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Transformation of hydroxylated derivatives of 2,5-dichlorobiphenyl and 2,4,6-trichlorobiphenyl by *Burkholderia xenovorans* LB400

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Abstract

The PCB-degrading bacterium, *Burkholderia xenovorans* LB400, was capable of transforming three hydroxylated derivatives of 2,5-dichlorobiphenyl (2,5-DCB) (2'-hydroxy-(2'-OH-), 3'-OH-, and 4'-OH-2,5-DCB) when biphenyl was used as the carbon source (i.e., biphenyl pathway-inducing condition), although only 2'-OH-2,5-DCB was transformed when the bacterium was growing on succinate (i.e., condition non-inductive of the biphenyl pathway). On the contrary, hydroxylated derivatives of 2,4,6-trichlorobiphenyl (2,4,6-TCB) (2'-OH-, 3'-OH-, and 4'-OH-2,4,6-TCB) were not significantly transformed by *B. xenovorans* LB400, regardless of the carbon source used. Gene expression analyses showed a clear correlation between the transformation of OH-2,5-DCBs and expression of genes of the biphenyl pathway (*bph*). The PCB metabolite, 2,5-dichlorobenzoic acid (2,5-DCBA), was produced following the transformation of OH-2,5-DCBs. 2,5-DCBA was not further transformed by *B. xenovorans* LB400. The present study is significant because it provides evidence that PCB-degrading bacteria are capable of transforming hydroxylated derivatives of PCBs, which are increasingly considered as a new class of environmental contaminants.

Keywords

Hydroxylated polychlorinated biphenyls; *Burkholderia xenovorans* LB400; dichlorobiphenyl; trichlorobiphenyl; biodegradation; biphenyl pathway

Introduction

Polychlorinated biphenyls (PCBs) are among the most dangerous environmental contaminants because of their toxicity, their widespread dispersion, and their recalcitrance to biodegradation. Because of their high chemical and physical stability and high dielectric constant, PCBs have been used for a variety of industrial applications, until recognition of toxicity led to their interdiction in most countries by the late seventies (Borja et al. 2005; Field and Sierra-Alvarez 2008). During about a half-century, the production, use, and disposal of PCBs resulted in their dispersion in every compartment of the environment,

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including air, water, soil, sediments, and living organisms (Pieper and Seeger 2008). PCBs exert various detrimental effects on wildlife and humans, including immunotoxicity, neurotoxicity, developmental toxicity, and reproductive toxicity. PCBs are classified as suspected carcinogens by the International Agency for Research on Cancer (IARC) (Takeuchi et al. 2011).

Bacterial degradation of PCBs is well documented. PCB congeners with four or more chlorine atoms are susceptible to anaerobic dechlorination, while PCB congeners with less than three chlorine atoms undergo preferentially aerobic oxidation by dioxygenases (Borja et al. 2005; Field and Sierra-Alvarez 2008; Pieper and Seeger 2008). Higher organisms, including mammals, plants, and fungi, are also capable of transforming PCBs through a sequence of reactions whose first phase frequently results in the formation of hydroxylated derivatives (Kaminski et al. 1981; Letcher et al. 2000; Schroder et al. 2001; Sietmann et al. 2006). The biological and abiotic transformation of PCBs generate primarily hydroxylated derivatives, which have been detected in a variety of environmental samples, including animal tissues and feces, water, and sediments. Because of their toxicity and widespread dispersion in the environment, OH-PCBs are today increasingly considered as a new class of environmental contaminants (Kawano et al. 2005; Ueno et al. 2007). Because of their high toxicity (e.g., generation of reactive oxygen species, thyroid hormone-disrupting activity, endocrine disrupting activity), OH-PCBs are increasingly considered as a new class of environmental contaminants (Cámara et al. 2004; Kawano et al. 2005; Kitamura et al. 2005).

However, little information is available about the bacterial metabolism of OH-PCBs. Two publications have reported the transformation of mono- and dichlorinated OH-PCBs by *Comamonas testosteroni* B-356 and *Burkholderia xenovorans* LB400 (Francova et al. 2004; Sondossi et al. 2004). However, these reports focused on congeners bearing hydroxyl and chlorine substituents located both on the same ring, even though hydroxylation of PCBs by higher organisms is known to occur on both chlorinated and non-chlorinated rings (Kaminski et al. 1981; Kucerovala et al. 2000; Sietmann et al. 2006; Rezek et al. 2007; Rezek et al. 2008; Zhai et al. 2010). In a previous report, we showed that derivatives of 4-chlorobiphenyl (4-CB) hydroxylated on the non-chlorinated ring (2'-hydroxy-(2'-OH-), 3'-OH-, and 4'-OH-4-CB) were transformed by *B. xenovorans* LB400 cells growing in the presence of biphenyl (Tehrani et al. 2012). In the present study, we have tested the capability of *B. xenovorans* LB400 to transform hydroxylated derivatives of higher-chlorinated biphenyls, including 2,5-dichlorobiphenyl (2,5-DCB) and 2,4,6-trichlorobiphenyl (2,4,6-TCB).

Materials and methods

Chemicals

Biphenyl (99% purity) was obtained from Acros Chemicals (Geel, Belgium). 2,5-Dichlorobiphenyl (2,5-DCB), 2,4,6-trichlorobiphenyl (2,4,6-TCB), 2'-hydroxy-2,4,6-trichlorobiphenyl (2'-OH-2,4,6-TCB), 3'-OH-2,4,6-TCB, and 4'-OH-2,4,6-TCB (99% purity) were purchased from Accustandard (New Haven, CT). 2'-Hydroxy-2,5-dichlorobiphenyl (2'-OH-2,5-DCB), 3'-OH-2,5-DCB, and 4'-OH-2,5-DCB were custom-synthesized in purity greater than 99% by the Superfund Research Program Synthesis Core

at the University of Iowa (Iowa City, IA) (Lehmle and Robertson 2001). Other chemicals were of analytical grade, solvents were of HPLC grade, and they were obtained from Acros Chemicals, Fischer Scientific (Pittsburgh, PA), or Sigma-Aldrich (St. Louis, MO).

Bacterium strain and culture conditions

B. xenovorans LB400 was obtained from the U.S. Department of Agriculture (Agricultural Research Service Culture Collection (NRRL), Peoria, IL). The bacterium was routinely maintained and propagated on Luria-Bertani (LB) Lennox medium. For the conduction of toxicity and biodegradation tests, the bacterium was acclimated on K1 mineral medium supplemented either with 10 mM (1,180 mg L⁻¹) sodium succinate (i.e., condition non-inductive of the biphenyl pathway) or 5 mM (770 mg L⁻¹) biphenyl (i.e., biphenyl pathway-inducing condition) as the carbon source as described by Denef et al. (2004). Cell suspensions were cultivated on an orbital shaker at 185 rpm, 30°C, aliquots were collected in late exponential phase (approx. 24 hours on succinate and 48 hours on biphenyl), and used to subculture the bacterium under the same conditions. Prior to conduct growth inhibition and biodegradation tests, the bacterial cells were acclimated to the carbon source by two successive sub-cultures on either succinate (1,180 mg L⁻¹) or biphenyl (770 mg L⁻¹) (Denef et al. 2004).

Growth inhibition and biodegradation experiments

The inhibitory effect of parent PCBs, OH-PCBs, and the metabolite 2,5-dichlorobenzoic acid (2,5-DCBA) was determined by cultivating the bacterium in 10-mL (16 × 100 mm) culture tubes (Kimble, Vineland, NJ) closed with polytetrafluoroethylene (PTFE)-lines stopper. The tubes contained 4-mL of K1 medium supplemented with succinate (1,180 mg L⁻¹) or biphenyl (770 mg L⁻¹) and they were dosed individually with increasing concentrations of 2,5-DCB and OH-2,5-DCBs (0.0 to 20 mg L⁻¹), 2,4,6-TCB and OH-2,4,6-TCBs (0.0 to 50 mg L⁻¹), and 2,5-DCBA (0.0 to 20 mg L⁻¹). The target compounds were supplemented in the form of a 5,000-mg L⁻¹ stock solution in acetone. A corresponding volume of pure acetone was added to the non-exposed controls (0.4% v/v). The tubes were inoculated with 0.5% v/v acclimated cells in late exponential phase and incubated on a rotary shaker at 185 rpm, 30°C for 96 hours. Experiments were conducted in triplicate. Bacterial growth was monitored by the optical density at 600 nm (OD₆₀₀).

For biodegradation experiments, cells were grown in 125-mL Erlenmeyer flasks closed with PTFE-lines stopper and containing 40 mL of K1 medium supplemented with succinate or biphenyl as described previously. The flasks were dosed individually with 5 mg L⁻¹ of 2,5-DCB, OH-2,5-DCBs, 2,4,6-TCB, OH-2,4,6-TCBs, and 2,5-DCBA, inoculated with 0.5% v/v acclimated cells, and incubated on an orbital shaker at 185 rpm, 30°C for 96 hours. Experiments were conducted in triplicate.

To determine the expression of key genes of the biphenyl pathway (*bph*), the bacterial cells were cultivated in 125-mL Erlenmeyer flasks containing 40 mL of K1 medium supplemented with the carbon source (succinate or biphenyl) as described above. The effect of biphenyl (known to be a strong inducer of the biphenyl pathway) was tested by conducting in parallel cultures using either biphenyl (770 mg L⁻¹) or succinate (1,180 mg

L⁻¹) as the carbon source. The effect of 2,5-DCB and its hydroxylated derivatives was tested by conducting cultures using succinate (1,180 mg L⁻¹) as the carbon source and exposed individually to 2,5-DCB, 2'-OH-, 3'-OH-, and 4'-OH-2,5-DCB (5 mg L⁻¹). In each case, the flasks were inoculated with 0.5% v/v of acclimated cells collected in the late exponential phase. Aliquots of the cell suspensions were collected after 18 hours of growth/exposure (mid-exponential phase), immediately mixed with two volumes of RNA Protect® (Qiagen, Carlsbad, CA), and frozen at -80°C. Gene expression experiments were conducted in triplicate.

Extraction and analysis of PCBs, OH-PCBs, and their metabolites

PCBs, OH-PCBs, and their metabolites were extracted and analyzed by GC-MS as described elsewhere (Tehrani et al, 2012). Briefly, parent PCBs, 2,5-DCB and 2,4,6-TCB, were extracted using a mixture of Triton X100 and *n*-hexane. For the extraction of hydroxylated PCBs, OH-2,5-DCBs and OH-2,4,6-TCBs, and their metabolites, 2,5-dichlorobenzoic acid (2,5-DCBA) and 2,4,6-trichlorobenzoic acid (2,4,6-TCBA), samples were acidified with 6 M hydrochloric acid (HCl) to pH 3, extracted with ethyl acetate, and derivatized using a mixture of BSTFA/1%-TMCS. Analyses were conducted using a 7890A GC equipped with a 5975C MSD (Agilent, Santa Clara, CA) and a HP-5MS high-efficiency capillary column (30 m × 0.25 mm, 0.25 µm; Agilent). The extraction efficiencies were estimated by the extraction and analysis of controls dosed with known concentrations of target compounds. Detailed extraction and analysis protocols are provided as supplementary information.

RNA extraction and gene expression analyses

Briefly, total RNA was extracted from RNA Protect®-treated cells using RNeasy® Tissue Mini Kit (Qiagen) and reverse-transcribed using SuperScript™ Reverse Transcriptase III (Invitrogen, Carlsbad, CA) following the manufacturers' recommendations. Quantification of the resulting complementary DNA (cDNA) was performed by real-time PCR on an ABI StepOne™ System using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). Cycling conditions were as prescribed by the manufacturer. The amplification levels of bph genes were normalized by the 16S rDNA (internal standard) and expressed by reference to controls non-exposed to the target compounds (ΔCT method, StepOne™ Software, version 2.1, Applied Biosystems). The amplification efficiency was determined for all primer sets using 6-log dilutions of the cDNA template according to standard protocols.

Data analyses

Inhibition of *B. xenovorans* LB400 was modeled using a first-order inactivation model with shoulder using the non-linear regression procedure in Prism 5.0 (La Jolla, CA). The efficiency of real-time PCR amplifications was calculated by plotting C_T values against log cDNA concentrations according to standard procedures. The statistical significance of the results (for biodegradation experiments and gene expression analyses) was assessed by performing unpaired *t*-tests at 95% confidence level.

Results

The half-maximum inhibitory concentration (IC₅₀) of the target compounds, 2,5-DCB, 2,4,6-TCB, and their hydroxylated derivatives (2'-OH-, 3'-OH-, and 4'-OH-), toward *B. xenovorans* LB400 was determined by performing growth inhibition tests in which the cells were individually exposed to increasing concentrations of the compounds. The bacterial growth was monitored by the OD₆₀₀. Overall, exposure to PCBs and OH-PCBs resulted in higher inhibition of cell growth when biphenyl was used as the carbon source as compared to succinate (Fig. 1 and Table 1).

The capability of *B. xenovorans* LB400 to metabolize 2,5-DCB, 2,4,6-TCB, and their hydroxylated derivatives (2'-OH-, 3'-OH-, and 4'-OH-) was tested by growing the bacterium on K1 mineral medium containing succinate or biphenyl and dosed with 5 mg L⁻¹ of target compounds. The residual concentrations of the compounds were determined after 96 hours of incubation (Table 2). In the presence of biphenyl, 2,5-DCB and the three OH-2,5-DCBs tested were significantly transformed after 4 days: 100% of 2,5-DCB and 2'-OH-2,5-DCB, 69.0 ± 3.3% of 3'-OH-2,5-DCB, and 60.7 ± 0.9 % of 4'-OH-2,5-DCB. In the presence of succinate, 100% of 2,5-DCB, 46.6 ± 0.6% of 2'-OH-2,5-DCB, and 19.6 ± 5.3% of 4'-OH-2,5-DCB were transformed over the time of the experiment, while no significant transformation of 3'-OH-2,5-DCB was recorded. The expected metabolite, 2,5-DCBA, was detected in all cultures where a significant degradation of 2,5-DCB and OH-2,5-DCBs occurred (Table 2, upper section). 2,5-DCBA was measured at concentrations suggesting almost quantitative conversion of 2,5-DCB and OH-2,5-DCBs into 2,5-DCBA, except for 2'-OH-2,5-DCB in the presence of succinate, whose recovery accounted for 82.7 ± 1.6% of the parent compound. The estimated extraction efficiencies (expressed as mean ± standard deviation of three replicates) were 99.7 ± 3.8% for 2,5-DCB, 95.2 ± 1.8% for 2,4,6-TCB, 105.8 ± 6.5 for 2,5-DCBA, 100.1 ± 0.4% for 2,4,6-TCBA, 89.4 ± 1.3% to 95.6 ± 2.8% for OH-2,5-DCBs, and 94.5 ± 4.0% to 95.2 ± 1.8% for OH-2,4,6-TCBs. On the other hand, neither 2,4,6-TCB nor any of the OH-2,4,6-TCBs tested, except one, were significantly transformed by *B. xenovorans* LB400 over the time of the experiment, regardless of the carbon source used. Only 3'-OH-2,4,6-TCB was slightly transformed by *B. xenovorans* LB400 in the presence of succinate (12.3 ± 2.7%). The predicted biphenyl pathway metabolite, 2,4,6-TCBA, was not detected (Table 2, bottom section).

Because 2,5-DCBA is the only metabolite detected from the transformation of 2,5-DCB and OH-2,5-DCBs, the inhibitory effect and biodegradation of 2,5-DCBA by *B. xenovorans* LB400 were tested in the presence of succinate and biphenyl as carbon sources. The corresponding dose-response curves are presented in Fig. 2. Biodegradation experiments did not show significant transformation of 2,5-DCBA regardless of the carbon source used (supplementary information, Table S2).

In order to understand further the metabolism of 2,5-DCB and its hydroxylated derivatives, the expression of key genes of the biphenyl pathway, *bphA*, *bphB*, *bphC*, and *bphD*, was determined using RT-qPCR (Table 3). The induction of *bph* genes by biphenyl was assessed by comparing their expression levels in cells growing in the presence of succinate (1,180 mg L⁻¹) and biphenyl (770 mg L⁻¹). Biphenyl was shown to result in significant overexpression

of *bph* genes as compared to succinate, with relative expression levels ranging from 6.5 ± 0.5 to 8.8 ± 1.2 . On the other hand, when cells were grown on succinate ($1,180 \text{ mg L}^{-1}$) as the carbon source, an overexpression of *bph* genes was observed in the presence of 5 mg L^{-1} of 2,5-DCB, and 2'-OH-2,5-DCB as compared to non-exposed controls, with relative expression levels ranging from 1.8 ± 0.0 to 2.5 ± 0.5 and 1.8 ± 0.3 to 2.0 ± 0.4 , respectively (Table 3). (Exposure to 5 mg L^{-1} of biphenyl resulted in expression levels of 2.7 ± 0.9 to 5.7 ± 2.3 .) On the contrary, a downregulation of *bph* genes was observed in the presence of 3'-OH-2,5-DCB and 4'-OH-2,5-DCB (5 mg L^{-1}). The expression levels of *bph* genes upon exposure to biphenyl, 2,5-DCB, and OH-2,5-DCBs relative to non-exposed controls were statistically significant for all genes tested ($p < 0.05$) except *bphD*. The amplification efficiencies for all sets of primers ranged from 91 to 101% (regression lines slopes between -3.5 and -3.3 , with R^2 higher than 0.99).

Discussion

In contrast to PCBs, very little is known about the bacterial metabolism of hydroxylated PCBs. In this study, we chose to study hydroxylated derivatives of a dichloro- and a trichlorobiphenyl: 2,5-DCB was selected because it is present in the commercial mixtures, Aroclor 1016 and 1242, and because hydroxylated isomers could be synthesized; 2,4,6-TCB is not detected in commercial mixtures but it was chosen based on the commercial availability its hydroxylated isomers. We showed that exposure of *B. xenovorans* LB400 to hydroxylated derivatives of 2,5-DCB and 2,4,6-TCB resulted in a stronger inhibition when the cells were growing in the presence of biphenyl as compared to succinate, suggesting either that induction of the biphenyl pathway produced toxic metabolites or that hydroxylated derivatives of 2,5-DCB and 2,4,6-TCB inhibited the biphenyl pathway. A few publications showed that hydroxylation of PCBs resulted in increasing toxicity toward bacteria. Sondossi et al. (2004) measured a significant decrease of the oxygen uptake rate in *C. testosteroni* B-356 exposed to hydroxylated PCBs as compared to hydroxybiphenyls and PCBs. Similarly, Cámara et al. (2004) reported decreasing cell viability in recombinant *E. coli* expressing subsets of *bph* genes following exposure to PCBs, which was attributed to the high toxicity of di-hydroxylated derivatives originating from the PCB metabolism. Exposure of *B. xenovorans* LB400 to the commercial mixture Aroclor 1242 was shown to result in the induction of detoxification genes under biphenyl pathway-inducing condition, again suggesting the generation of toxic metabolites (Parnell et al. 2006). We have recently showed that exposure to hydroxylated metabolites of 4-CB resulted in higher inhibition of *B. xenovorans* LB400 under condition inductive of the biphenyl pathway. Besides specific toxic mechanisms, hydroxylation is known to result in increasing solubility and bioavailability of the molecules susceptible to explain higher toxicity (Cámara et al. 2004).

Our results also showed that the extent of transformation of 2,5-DCB and its hydroxylated derivatives was consistent with the pattern of expression of genes of the upper biphenyl pathway (*bph*). When cells were growing on biphenyl, a strong induction of *bph* genes was recorded in the presence of all target compounds, which resulted in significant transformation of 2,5-DCB and the three OH-2,5-DCBs. On the other hand, when cells were growing on succinate, only 2,5-DCB and 2'-OH-2,5-DCB slightly induced expression of *bph* genes, leading to transformation of these two compounds, while 3'-OH- and 4'-OH-2,5-

DCB were left unchanged. These results strongly suggest that the metabolism of OH-2,5-DCBs occurred through the biphenyl pathway. Biphenyl, as the natural substrate of the biphenyl pathway, is known as a strong inducer of *bph* genes (Denef et al. 2004; Denef et al. 2005; Parnell et al. 2010). Previous publications have shown that lesser-chlorinated OH-PCBs were transformed through the biphenyl pathway. A variety of hydroxylated biphenyls (2-, 3-, and 4-OH-biphenyl) and PCBs (4-OH-2-CB, 4-OH-3-CB, and 4-OH-5-CB) was shown to be transformed by *C. testosteroni* B-356 and by a recombinant *Pseudomonas putida* strain harboring the biphenyl pathway system (Sondossi et al. 2004). As it was observed with most PCBs, the metabolism of these compounds involved dihydroxylation in positions 2,3 of the unsubstituted ring followed by *meta*-cleavage in position 1,2. Francova et al. (2004) reported the transformation of a series of *ortho*-substituted hydroxylated PCBs (2-OH-3-CB, 2-OH-5-CB, and 2-OH-3,5-DCB) by biphenyl dioxygenases of *B. xenovorans* LB400 and *C. testosteroni* B-356. However, these reports focused on congeners bearing hydroxyl and chlorine substituents located on the same ring, even though hydroxylation of PCBs has been reported on both chlorinated and non-chlorinated rings (Kaminski et al. 1981; Kucerova et al. 2000; Sietmann et al. 2006; Rezek et al. 2007; Rezek et al. 2008; Zhai et al. 2010). We have recently showed that *B. xenovorans* LB400 was capable of transforming 4-CB and three of its derivatives hydroxylated on the *non*-chlorinated ring (2'-OH-, 3'-OH-, and 4'-OH-4-CB) (Tehrani et al., 2012). The specific contribution of the present study is to provide evidence of the metabolism of higher-chlorinated biphenyl derivatives that are hydroxylated on the *non*-chlorinated ring (i.e., 2'-OH-, 3'-OH-, and 4'-OH-2,5-DCB).

Our results showed that transformation of 2,5-DCB and OH-2,5-DCBs was associated with the accumulation of the PCB metabolite, 2,5-dichlorobenzoate (2,5-DCBA), which accounted for a large fraction of the parent compounds, suggesting that the initial attack by dioxygenases occurred primarily on the non-chlorinated ring. Our results showed that transformation of 2,5-DCB and OH-2,5-DCBs was associated with the accumulation of the PCB metabolite, 2,5-dichlorobenzoate (2,5-DCBA), which accounted for a large fraction of the parent compounds, suggesting that the initial attack by dioxygenases occurred primarily on the non-chlorinated ring. Indeed, the biphenyl 2,3-dioxygenase (BphA) of *B. xenovorans* LB400, which catalyzes the initial hydroxylation of the biphenyl core, was shown to oxidize preferentially the non-chlorinated ring. Moreover, oxidation of 2,5-dichlorinated rings by BphA of *B. xenovorans* LB400 (3,4-dioxygenation) is known to produce dead-end metabolites, which were not detected in our experiments (Peeper and Seeger, 2008). Our results also suggest that 2,5-DCBA was not efficiently transformed by *B. xenovorans* LB400. Some chlorobenzoic acids are known to be dead-end products of the bacterial metabolism of PCBs and their toxicity has been suggested to be responsible for the limited degradation of PCBs observed in the environment (Billingsley et al. 1999; Flanagan and May 1993; Cámara et al. 2004; Martínez et al. 2007). In order to test this hypothesis, we measured the toxicity and biodegradability of 2,5-DCBA by *B. xenovorans* LB400 and observed that the compound was not significantly transformed by the bacterium, even under biphenyl pathway-inducing condition, nor did it result in significant inhibition of cell growth at concentration up to 20 mg L⁻¹. As for PCBs, biodegradation of chlorobenzoic acids by bacteria depends largely on the degree and pattern of chlorine substitution of the aromatic

ring. Billingsley et al. (1999) have reported that *B. xenovorans* LB400 growing on biphenyl could transform some chlorobenzoic acids (i.e., 2-CB, 2,3-DCBA), while others were not metabolized (i.e., 4-CBA, 2,4-DCBA). On the other hand, a more recent study reported that the same strain was not capable of metabolizing 2- and 4-CBA when using glucose as the carbon source (Martinez et al. 2007). In agreement with our results, Cámara et al. (2004) reported a mild toxicity of 2,3-DCBA for *Escherichia coli* cells.

Our results also showed that *B. xenovorans* LB400 was not capable of transforming efficiently 2,4,6-TCB or any of its hydroxylated derivatives over the time of the experiment, which could be explained by the inability of these compounds to induce the biphenyl pathway. Alternatively, the recalcitrance of 2,4,6-trichlorinated congeners to degradation by *B. xenovorans* may be related to the specificity of the biphenyl 2,3-dioxygenase (BphA) known to be highly dependent on the substitution pattern of the substrate (Peeper and Seeger, 2008). Our findings are also consistent with the general observation that higher-chlorinated PCBs are less susceptible to oxidative transformation, which is explained by the electron-withdrawing effect of the chlorine atom, reducing the electron density of the aromatic rings (Borja et al., 2005).

Our results may have important implications for the natural attenuation of PCBs because hydroxylated PCBs are the most common PCB metabolites generated by higher organisms and they have been detected in the environment (Flanagan and May 1993; Jansson et al. 1975; Kawano et al. 2005; Ueno et al. 2007). In addition, the recalcitrance to biodegradation and toxicity of some hydroxylated PCBs may provide an explanation for the persistence of PCBs in the environment (Cámara et al. 2004).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Billingsley KA, Backus SM, Ward OM. Production of metabolites from chlorobiphenyls by resting cells of *Pseudomonas* strain LB400 after growth on different carbon sources. *Can J Microbiol.* 1999; 45:178–184.
- Borja J, Taleon DM, Aurensenia J, Gallardo S. Polychlorinated biphenyls and their biodegradation. *Process Biochem.* 2005; 40:1999–2013.
- Cámara B, Herrera C, González M, Couve E, Hofer B, Seeger M. From PCBs to highly toxic metabolites by the biphenyl pathway. *Environ Microbiol.* 2004; 6:842–850. [PubMed: 15250886]
- Denef VJ, Park J, Tsoi TV, Rouillard JM, Zhang H, Wibbenmeyer JA, Verstraete W, Gulari E, Hashsham SA, Tiedje JM. Biphenyl and benzoate metabolism in a genomic context: Outlining genome-wide metabolic networks in *Burkholderia xenovorans* LB400. *Appl Environ Microbiol.* 2004; 70:4961–4970. [PubMed: 15294836]

- Denef VJ, Patrauchan MA, Florizone C, Park J, Tsoi TV, Verstraete W, Tiedje JM, Eltis LD. Growth substrate- and phase-specific expression of biphenyl, benzoate, and C-1 metabolic pathways in *Burkholderia xenovorans* LB400. *J Bacteriol.* 2005; 187:7996–8005. [PubMed: 16291673]
- Field JA, Sierra-Alvarez R. Microbial transformation and degradation of polychlorinated biphenyls. *Environ Pollut.* 2008; 155:1–12. [PubMed: 18035460]
- Flanagan WP, May RJ. Metabolite detection as evidence for naturally occurring aerobic PCB biodegradation in Hudson River sediments. *Environ Sci Technol.* 1993; 27:2207–2212.
- Francova K, Mackova M, Macek T, Sylvestre M. Ability of bacterial biphenyl dioxygenases from *Burkholderia* sp. LB400 and *Comamonas testosteroni* B-356 to catalyse oxygenation of ortho-hydroxychlorobiphenyls formed from PCBs by plants. *Environ Pollut.* 2004; 127:41–48. [PubMed: 14553993]
- Jansson B, Jensen S, Olsson M, Renberg L, Sundström G, Vaz R. Identification by GC-MS of phenolic metabolites of PCB and p,p'-DDE isolated from Baltic guillemot and seal. *Ambio.* 1975; 4:93–97.
- Kaminski L, Kennedy MW, Adams SM, Guengerich FP. Metabolism of dichlorobiphenyls by highly purified isozymes of rat liver cytochrome P-450. *Biochemistry.* 1981; 20:7379–7384. [PubMed: 6798990]
- Kawano M, Hasegawa J, Enomoto T, Onishi H, Nishio Y, Matsuda M, Wakimoto T. Hydroxylated polychlorinated biphenyls (OH-PCBs): Recent advances in wildlife contamination study. *Environ Sci.* 2005; 12:315–324. [PubMed: 16609671]
- Kitamura S, Jinno N, Suzuki T, Sugihara K, Ohta S, Kuroki H, Fujimoto N. Thyroid hormone-like and estrogenic activity of hydroxylated PCBs in cell culture. *Toxicology.* 2005; 208:377–387. [PubMed: 15695023]
- Kucerova P, Mackova M, Chroma L, Burkhard J, Triska J, Demnerova K, Macek T. Metabolism of polychlorinated biphenyls by *Solanum nigrum* hairy root clone SNC-90 and analysis of transformation products RID B-1876-2008. *Plant Soil.* 2000; 225:109–115.
- Lehmleer HJ, Robertson LW. Synthesis of hydroxylated PCB metabolites with the Suzuki-coupling. *Chemosphere.* 2001; 45:1119–1127. [PubMed: 11695625]
- Letcher, RJ.; Klasson-Wehler, E.; Bergman, A. Methyl sulfone and hydroxylated metabolites of polychlorinated biphenyls. In: Hutzinger, O.; Paasivirta, J., editors. *Earth and Environmental Science. Vol. 3, Anthropogenic Compounds.* Springer; Berlin: 2000. p. 315-359.
- Martínez P, Agulló L, Hernández M, Seeger M. Chlorobenzoate inhibits growth and induces stress proteins in the PCB-degrading bacterium *Burkholderia xenovorans* LB400. *Arch Microbiol.* 2007; 188:289–297. [PubMed: 17522847]
- Parnell JJ, Park J, Denef V, Tsoi T, Hashsham S, Quensen J, Tiedje JA. Coping with polychlorinated biphenyl (PCB) toxicity: Physiological and genome-wide responses of *Burkholderia xenovorans* LB400 to PCB-mediated stress. *Appl Environ Microbiol.* 2006; 72:6607–6614. [PubMed: 17021212]
- Parnell JJ, Denef VJ, Park J, Tsoi TV, Tiedje JM. Environmentally relevant parameters affecting PCB degradation: Carbon source- and growth phase-mitigated effects on the expression of the biphenyl pathway and associated genes in *Burkholderia xenovorans* LB400. *Biodegradation.* 2010; 21:147–156. [PubMed: 19672561]
- Pieper DH, Seeger M. Bacterial metabolism of polychlorinated biphenyls. *J Mol Microbiol Biotechnol.* 2008; 15:121–138. [PubMed: 18685266]
- Rezek J, Macek T, Mackova M, Triska J. Plant metabolites of polychlorinated biphenyls in hairy root culture of black nightshade *Solanum nigrum* SNC-90. *Chemosphere.* 2007; 69:1221–1227. [PubMed: 17640705]
- Rezek J, Macek T, Mackova M, Triska J, Ruzickova K. Hydroxy-PCBs, methoxy-PCBs and hydroxy-methoxy-PCBs: Metabolites of polychlorinated biphenyls formed *in vitro* by tobacco cells. *Environ Sci Technol.* 2008; 42:5746–5751. [PubMed: 18754503]
- Sietmann R, Gesell M, Hammer E, Schauer F. Oxidative ring cleavage of low chlorinated biphenyl derivatives by fungi leads to the formation of chlorinated lactone derivatives. *Chemosphere.* 2006; 64:672–685. [PubMed: 16352329]

- Sondossi M, Barriault D, Sylvestre M. Metabolism of 2,2'- and 3,3'-dihydroxybiphenyl by the diphenyl catabolic pathway of *Comamonas testosteroni* B-356. *Appl Environ Microbiol.* 2004; 70:174–181. [PubMed: 14711640]
- Schroder P, Scheer C, Belford BJD. Metabolism of organic xenobiotics in plants: Conjugating enzymes and metabolic end points. *Minerva Biotechnologica.* 2001; 13:85–91.
- Takeuchi S, Shiraishi F, Kitamura S, Kuroki H, Jin K, Kojima H. Characterization of steroid hormone receptor activities in 100 hydroxylated polychlorinated biphenyls, including congeners identified in humans. *Toxicology.* 2011; 289:112–121. [PubMed: 21843587]
- Tehrani R, Lyv MM, Kaveh R, Schnoor JL, Van Aken B. Biodegradation of mono-hydroxylated PCBs by *Burkholderia xenovorans*. *Biotechnol lett.* 2012; 34:2247–2252. [PubMed: 22918793]
- Ueno D, Darling C, Alaei M, Campbell L, Pacepavicius G, Teixeira C, Muir D. Detection of hydroxylated polychlorinated biphenyls (OH-PCBs) in the abiotic environment: Surface water and precipitation from Ontario. *Canada Environ Sci Technol.* 2007; 41:1841–1848.
- Zhai G, Lehmler H, Schnoor JL. Hydroxylated metabolites of 4-monochlorobiphenyl and its metabolic pathway in whole poplar plants. *Environ Sci Technol.* 2010; 44:3901–3907. [PubMed: 20402517]

Fig. 1.

Dose-response curves showing the effect of 2,5-dichlorobiphenyl (2,5-DCB), 2,4,6-trichlorobiphenyl (2,4,6-TCB), and their hydroxylated derivatives on the growth of *B. xenovorans* LB400. The endpoint used for the measurement of growth inhibition was the cell concentration (expressed as the optical density at 600 nm - OD₆₀₀) that was reached in the early stationary phase. Solid lines show fitting to a first-order inactivation model with shoulder. **Panel a:** Exposure to 2,5-DCB, 2'-hydroxy-(2'-OH-), 3'-OH-, and 4'-OH-2,5-DCB (0.0 to 20 mg L⁻¹) with succinate (1,180 mg L⁻¹) as the carbon source. **Panel b:** Exposure to 2,5-DCB, 2'-OH-, 3'-OH-, and 4'-OH-2,5-DCB (0.0 to 20 mg L⁻¹) with biphenyl (770 mg L⁻¹) as the carbon source. **Panel c:** Exposure to 2,4,6-TCB, 2'-OH-, 3'-OH-, and 4'-OH-2,4,6-TCB (0.0 to 50 mg L⁻¹) with succinate (1,180 mg L⁻¹) as the carbon source. **Panel d:** Exposure to 2,4,6-TCB, 2'-OH-, 3'-OH-, and 4'-OH-2,4,6-TCB (0.0 to 50 mg L⁻¹) with biphenyl (770 mg L⁻¹) as the carbon source. Errors bars show the standard deviation between three replicates.

Fig. 2.

Dose-response curves showing the effect of 2,5-dichlorobenzoic acid (2,5-DCBA) (0.0 to 50 mg L⁻¹) on the growth of *B. xenovorans* LB400 growing on succinate (1,180 mg L⁻¹) and biphenyl (770 mg L⁻¹). Bacterial growth was expressed by the optical density at 600 nm (OD₆₀₀) that was reached in the early stationary phase. Solid lines show fitting to a first-order inactivation model with shoulder. Errors bars show the standard deviation between three replicates.

Table 1

Half-maximum inhibitory concentrations (IC₅₀) of *B. xenovorans* LB400 exposed to 2,5-dichlorobiphenyl (2,5-DCB), 2,4,6-trichlorobiphenyl (2,4,6-TCB), and their respective 2'-hydroxy- (2'-OH-), 3'-hydroxy- (3'-OH-), and 4'-hydroxy- (4'-OH-) derivatives

Compounds	Succinate ^a				Biphenyl ^b			
	2,5-DCB ^c	2'-OH-	3'-OH-	4'-OH-	2,5-DCB ^c	2'-OH-	3'-OH-	4'-OH-
IC ₅₀ ^e (mg L ⁻¹) ^g	N.D. ^f	6.9	16.0	14.9	N.D. ^f	5.7	5.2	11.4
CI 95% (mg L ⁻¹) ^h	-	-	-	-	-	-	-	-
R ²		1.00	1.00	1.00	1.00	1.00	1.00	1.00
Compounds	2,4,6-TCB ^d	2'-OH-	3'-OH-	4'-OH ^c	2,4,6-TCB ^d	2'-OH-	3'-OH-	4'-OH-
	2,4,6-TCB ^d	2'-OH-	3'-OH-	4'-OH ^c	2,4,6-TCB ^d	2'-OH-	3'-OH-	4'-OH-
IC ₅₀ ^e (mg L ⁻¹) ^g	N.D. ^f	19.3±10.6	13.5±8.7	15.7±4.8	14.3±2.7	4.2±1.2	2.7±0.0	2.7±0.0
CI 95% (mg L ⁻¹) ^h	-	-	-	7.8-30.2	-	-	2.6-2.8	2.7-2.7
R ²	0.96	0.98	0.97	0.99	0.98	0.99	1.0	1.0

^a *B. xenovorans* LB400 was growing using succinate (10 mM, 1,180 mg L⁻¹) as the carbon source.

^b *B. xenovorans* LB400 was growing using biphenyl (5 mM, 770 mg L⁻¹) as the carbon source.

^c 2,5-DCB and OH-2,5-DCBs were dosed at concentration ranging from 0 to 20 mg L⁻¹.

^d 2,4,6-TCB and OH-2,4,6-TCBs were dosed at concentration ranging from 0 to 50 mg L⁻¹.

^e Half-maximum inhibitory concentrations (IC₅₀) were estimated using a first-order inactivation model with shoulder. R² is the coefficient of determination of the curve fitting.

^f The IC₅₀ of these compounds was not determined (N.D.) because they did not exhibited a significant toxicity.

^g The standard deviation could not be calculated for several compounds because the first-order decay moiety of the curve did not include enough data points.

^h The 95%-confidence intervals (CI 95%) of IC₅₀ could not be calculated for several compounds because the first-order decay moiety of the curve did not include enough data points.

Table 2

Transformation of 2,5-dichlorobiphenyl (2,5-DCB), 2,4,6-trichlorobiphenyl (2,4,6-TCB) and their respective 2'-hydroxy- (2'-OH-), 3'-OH-, and 4'-OH- derivatives by *B. xenovorans* LB400 and formation of their potential metabolites, 2,5-dichlorobenzoic acid (2,5-DCBA) and 2,4,6-trichlorobenzoic acid (2,4,6-TCBA)

Compound	Succinate ^a			Biphenyl ^b		
	Initial ^c (%) (0 h)	Final ^c (%) (96 h)	CBA ^d (%) (96 h)	Initial ^c (%) (0 h)	Final ^c (%) (96 h)	CBA ^d (%) (96 h)
2,5-DCB	101.53±0.46	N.D. ^e	82.70±1.62	98.74±0.87	1.00±1.13	100.83±4.12
<i>P</i> -Value ^f		0.003			0.000	
2'-OH-	99.71±1.35	53.40±0.64	47.07±0.18	98.94±0.42	N.D. ^e	99.01±3.4
<i>P</i> -Value ^f		0.000			0.000	
3'-OH-	100.91±0.68	99.23±3.16	N.D. ^e	100.65±0.73	30.97±3.30	66.34±0.29
<i>P</i> -Value ^f		0.377			0.004	
4'-OH-	100.13±0.19	80.41±5.34	N.D. ^e	100.20±1.09	39.33±0.92	60.33±2.41
<i>P</i> -Value ^f		0.022			0.007	
2,4,6-TCB	98.09±3.00	90.01±5.80	N.D. ^e	100.62±0.86	104.16±4.17	N.D. ^e
<i>P</i> -Value ^f		0.098			0.220	
2'-OH-	101.10±5.62	94.27±6.73	N.D. ^e	105.98±1.38	94.63±3.16	N.D. ^e
<i>P</i> -Value ^f		0.269			0.379	
3'-OH-	105.65±3.26	87.69±2.68	N.D. ^e	103.84±1.40	94.74±5.39	N.D. ^e
<i>P</i> -Value ^f		0.020			0.754	
4'-OH-	102.38±4.09	90.72±4.34	N.D. ^e	107.15±0.25	94.73±2.01	N.D. ^e
<i>P</i> -Value ^f		0.075			0.498	

^a *B. xenovorans* LB400 was growing using succinate (10 mM, 1,180 mg L⁻¹) as the carbon source.

^b *B. xenovorans* LB400 was growing using biphenyl (5 mM, 770 mg L⁻¹) as the carbon source.

^c 2,5-DCB, 2,4,6-TCB, and their hydroxylated derivatives are expressed as percentage of the initial concentration (5 mg L⁻¹). Results are presented as mean and standard deviation of three replicates.

^d Chlorobenzoic acids (CBAs) are expressed as percentage of the maximum theoretical amount that could be produced given the initial concentration of PCBs and OH-PCBs. 2,5-DCBA and 2,4,6-TCBA are produced from the transformation of 2,5-DCB and OH-2,5-DCBs, and 2,4,6-TCB and OH-2,4,6-TCBs, respectively.

^e Not detected (N.D.).

^f The statistical significance of the transformations was assessed by comparing the initial and final concentrations using unpaired *t*-tests at 95% confidence level. Not detected (N.D.) concentrations were counted as zero.

Table 3

Level of expression of genes of the biphenyl pathway, *bphA*, *bphB*, *bphC*, and *bphD*, in *B. xenovorans* LB400 exposed to biphenyl (BP), 2,5-dichlorobiphenyl (2,5-DCB), and its hydroxylated derivatives, 2'-hydroxy- (2'-OH-), 3'-OH-, and 4'-OH-DCB.

Biphenyl^a		Succinate^b				
Genes	BP^c (770 mg L⁻¹)	BP^{c,d} (5 mg L⁻¹)	2,5-DCB^{c,d} (5 mg L⁻¹)	2'-OH-^{c,d} (5 mg L⁻¹)	3'-OH-^{c,d} (5 mg L⁻¹)	4'-OH-^{c,d} (5 mg L⁻¹)
<i>bphA</i>	6.50±0.53	2.70±0.91	2.23±0.10	2.03±0.39	0.40±0.10	0.20±0.03
<i>P</i> -Value ^e	0.001	0.003	0.001	0.014	0.011	0.001
<i>bphB</i>	6.81±1.97	5.11±3.41	2.53±0.53	1.98±0.40	0.39±0.13	0.25±0.08
<i>P</i> -Value ^e	0.005	0.027	0.009	0.015	0.018	0.018
<i>bphC</i>	7.55±0.14	5.66±2.32	2.25±0.38	1.89±0.17	0.46±0.17	0.25±0.06
<i>P</i> -Value ^e	0.001	0.014	0.015	0.033	0.05	0.013
<i>bphD</i>	8.79±1.18	4.79±1.87	1.86±0.04	1.78±0.27	0.52±0.10	0.20±0.11
<i>P</i> -Value ^e	0.001	0.089	0.286	0.312	0.253	0.154

^a *B. xenovorans* LB400 was growing using biphenyl (5 mM, 770 mg L⁻¹) as the carbon source.

^b *B. xenovorans* LB400 was growing using succinate (10 mM, 1,180 mg L⁻¹) as the carbon source.

^c Gene expression levels were recorded after 18 hours of exposure and were normalized by the expression level of 16S ribosomal DNA (rDNA) gene. Results are presented as mean and standard deviation of three replicates.

^d Biphenyl (BP), 2,5-DCB, 2'-OH-, 3'-OH-, and 4'-OH-2,5-DCB were dosed at 5 mg L⁻¹.

^e The statistical significance was assessed by comparing the genes expression levels in exposed and non-exposed cells using unpaired *t*-tests at 95% confidence level.