2. Percent depletion of nine PBDEs in separate assays by RHA1 grown on different substrates. The bromine substitution pattern for each congener is listed in parentheses. Error bars represent the total error of triplicate controls and samples of cell suspensions.

transformation activities of the two enzymes, two recombinant strains were created, expressing BPDO and EBDO, respectively, with the non-PBDE transforming strain *R. erythropolis* IAM1399 as a host. To demonstrate that the genes inserted into the recombinant strains produced functional proteins, cells were exposed to PCB 3 (4-chlorobiphenyl), which the recombinant IAM1399 strains had been previously demonstrated to degrade (Iwasaki et al., 2007). Both recombinant strains transformed PCB 3 effectively, with 100% and 83% of the chemical depleted by the *etb* and *bph* recombinants, respectively, indicating that the recombinant IAM1399 strains expressed the *bph* and *etb* genes and produced functional BPDO and EBDO. Figure 3 shows the percent depletion of the tested PBDE congeners by the IAM1399 host and both recombinant strains after a 3-day incubation. While the host IAM1399 strain was not active towards PBDEs, both recombinants degraded a variety of congeners, with the Bph recombinant degrading a more restricted set of PBDEs than the Etb recombinant or the wild-type RHA1. In particular, BPDO failed to transform the two tri-BDEs or the two penta-BDEs tested. BPDO may have transformed trace amounts of tetra-BDE 47 and penta-BDE 99, as there was a small but not statistically significant decrease (3–4%) in

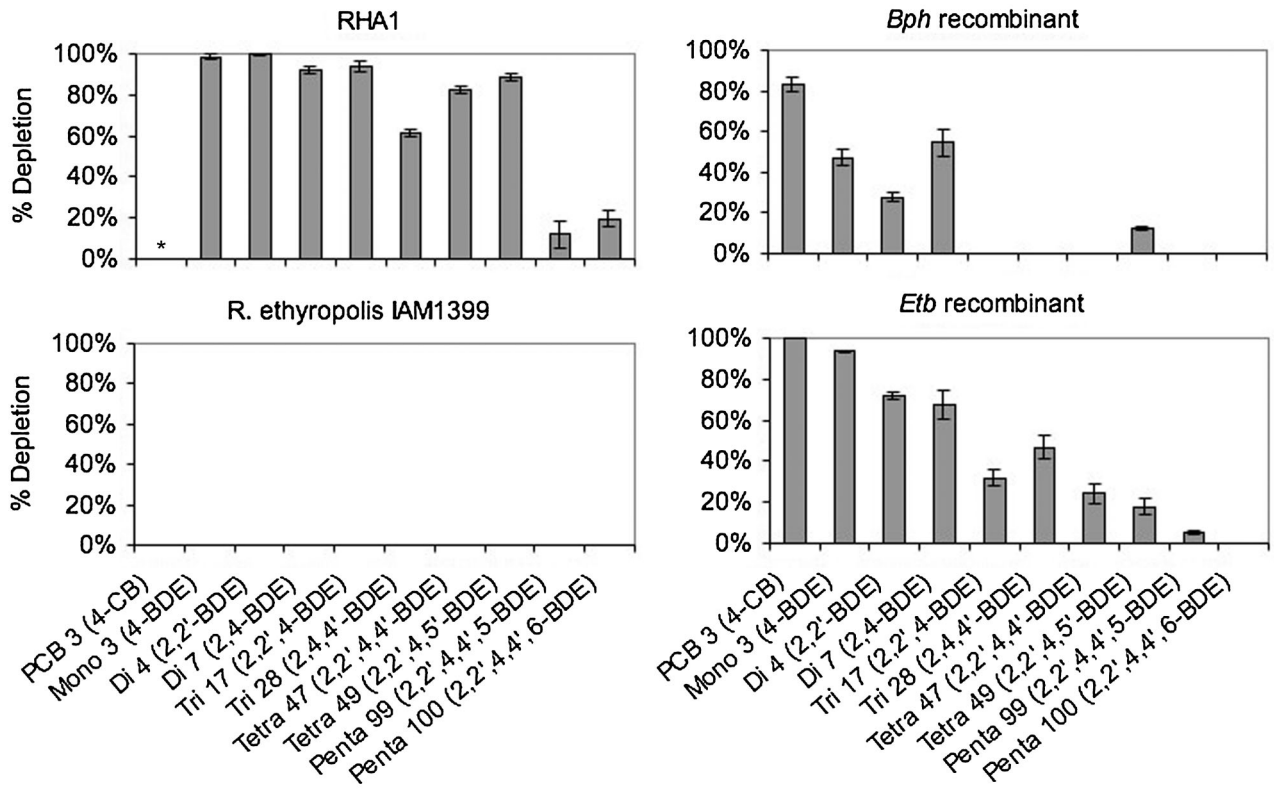

Figure 3. Percent depletion of 9 PBDE and one PCB congeners by *R. erythropolis* IAM1399 recombinant strains containing either *bphAaAbAcAd* or *etbAa1Ab1AcAd* after 3 days incubation. Values are averages of triplicate samples and error bars represent the total error. Asterisk indicates that the depletion of this congener was not tested.

their concentrations compared to the controls. In contrast, EBDO was active towards nearly all of the congeners depleted by wild-type RHA1. As with RHA1, the degree of depletion of individual congeners by EBDO was inversely proportional to the number of bromine substituents, decreasing from 94% removal of mono-BDE 3 to 5%, of penta-BDE 99. The only congener depleted by RHA1 but not by the *etb* recombinant is penta-BDE 100.

Identification of Metabolites

The recombinant strains exposed to mono-BDE were analyzed for the presence of metabolites by derivatization with heptafluorobutyrate anhydride. Derivatized dihydroxy-monobromodiphenyl ether was detected based on equal mass/ion ratios of 672 and 674 in the extracts of both the *bph* and *etb* recombinants as expected for a brominated compound. The bromine ions 79 and 81 were present in equal amounts in the MS spectrum and the fragment 197 was also observed, corresponding to the mass of the heptafluorinated derivatizing chain. No significant concentrations of a derivatized brominated dihydrodiol were detected. Monohydroxylated-monobromodiphenyl ethers were also not detected, nor were brominated phenols or any other brominated compounds. The MS spectrum and a potential chemical structure of the derivatized metabolite are shown on Figure 4. The specific location of the hydroxyl groups on the ring is unknown as no standards are available. However, since monohydroxy-monobromodiphenyl ethers were not detected, there is no evidence that the biphenyl and ethylbenzene diooxygenases were functioning as monooxygenases and attacking single carbons on the ring structures. However, the predominant 3-chlorobiphenyl metabolites detected by Iwasaki et al. (2007) were 4-chloro-2′,3′-dihydrodiol and 2′,3′-dihydroxy-4-chlorobiphenyl, so the analogous 2′,3′-dihydroxy-4-bromodiphenyl ether is likely.

Discussion

The range of PBDEs depleted by RHA1 and the extent of their depletion vary dramatically depending on the growth substrate (Fig. 2). Extensive PBDE depletion was seen when RHA1 was grown on biphenyl, propane, styrene, or ethylbenzene, with all tested mono through penta congeners transformed to some extent, whereas only limited mono through tri congeners were depleted when cells were grown on pyruvate or benzoate. It is unclear why the PBDE-depletion pattern is different between pyruvate- and

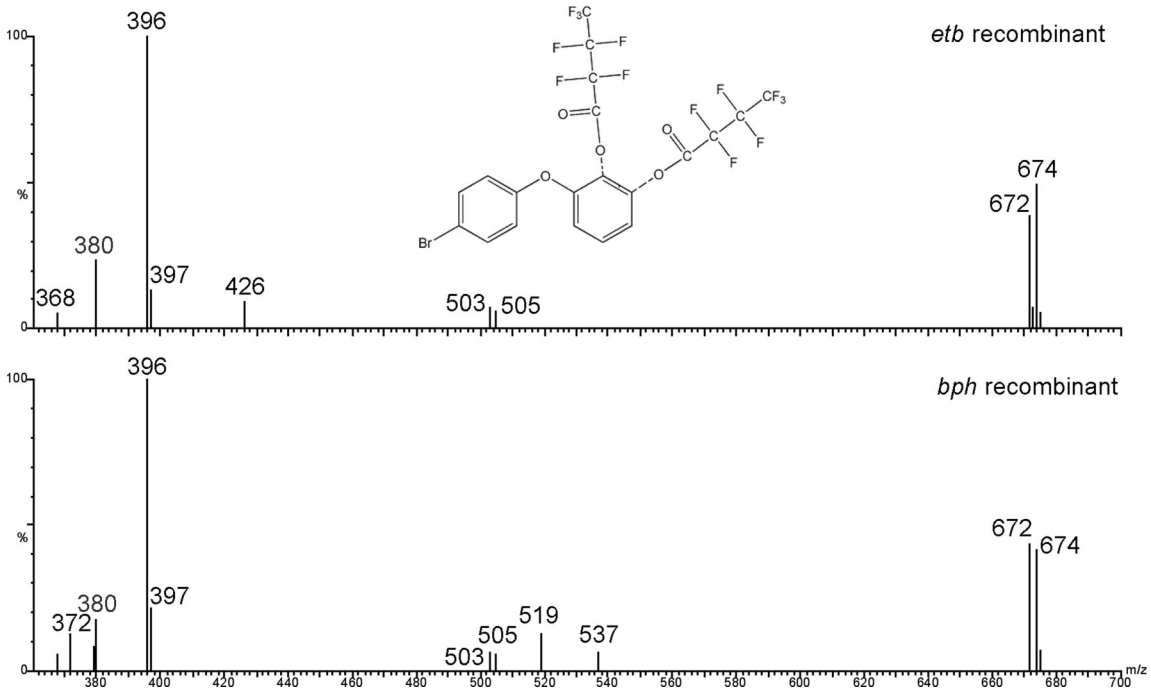

Figure 4. Mass spectrum and the proposed chemical structure of the derivatized mono-BDE metabolite detected in extracts from the *bph* and *etb* recombinants. The exact locations of the hydroxyl groups on the ring are unknown, as indicated by dotted lines, but this speculative structure is presented for comparison with the mass spectrum.

benzoate-grown cells since the gene expression patterns are essentially identical. We also saw a more subtle difference in the relative extent of removal of particular congeners between RHA1 cells grown on the different inducing substrates. Cells grown on styrene had the most distinct PBDE removal profile. Similar studies with *Sphingomonas* strains found that diphenyl ether was required for transformation of PBDEs, as no activity was observed under acetate growth conditions (Kim et al., 2007; Schmidt et al., 1993). Under substrate conditions in which many PBDE congeners were transformed, the extent of transformation decreased with an increasing number of bromines. This is likely due to the bioavailability of these compounds, as they become less soluble with increasing bromine content.

Both BPDO and EBDO of RHA1 degrade PCBs, as established by studies using both gene disruption mutants and recombinant strains (Iwasaki et al., 2006, 2007). RT-qPCR analysis shows that their expression levels reflect the depletion pattern, lending evidence to their involvement in PBDE degradation. These genes were previously shown to be co-regulated and induced by biphenyl or ethylbenzene in RHA1 (Gonçalves et al., 2006). The Bph pathway is utilized for growth on biphenyl, ethylbenzene, or styrene, while the Etb pathway is utilized for growth on biphenyl or ethylbenzene (Patrauchan et al., 2008), which explains their high expression with these substrates. Not surprisingly, the expression of *bphAa* was highest with styrene-grown cells since only BPDO degrades styrene. RHA1 utilizes a different oxygenase—propane monooxygenase—for growth on propane, so the high expression of *bph* and *etb* when grown on propane is interesting, although it had been previously noted (Sharp et al., 2007). Oddly, *bphA* was not differentially expressed in the previous study compared to other highly expressed genes in the biphenyl pathway (Sharp et al., 2007), while we observed 10-fold higher expression in *bphA* expression in propane-grown RHA1 cells, which may be due to differences in minimal growth medium and experimental techniques.

Expression of either BPDO or EBDO in strain IAM1399 enabled this strain to deplete PBDEs. This result demonstrates that both dioxygenases are capable of transforming PBDEs and explains the depletion patterns seen with cells grown on substrates that induce high expression of the *bph* and *etb* genes. This result aligns with previous research that found that dioxygenase enzymes are responsible for degradation of diphenyl ether and may be responsible for degradation of less brominated PBDEs (Pfeifer et al., 1993; Schmidt et al., 1992). The *etb* recombinant transformed all PBDEs tested but penta-BDE 100, while the *bph* recombinant had more limited transformation ability, transforming only the less brominated mono-BDE, di-BDEs, and tetra-BDE 49. Penta-BDE 100 removal by RHA1 was minimal, and the *etb* recombinant removed lesser amounts than RHA1 of all highly brominated congeners, perhaps due to slower rates of transformation or transport across the cell membrane, so the failure to detect penta-BDE 100 removal

by the *etb* recombinant does not necessarily prove that the enzyme cannot transform that congener. The PBDE transformation patterns of the recombinant strains are consistent with the PCB transformation results reported by Iwasaki et al. (2007). EBDO was better at degrading the higher PCB congeners than BPDO. Results with RHA1 disruption mutants were similar. A disruption mutant of *etbAa1*—in which the *bph* genes were still active—degraded PCBs far less extensively than the *bphAa* disruption mutant in which the *etb* genes were active (Iwasaki et al., 2006). The pattern of PBDE deletion in this study is also consistent with the substrate preferences of BPDO and EBDO exhibited with other aromatic compounds. In general, BPDO is better at catalyzing the oxidation of smaller aromatic compounds such as toluene and styrene, while EBDO prefers larger substrates such as naphthalene, phenanthrene, and dibenzo-*p*-dioxin (Iwasaki et al., 2007; Patrauchan et al., 2008). Phylogenetically, the BPDO enzyme clusters with other toluene and BPDOs, whereas EBDO clusters with naphthalene dioxygenases (Pieper 2005).

The substrate preference of BPDO appears complex. With the *bph* recombinant, no transformation of the tri-BDEs or tetra-BDE 47 (2,2′,4,4′-BDE) was observed, while 13% transformation of the tetra-BDE 49 (2,2′,4,5′-BDE) was observed. Although there is very little overlap between the tested PCB and PBDE congeners, heterologously expressed BPDO did transform 100% of the one tri-CB to which it was exposed as well as all the tetra-CBs including a very small amount of the tetra-CB 47 (Iwasaki et al., 2007). In general, Iwasaki et al. found that BPDO did not transform congeners in a predominantly para-chlorinated PCB mixture compared to an ortho-chlorinated PCB mixture, which is consistent with BPDO's relative inability to transform the doubly para substituted tetra-BDE 47 compared to the singly para substituted tetra-BDE 49 in the current study.

A catecholic-monobromodiphenyl ether metabolite was detected in the extracts from both recombinant strains instead of the expected dihydrodiol that should have been formed by the recombinant strains according to the biphenyl pathway. This suggests that a native enzyme present in IAM1399 is able to catalyze the dehydrogenation reaction, as was observed with the formation of a catecholic PCB metabolite in analogous PCB transformation experiments by Iwasaki et al. (2007) instead of a dihydrodiol. The chemical structure is speculative since no standards are available for this compound and it is not clear from the MS fragmentation pattern whether the dihydroxylation occurs on the non-brominated ring nor whether the dihydroxylation occurs specifically at the ortho and meta positions. Given the slight differences between the MS spectra for the *bph* and *etb* recombinants, it is possible that the two metabolites have hydroxyl groups located in different positions. Furthermore, the hydroxyl groups may not be located on vicinal carbons on the ring.

The current study is the first to demonstrate that both the 2,3-dioxygenases BPDO and EBDO transform PBDEs in RHA1. Given the structural similarity between PCBs and PBDEs, this is not surprising. The possibility; however, that other enzymes may also be involved in PBDE transformation by RHA1 cannot be excluded. When grown on pyruvate, RHA1 transformed only the less brominated congeners, perhaps by constitutive low-levels of BPDO and EBDO. However, neither of these enzymes were detected in proteomic analyses of pyruvate-grown cells (Patrauchan et al., 2008). The substantially lower PBDE removal by benzoate-grown cells versus pyruvate-grown cells (Fig. 2) cannot be explained by measured levels of gene expression, which were not significantly different on the two substrates (Fig. 3). However, a previous microarray study (Gonçalves et al., 2006) was consistent with the substrate removal results, finding lower levels of expression of all the *bph* and *etb* genes on benzoate versus pyruvate. Furthermore, benzoate-grown RHA1 cells did not transform mono-BDE, which was easily depleted under all other growth conditions. Given the low expression of BPDO and EBDO in cells grown on pyruvate and benzoate and unusual transformation patterns, it is possible that an entirely different enzyme or multiple enzymes produced under these growth conditions were responsible for PBDE transformation.

PBDEs do not appear to induce the enzymes required for their transformation. It was shown that when the recalcitrant tetra-BDEs and the transformable mono-BDE were exposed together to RHA1 cells growing on pyruvate, tetra-BDE remained untransformed. This has important implications for the environmental fate of these compounds, as the enzymes necessary for PBDE degradation might not be produced in the environment in the absence of appropriate inducing substrates. *R. jostii* RHA1 is a promising degrader of the environmentally prevalent tetra- and penta-BDEs, since it is capable of completely transforming PBDEs and producing stoichiometric quantities of bromide (Robrock et al., 2009). However, for organisms with multiple dioxygenase systems, like RHA1, inducing substrates can influence which PBDEs are degraded. Therefore, substrate availability will need to be judiciously controlled in systems for bioremediation of PBDEs. Biphenyl-enhanced biodegradation of PCBs has been demonstrated as a successful remediation technique in the environment and appears to be a promising strategy for PBDEs (Brunner et al., 1985). Future research of the ability of RHA1 to transform PBDEs under natural conditions is required as bioavailability may be a limiting aspect to bioremediation of PBDEs. Recent work; however, has demonstrated transformation of the more hydrophobic octa-BDEs in soils and sediments and therefore, aerobic transformation of tetra- and penta-BDEs in contaminated soils should be possible (Lee and He 2010).

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