Bacterial Metabolism of Polychlorinated Biphenyls

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\textbf{Abstract}
Microbial metabolism is responsible for the removal of persistent organic pollutants including PCBs from the environment. Anaerobic dehalogenation of highly chlorinated congeners in aquatic sediments is an important process, and recent evidence has indicated that \textit{Dehalococcoides} and related organisms are predominantly responsible for this process. Such anaerobic dehalogenation generates lower chlorinated congeners which are easily degraded aerobically by enzymes of the biphenyl upper pathway (bph). Initial biphenyl 2,3-dioxygenases are generally considered the key enzymes of this pathway which determine substrate range and extent of PCB degradation. These enzymes have been subject to different protein evolution strategies, and subsequent enzymes have been considered as crucial for metabolism. Significant advances have been made regarding the mechanistic understanding of these enzymes, which has also included elucidation of the function of BphK glutathione transferase. So far, the genomes of two important PCB-metabolizing organisms, namely \textit{Burkholderia xenovorans} strain LB400 and \textit{Rhodococcus} sp. strain RHA1, have been sequenced, with the rational to better understand their overall physiology and evolution. Genomic and proteomic analysis also allowed a better evaluation of PCB toxicity. Like all \textit{bph} gene clusters which have been characterized in detail, particularly in strains LB400 and RHA1, these genes were localized on mobile genetic elements endowing single strains and microbial communities with a high flexibility and adaptability. However, studies show that our knowledge on enzymes and genes involved in PCB metabolism is still rather fragmentary and that the diversity of bacterial strategies is highly underestimated. Overall, metabolism of biphenyl and PCBs should not be regarded as a simple linear pathway, but as a complex interplay between different catabolic gene modules.

\textbf{Introduction}

The concern about environmental pollution with persistent organic pollutants is increasing due to their toxicity, bioaccumulation, extensive distribution and recalcitrance. Polychlorinated biphenyls (PCBs) are a class of compounds which have been used since 1929 for various industrial and commercial purposes such as dielectric, heat transfer, hydraulic fluids, plasticizers and fire retardants. PCBs have been sold under trade names such as Aroclor (Monsanto, USA, Canada, and UK), Phenoclor (Prodelec, France and Spain), Clophen (Bayer, Germany), Sovol and Sovtol (Orgsteklo, Orgsintez, former Soviet Union) and Kanechlor (Kanegafuchi, Japan). Typically,
commercial PCB mixtures contain between 20 and 70 of the 209 theoretically possible congeners. It is estimated that more than 1.5 million tons of PCBs have been manufactured worldwide [Faroon et al., 2003], where a significant amount has been released into the environment and accumulated in soils and sediments [Nogales et al., 1999; Sericano et al., 1995]. The lipophilicity of PCBs contributes to their magnification in the food chain. Although adverse health effects were first recorded in the 1930s [Drinker et al., 1937], PCBs continued to be used for decades. Since then, PCBs have been shown to cause cancer [Mayes et al., 1998] and a number of serious effects on the immune, reproductive, nervous and endocrine system [Aoki, 2001; ATSDR, 2000; Faroon et al., 2001].

Bacteria play a fundamental role in the removal of waste chemical compounds from the environment. Bioremediation is a promising technology for the treatment of PCB-contaminated environments [Harkness et al., 1993; Pieper, 2005]. However, the process of bioremediation of PCBs is still not well understood and its microbial and molecular basis has to be further studied.

Reductive Dehalogenation

Although PCBs are highly stable environmental pollutants, their metabolism has been known for a few decades. Since the first report on bacterial metabolism of PCBs under anaerobic conditions in sediments of the Hudson River [Brown et al., 1987] numerous studies have since reported on the microbial dechlorination of PCBs in situ and in laboratory experiments with sediment slurries. Various dechlorination patterns in environmental and laboratory samples have been described. Typically, meta and/or para chlorines are removed to generate primarily ortho-substituted chlorobiphenyls, but ortho dechlorination of several PCB congeners has also been reported [Wiegel and Wu, 2000].

It has been known for more than one decade that chloroaromatics can function as an alternative electron acceptor in anaerobic respiration [Mohn and Tiedje, 1990]. Several anaerobic bacteria, such as the Dehalococcoides [Maymo-Gatell et al., 1999], the low GC Gram-positive bacteria Desulfotobacterium [Sanford et al., 1996], Dehalobacter [Holliger et al., 1998], the proteobacteria Desulfomonaile [de Weerd and Suflita, 1990], Desulfiuromonas [Krumholz, 1997], and Sulfospirillum [Boyle et al., 1999], have been identified as being able to reductively dechlorinate chlorinated phenols, benzoates, and trichloroethene and to couple this reaction to the synthesis of ATP via a chemiosmotic mechanism [Mohn and Tiedje, 1991].

Organisms able to mediate the reductive dechlorination of PCBs have been difficult to identify by traditional isolation techniques. Using an approach that combined classical enrichment protocols in a defined sediment-free medium with genetic screening of the microbial communities, two anaerobic PCB-dechlorinating microorganisms, o-17 and DF-1, both distantly related to Dehalococcoides (<90% 16S rDNA sequence similarity, while sequence similarity among strains was very high, >98%) were identified as able to perform a reductive dehalogenation. While strain DF-1 specifically dechlorinates congeners with doubly flanked chlorines such as 2,3,4,5-tetrachlorobiphenyl (2,3,4,5-CB) which is dechlorinated at the para position [Wu et al., 2002], the o-17 strain was capable of ortho dechlorination of 2,3,5,6-chlorobiphenyl to 2,3,5-trichloro- and 3,5-dichlorobiphenyl [Cutler et al., 2001] (fig. 1), a capability which is rarely observed in the environment. The o-17 strain culture was assayed with 26 PCB congeners and revealed that 8 PCBs could be dechlorinated [May et al., 2006], including single-flanked ortho PCB chlorines. However, double-flanked chlorines were preferentially dechlorinated, and the dechlorination of three congeners could be carried out by various sequential transfers. Nevertheless, the ability to dechlorinate more extensively chlorinated congeners was limited and some PCB congeners were shown to inhibit the process.

Dehalococcoides ethenogenes 195 was the first Dehalococcoides isolated based on its capability to dehalogenate tetrachloroethene to ethene [Maymo-Gatell et al., 1999]. The genome of strain 195 has been sequenced and 17 possible reductive dehalogenase genes were observed, suggesting a diverse dehalogenation ability [Seshadri et al., 2005]. However, only one dehalogenase encoded by tceA and responsible for the dechlorination of trichloroethene, dichloroethene and vinylchloride has been isolated and characterized [Magnuson et al., 2000]. In fact, analysis of the capabilities of strain 195 revealed that this strain can dehalogenate different types of chlorinated aromatics in addition to its known chloroethene electron acceptors, including PCBs [Fennel et al., 2004]. Double-flanked chlorines could be dechlorinated by D. ethenogenes 195 (fig. 1), as in the case of strain DF-1. Dehalococcoides have also been implicated in the removal of double-flanked meta and para chlorines from 2,3,4,5-tetrachlorobiphenyl to form 2,3,5-trichloro- and 2,4,5-trichlorobiphenyl, respectively, in different enrichment cultures [Yan et al., 2006]. Fagervold et al. [2005] provided evidence for the

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involvement of two types of microorganisms belonging to the *Dehalococcoides* and o-17/DF-1 group, during the reductive dehalogenation of 2,2',3,3',4,6'-hexachlorobiphenyl to 2,2',4,6-tetrachlorobiphenyl. However, all the studies mentioned above analyzed the metabolism of only a few PCB congeners and do not reflect reductive dehalogenation processes observed in the environment. Only recently, a stable sediment-free culture showed to be able to dechlorinate Aroclor 1260 [Bedard et al., 2006]. Sixty-four PCB congeners were confirmed as substrates for this consortium, in which 47 congeners contained 6 or more chlorides. It was speculated that two or more *Dehalococcoides* strains with different sets of reductive dehalogenase genes act together to achieve the broad congener specificity observed (fig. 1). Considering that the isolated *Dehalococcoides* strains 195, FL2, BAV1, and CBDB1 contain 18, 14, 7 and 32 non-identical reductive dehalogenase genes, respectively [Holscher et al., 2004], there is a significant lack of knowledge on substrate specificity and distribution of such dehalogenases genes and thus their importance for bioremediation.

**Aerobic Degradation**

Since the pioneering studies of Lunt and Evans [1970], Catelani et al. [1971] and Ahmed and Focht [1973a, b], several bacteria able to use biphenyl as a sole source of carbon and energy (predominantly *Pseudomonas*, *Burkholderia*, *Comamonas*, *Capriavidus*, *Sphingomonas*, *Acidovorax*, *Rhodococcus* and *Bacillus* strains) have been isolated and their capability to transform PCB congeners has been evaluated. The degradation of biphenyl and transformation of PCBs is usually catalyzed by enzymes encoded by the so-called biphenyl upper (bph) pathway.

**The Biphenyl Upper (bph) Pathway**

A major interest for analyzing aerobic biphenyl degrading bacteria is because of their capability to transform PCB congeners. Based on the analysis of various biphenyl degrading isolates, it could be deduced that, in general, lower chlorinated congeners are more easily transformed compared to higher chlorinated congeners and that PCB congeners with chlorines only on one aro-
matic ring are more easily degraded, when compared with those bearing chlorine substituents on both aromatic rings. However, each isolate exhibits a particular activity spectrum with regard to the type and extent of PCB congeners metabolized. Some strains have a narrow spectrum and others, notably *Burkholderia xenovorans* LB400 [Mondello, 1989], are able to transform a broad range of congeners [Bopp, 1986; Haddock et al., 1995; Mondello, 1989; Seeger et al., 1995a, b, 1997, 1999].

**Biphenyl 2,3-Dioxygenases**

The aerobic degradation of aromatic compounds is frequently initiated by Rieske non-heme iron oxygenases, which catalyze the incorporation of two oxygen atoms into the aromatic ring to form arene cis-diols [Gibson and Parales, 2000]. Rieske non-heme iron oxygenases are multicomponent enzyme complexes composed of a terminal oxygenase component (iron-sulfur protein [ISP]) and different electron transport proteins (a ferredoxin and a reductase or a combined ferredoxin-NADH-reductase) [Butler and Mason, 1997]. The catalytic iron-sulfur proteins are heteromultimers, comprising a large (α) and a small (β) subunit, with the former containing a Rieske-type [2Fe-2S] cluster, a mononuclear non-heme iron oxygen activation center, and a substrate-binding site [Butler and Mason, 1997; Furusawa et al., 2004] which is responsible for substrate specificity [Gibson and Parales, 2000]. Comparison of the amino acid sequences of the terminal oxygenase α subunits revealed that they form a family of diverse but evolutionarily related sequences, and distinct major lineages have been identified [Gibson and Parales, 2000]. Although none of the enzymes are strictly specific, a correlation between the subfamilies (e.g. toluene/biphenyl, naphthalene, benzoate, or phthalate subfamilies) and the oxidized substrates can be observed.

Biphenyl 2,3-dioxygenases (BphA) usually belong to the toluene/biphenyl branch of Rieske non-heme iron oxygenases [Gibson and Parales, 2000] where a ferredoxin and a ferredoxin reductase act as an electron transport system to transfer electrons from NADH to the terminal oxygenase. The biphenyl 2,3-dioxygenases are of crucial importance for the successful metabolism of PCBs (fig. 2). On the one hand, their dioxygenation regiospecificity determines the sites of attack by the subsequent metabolic pathway while, on the other hand, their specificity determines the spectrum of PCB congeners that can be transformed by an organism.

Studies on various biphenyl 2,3-dioxygenases have revealed considerable differences in their congener selectivity patterns, as well as their preference of the attacked ring [Kimura et al., 1997; McKay et al., 1997; Seeger et al., 1999]. The biphenyl pathway of strain LB400 oxidizes an unusually wide range of PCB congeners and BphA is able to dihydroxylate from mono-chlorobiphenyls to 2,3,4,5,2′,5′-hexachlorobiphenyl [Haddock et al., 1995; Seeger et al., 1995a, b, 1997, 1999]. The following order of
preference for dioxygenation has been observed: unsubstituted > 2-chloro > 2,5-dichloro > 2,4-dichloro ~ 3-chloro > 4-chloro > 2,3-dichloro. Most primary catabolites that are dioxygenated by BphA from strain LB400 at ortho and meta carbons are further metabolized by the other enzymes of the upper pathway. In contrast, dioxygenation at meta and para positions results in channeling into a dead-end pathway. In addition to PCBs, BphA from strain LB400 is able to oxidize other substituted biphenyls, unsubstituted dibenzofuran and dibenzodioxin [Seeger et al., 2001]. Dehalogenation by BphA of ortho-chlorinated, -brominated and -fluorinated biphenyls has been observed [Haddock et al., 1995; Seeger et al., 1995b, 1997, 2001], in addition to denitration and dehydroxylation [Seeger et al., 2001]. Interestingly, BphA of LB400 is also able to transform different isoflavonoids [Seeger et al., 2003], compounds which are found in nature as secondary metabolites of plants. The isoflavonoid daidzein, a major phytoestrogen present in soy beans is dioxygenated by BphA, generating 7,2',4'-trihydroxyisoflavone after spontaneous dehydration. This has previously been used for chemical synthesis of substituted pterocarpans [Visser and Lane, 1987], plant metabolites which are suggested to be superior anti-tumor agents. The cis-2,3-dihydroxybiphenyl dehydrogenase (BphB, see below) from strain LB400 is able to rearomatize isoflavonoids dihydroxylated by BphA and the resulting products are assumed to have improved antioxidant properties [Arora et al., 1998].

Pollution by PCBs consists of mixtures of congeners and only a fraction of these can be transformed by BphAs. Therefore, for improved PCB catabolic pathways, recruitment or generation of biphenyl 2,3-dioxygenases with broadened and/or altered substrate ranges is required. Two main approaches have been employed to obtain novel BphA activities. One approach is to isolate naturally occurring bphA genes or gene segments from bacterial isolates [Camara et al., 2007] or from environmental DNA [Kahl and Hofer, 2003]. As an example, a hybrid BphA based on the LB400 enzyme but harboring the core segment of an oxygenase from Pseudomonas sp. strain B4-Magdeburg showed (depending on the congener considered) complementing or improved degradative properties [Camara et al., 2007]. The other approach is to generate altered enzymes through protein engineering and strategies of artificial evolution [Furukawa et al., 2004; Zielinski et al., 2006]. The construction of chimeric BphA derivatives, generated by the combination of gene segments of well known PCB degraders, enabled the identification of key domains of these oxygenases [Kimura et al., 1997; Kumamaru et al., 1998] and generated biphenyl 2,3-dioxygenases with improved capacities [Erickson and Mondello, 1993; Mondello et al., 1997; Suenaga et al., 1999, 2002]. A directed evolution approach using random mutagenesis to these specific segments allowed generating BphAs with increased catalytic turnover on PCBs, that were recalcitrant for the parental BphA from strain LB400 [Zielinski et al., 2006]. Recent studies have combined both approaches; the broad natural diversity and methods of artificial evolution by family shuffling of soil DNA encoding BphA segments to generate BphA variants with novel regioselectivities [Vezina et al., 2007].

cis-2,3-Dihydro-2,3-Dihydroxybiphenyl Dehydrogenases

The second step in the metabolic pathway, the dehydrogenation of (chlorinated) cis-2,3-dihydro-2,3-dihydroxybiphenyls (biphenyl 2,3-dihydrodiol) producing (chlorinated) 2,3-dihydroxybiphenyl, is catalyzed by cis-2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenases (BphB, fig. 2). cis-Dihydrodiol dehydrogenases are involved in various aromatic degradation pathways and exhibit common features such as an absolute requirement for NAD⁺. They are usually members of the family of short-chain alcohol dehydrogenases, showing a broad substrate specificity and able to transform several cis-dihydrodiol substrates [Jouanneau and Meyer, 2006; Patel and Gibson, 1974; Raschke et al., 1999; Rogers and Gibson, 1977]. As examples, NahB, involved in the degradation of naphthalene by P. putida G7, and BphB, involved in biphenyl degradation by C. testosteroni B-356, catalyze the transformation of biphenyl 2,3-dihydrodiol and naphthalene 1,2-dihydrodiol, although they only share 37% of sequence identity [Barriault et al., 1999]. In addition, both enzymes were able to catalyze the dehydrogenation of the 2,2',5,5'-tetrachlorinated derivative of biphenyl 3,4-dihydrodiol. The broad substrate specificity of dihydrodiol dehydrogenases is further underlined by the fact that all biphenyl 2,3-dihydrodiols chlorinated at the non-oxidized ring, except for 2',6'-substituted compounds, and various dihydrodiols chlorosubstituted at both aromatic rings were efficiently dehydrogenated by BphB of strain LB400 [Hülsmeyer et al., 1998].

2,3-Dihydroxybiphenyl 1,2-Dioxygenases

The ring-cleavage of aromatic intermediates possessing hydroxyl substituents on adjacent carbon atoms can be catalyzed by enzymes from one of two structurally and mechanistically distinct enzyme classes. While intradiol
dioxigenases, which cleave the aromatic nucleus between the hydroxyl substituents (ortho-cleavage) use non-heme Fe(III), extradiol dioxigenases, that cleave the aromatic nucleus adjacent to the hydroxyl substituents (meta-cleavage) use non-heme Fe(II) in the reaction [Harayama and Rekik, 1989]. However, Mn(II)-dependent extradiol dioxigenases showing high sequence similarity to the Fe(II)-dependent enzymes have been reported [Hatta et al., 2003]. Based on the sequence alignments, the extradiol group of dioxigenases has been divided in three families [Vaillancourt et al., 2003]. The type II extradiol dioxigenases include enzymes such as protocatechuate 4,5-dioxygenase (LigAB) from *Pseudomonas paucimobilis* [Noda et al., 1990] which has two different types of subunits. The type III extradiol dioxigenases, including 1-hydroxy-2-naphthoate dioxygenase from *Nocardioioides* sp. strain KP7 [Iwabuchi and Harayama, 1998], belong to the cupin superfamily [Dunwell et al., 2001]. The type I extradiol dioxigenases belong to the vicinal oxygen clelate superfamily [Gerlt and Babbitt, 2001]. Both, one- and two-domain type I enzymes have been described, and their phylogenetic analysis indicates that these enzymes share a common one-domain ancestor [Eltis and Bolin, 1996]. Divergence has since resulted in several subfamilies. 2,3-Dihydroxybiphenyl 1,2-dioxygenases (BphC), and extradiol dioxigenases, which are involved in biphenyl degradation, usually belong to the 1.3.A subfamily [Eltis and Bolin, 1996] and are specialized for transformation of 2,3-dihydroxybiphenyls (fig. 2).

The special interest on 2,3-dihydroxybiphenyl 1,2-dioxygenases is due to the fact that various studies indicate that activities of these enzymes limit the degradation of certain PCB congeners [Furukawa et al., 1979; Seeger et al., 1995a, 1997]. More detailed studies then revealed that those enzymes differ in substrate specificity, but seem to be generally capable of transforming various chlorosubstituted derivatives [Dai et al., 2002; Hein et al., 1998; McKay et al., 2003]. However, both 3,4-dihydroxybiphenyl as well as 2'-chlorosubstituted 2,3-dihydroxybiphenyls strongly inhibit 2,3-dihydroxybiphenyl 1,2-dioxygenases [Lloyd-Jones et al., 1995; McKay et al., 2003]. 2'-Chlorosubstituted 2,3-dihydroxybiphenyls promote suicide inactivation, which involves the release of superoxide radicals during catalysis and then the oxidation of the active site Fe(II) [Dai et al., 2002]. However, significant differences between different isoenzymes were observed [Fortin et al., 2005a] and specifically single domain extradiol dioxigenases have been shown to be adapted to the transformation of 2'-chlorosubstituted 2,3-dihydroxybiphenyls [McKay et al., 2003].

A second feature of extradiol dioxigenases is their rapid inactivation during turnover of 3-chlorocatechol [Bartels et al., 1984]. 3-Chlorocatechol is a metabolic intermediate of 3-chlorobiphenyl degradation, which severely affect the PCB metabolism [Sondossi et al., 1992]. Efforts were directed towards the possible optimization of extradiol dioxigenases through directed evolution [Fortin et al., 2005b]. However, although variants with increased resistance to 3-chlorocatechol could be obtained [Ohnishi et al., 2004], this increase was only two-fold and not comparable to natural variants of catechol 2,3-dioxygenases previously observed in chlorobenzene degrading *Pseudomonas* strains which are highly resistant to 3-chlorocatechol mediated inactivation [Göbel et al., 2004; Kaschabek et al., 1998].

2-Hydroxy-6-Phenyl-6-Oxohexa-2,4-Dieneoate (HOPDA) Hydrolases

The fourth step in the *bph* pathway is catalyzed by 2-hydroxy-6-phenyl-6-oxohexa-2,4-dieneoate (HOPDA) hydrolase BphD, which hydrolyzes HOPDA to yield 2-hydroxypenta-2,4-dieneoate and benzoate (fig. 2). HOPDA hydrolases belong to the α/β-hydrolase superfamily which include diverse hydrolytic enzymes sharing a similar structural fold [Ollis et al., 1992]. Most members of this superfamily are esterase and lipase enzymes that catalyze ester hydrolysis reactions by a serine-histidine-aspartate catalytic triad [Nardini and Dijkstra, 1999]. This superfamily also contains enzymes catalyzing a broad range of reactions, including a family of C-C hydrolase enzymes, and among them HOPDA hydrolases. Only limited information is available on the diversity of these hydrolases regarding their capability to transform chlorinated derivatives. Studies on *Burkholderia xenovorans* LB400 and *Rhodococcus globerulus* P6 BphDs, however have revealed that this enzyme may be a bottleneck for the metabolism of certain PCB congeners [Seah et al., 2000, 2001]. Although some differences in turnover were observed, both enzymes were similar in that HOPDAs bearing chlorine substituents at the phenyl moiety were efficiently transformed, whereas HOPDAs bearing chlorine substituents on the dienoate moiety were poor substrates and competitively inhibit BphD. Currently, more detailed mechanistic studies are being performed on these enzymes with the aim of use protein engineering approaches [Horsman et al., 2006; Li et al., 2006a, b].

BphK Glutathione-S-Transferase

BphK is a glutathione-S-transferase (GST) with an unclear function that occurs in some *bph* pathways [Bartels et al., 2000].
et al., 1999]. GSTs transform a wide range of electrophilic compounds in a reaction typically involving glutathione (γ-L-Glu-L-Cys-Gly) conjugation [Armstrong, 1997]. Bacterial GSTs are integrated in a wide range of catabolic pathways, such as in the degradation of gentisate, homogentisate or pentachlorophenol. In the described pathway of *Sphingobium chlorophenolicum*, tetrachlorohydroquinone dehalogenase, which appears to have been recently recruited for pentachlorophenol degradation [Copley, 2000], catalyze the reductive dehalogenation of the substrate via trichlorohydroquinone to dichlorohydroquinone [Kiefer and Copley, 2002; Warner et al., 2005]. It was thus proposed that BphK could be involved in dechlorination of PCB catabolic intermediates [Hofer et al., 1994; Seeger et al., 1997]. BphK was shown not to be essential for degradation of biphenyl [Bartels et al., 1999]; however, Gilmartin et al. (2003) revealed that this enzyme can catalyze dehalogenation of 4-chlorobenzoate, the same product found from 4-chlorobiphenyl degradation by the enzymes BphA, BphB, BphC and BphD. Therefore, it was suggested that BphK was recruited to facilitate the degradation of PCBs. Recent studies revealed that BphK catalyzes the dehalogenation of 3-chloro-2-hydroxy-6-oxo-6-phenyl-2,4-dieneoates (and some other chlorosubstituted derivatives), compounds that are produced by the cometabolism of PCBs by BphA, BphB and BphC [Fortin et al., 2006] and that inhibit BphD. In fact, 3-chloro-2-hydroxy-6-oxo-6-phenyl-2,4-dieneoates were significantly better substrates for the enzyme compared to 4-chlorobenzoate and it was proposed that BphK contributes to the superior PCB metabolizing activities of LB400 by decreasing the inhibition of BphD by chlorinated HOPDAs. Current efforts are being directed towards the mechanistic understanding of BphK catalysis [Tocheva et al., 2006].

**Lower Pathways for the Degradation of 2-Hydroxypenta-2,4-Dienoates and Benzoates**

The metabolism of (chloro)biphenyls by the biphenyl upper pathway results in the formation of (chlorinated) 2-hydroxypenta-2,4-dienoates and (chlorinated) benzoates (fig. 2). 2-Hydroxypenta-2,4-dienoate is transformed by 2-hydroxypenta-2,4-dienoate hydratase (*bphH*), an acylating acetaldehyde dehydrogenase (*bphL*) and 4-hydroxy-2-oxovalerate aldolase (*bphL*) to acetyl-CoA, which then can enter the Krebs cycle. Thus, these enzymes should allow growth of bacterial strains on chlorinated biphenyls chlorinated only at one aromatic ring, which yield chlorinated benzoates as dead-end metabolites and unchlorinated 2-hydroxypenta-2,4-dienoate. Still, the transformation of chlorinated 2-hydroxypenta-2,4-dienoate should be elucidated.

The 2-hydroxypenta-2,4-dienoate transforming enzymes have been described from different PCB-degrading strains such as *B. xenovorans* LB400 and *Rhodococcus* sp. strain RHA1. The encoding genes are typically organized in a *bphHIJ* (also termed *bphEGF*) or *bphHIJ* cluster, respectively (fig. 2). However, while in LB400 and KF707 strains these genes are integrated in the upper pathway gene cluster [Hofer et al., 1994] (see below), in strain RHA1 (and others), they are separated from the archetype upper pathway gene cluster and encoded by eight (partially incomplete) clusters [Goncalves et al., 2006; McLeod et al., 2006], with only three of them being expressed during growth on biphenyl [Goncalves et al., 2006]. As homologous enzymes are integral parts of archetype catechol meta-cleavage pathways [Williams and Sayers, 1994], the remaining genes are probably involved in such central metabolic routes.

Besides 2-hydroxypenta-2,4-dienoates, benzoates are generated during BphD catalyzed hydrolysis of HOPDAs (fig. 2). Classically, benzoate is assumed to be mineralized via catechol and a 3-oxoalipate pathway [Harwood and Parales, 1996] (fig. 2). Recently, a new and unusual pathway for aerobic benzoate oxidation has been described in various bacteria including strain LB400 [Denef et al., 2004; Gescher et al., 2002; Zaar et al., 2001]. Benzoate is first transformed by benzoate-CoA ligase into benzoic-CoA (fig. 2). Then, benzoic-CoA is transformed by a benzoic-CoA dioxygenase to 2,3-dihydroxydihydrobenzoic-CoA followed by nonoxigenolytic cleavage of the aromatic ring and a β-oxidation-like pathway of the ring-cleavage product. This *box* pathway generates 3-hydroxydipropionate-CoA and then 3-keto dipropionate-CoA, which is finally cleaved into succinyl-CoA and acetyl-CoA. Operon-like structures of *box* genes encoding both the initial ligase as well as the benzoyl-CoA pathway are present in LB400 strain in two copies on the major chromosome and on the mega plasmid [Chain et al., 2006]. However, this *box* pathway was not found in strain RHA1 [McLeod et al., 2006].

In addition to the *box* pathway, strain LB400 harbors an archetype benzoate degradation pathway via catechol and their respective functions were evaluated by proteomic and genomic studies [Denef et al., 2004, 2006]. It was established that benzoate, when supplied as a carbon source, was mainly metabolized via catechol, whereas benzoate generated from biphenyl was mainly metabolized via the box pathway. Thus, it was proposed that the *box* pathway is of advantage under oxygen limiting con-
ditions, as it consumes less oxygen compared to the degradation via catechol due to the nonoxygenolytic ring-cleavage [Denef et al., 2006; Gescher et al., 2005].

Although chlorobenzoates themselves are not very toxic to bacteria [Agulló et al., 2007; Martínez et al., 2007], negative effects of chlorobenzoate metabolism on chlorobiphenyl degradation have been reported [Havel and Reineke, 1992; Sondossi et al., 1992]. This is due to the channeling of chlorobenzoates via chlorocatechols into inappropriate pathways and includes the formation of toxic protoanemonin from 4-chlorocatechol by enzymes of the wide-spread 3-oxo-adipate pathway [Blasco et al., 1995, 1997] and the suicide inactivation during the transformation of 3-chlorocatechol by extradiol dioxygenases [Bartels et al., 1984; Vaillancourt et al., 2002]. Bacteria have evolved various strategies to degrade chlorobenzoates. The degradation via chlorocatechol and the chlorocatechol ortho-cleavage pathway (clc pathway and others) are the most intensively studied [Dorn et al., 1974]. However, various alternative strategies have been described such as: (1) the hydrolytic dehalogenation of 4-chlorobenzoate to give 4-hydroxybenzoate (fcb pathway) [Klages and Linges, 1979], (2) the 4,5-dioxygenation of 3-chlorobenzoate and 3,4-dichlorobenzoate to form 5-chloroprotocatechuate [Nakatsu and Wyndham, 1993], (3) the degradation of 3-chlorocatechol by variants of the catechol meta-cleavage pathway [Mars et al., 1997], and (4) chlorocatechols by novel variants of chlorocatechol ortho-cleavage routes [Moiseeva et al., 2002; Nikodem et al., 2003] [for details on the degradation of chlorobenzoates, see Pieper, 2005]. To overcome restrictions due to poisoning caused by the production of toxic metabolites, the application of co-cultures and the construction of hybrid strains capable of mineralizing some lower chlorinated biphenyls by an appropriate combination of pathway segments have been considered [Brenner et al., 1994].

Genomic Studies

Burkholderia xenovorans strain LB400 and Rhodococcus sp. strain RHA1 are two model microorganisms which have been extensively studied due to their ability to metabolize a broad range of PCBs [Bopp, 1986; Seeger, et al., 1995a; Seto et al., 1995]. Both genomes have recently been sequenced [Chain et al., 2006; McLeod et al., 2006] with the rationale to better understand their overall physiology and to foster their applicability for bioremediation purposes. The LB400 genome has a size of 9.73 Mb and comprises two circular chromosomes (4.90 and 3.36 Mb, respectively) and a circular mega plasmid (1.47 Mb). The RHA1 genome comprises 9.70 Mb arranged on a linear chromosome (7.80 Mb) and three linear plasmids (1.12, 044 and 0.33 Mb, respectively). Each of these genomes harbors ~9,000 coding sequences and both strains reside in soil and plant rhizosphere niches. These ecologically similar bacteria have evolved their large genomes by different means. More than 20% of the genome of strain LB400 was recently acquired via horizontal gene transfer (HGT). In contrast, strain RHA1 evolved through ancient acquisition or gene duplication and acquired far fewer genes by recent HGT than LB400 [McLeod et al., 2006].

Strains LB400 and RHA1 both have the potential to degrade a wide range of aromatic compounds, and the genome sequences indicate unusually high metabolic versatilities. A broad range of xenobiotic and natural compounds are funneled by a large number of ‘peripheral aromatic’ pathways (20 and 26, respectively) into fewer ‘central aromatic’ pathways (11 and 8, respectively), significantly exceeding the degradative versatility of other sequenced aromatic degrading bacteria such as Pseudomonas putida KT2440 [Jimenez et al., 2002] or Aromatoleum EbN1 [Rabus et al., 2005].

The genes encoding enzymes of the biphenyl upper bph pathway are located in both strains on acquired and mobile genetic elements. In LB400 they are encoded by a genomic island on the mega plasmid, indicating that these genes were acquired via HGT. Genomic islands also provide other catabolic capacities such as the abilities to degrade 2-aminophenol or 3-chlorocatechol to strain LB400. In strain RHA1, the bph genes are, like 11 of the 26 peripheral aromatic pathways, located on the plasmids [McLeod et al., 2006].

Toxicity and Stress

The biodegradation performance of bacteria can be affected by the toxicity of the pollutants or metabolites derived from them [Blasco et al., 1997; Erb et al., 1997]. Due to their lipophilicity, PCBs are expected to accumulate in membranes [Sikkema et al., 1995] and, in fact PCB congeners, specifically lower chlorinated ones significantly reduce the viability of E. coli cells [Cámara et al., 2004]. Also, Park et al. [2000] indicated that PCBs decrease survival. Other studies suggested that PCB metabolites drastically affect the cell viability [Blasco et al., 1997; Cámara et al., 2004; Sondossi et al., 1992; Vaillancourt et al., 2002]. As described above, depending on the PCB congener and the bph enzymes harbored by a given organism, different pathway steps may constitute metabolic bottlenecks, resulting in the accumulation of the respective metabolites. Specifically, biphenyl dihydrodi-
ols (formed by BphA) and dihydroxybiphenyls (formed by the subsequent action of BphA and BphB; fig. 2) were reported to be highly toxic to both E. coli and LB400 cells, affecting the cell viability much more than PCBs [Cámara et al., 2004]. Hydroxylated PCB metabolites affected the DNA content of Comamonas testosteroni TK102 and inhibited cell separation [Hiraoaka et al., 2002]. A recent study differentiated the effects of PCBs themselves and the possible metabolites on the performance of two fully sequenced potent PCB degraders, strains LB400 and RHA1 [Parnell et al., 2006]. Although PCBs were shown to partition to the cell fraction of cultures, no significant effects were observed regarding viability or growth rate in either strain under non PCB-degrading conditions. Strain LB400 was among the most potent PCB degraders exhibiting a high tolerance to PCB metabolites toxicity, while strain RHA1 showed to be highly sensitive.

The better performance of strain LB400 and the availability of the complete genome sequence make possible proteomic and transcriptomic studies in order to evaluate molecular defenses against PCB toxicity. During exposure of strain LB400 cells to 4-chlorobiphenyl, enlargement of the periplasmic space at the poles and electron-dense granules in the cytoplasm have been described [Agulló et al., 2007]. These electron-dense granules correspond to polyphosphates [Chavez et al., 2004] which are accumulated by bacteria exposed to stress conditions [Kulaev and Kulakovskaya, 2000].

In addition, the induction of the molecular chaperones DnaK and GroEL by the presence of 4-chlorobiphenyl and biphenyl in LB400 [Agulló et al., 2007] suggests that the presence, and perhaps metabolism, of these compounds constitutes a stress. During growth on biphenyl, oxidative stress was evident by the induction of proteins such as alkyl hydroperoxide reductase AhpC [Agulló et al., 2007; Denef et al., 2005] which detoxify peroxides. LB400 cells grown on glucose and exposed to biphenyl showed increased levels of reactive oxygen species [Agulló et al., 2007], which may result from the action of enzymes involved in the transformation of 2-hydroxyphenylaceta-2,4-dienoate (which is released during hydrolysis of HOPDA to form benzoate) that are localized between bphC and bphD [Hofer et al., 1994] (fig. 3). Regulation of these clusters is assumed to be mediated by the orf0 gene product, which belongs to the GntR family of transcriptional regulators [Beltrametti et al., 2001; Watanabe et al., 2000]. P. putida KF715 contains a bphABCD gene cluster [Hayase et al., 1990] (fig. 4) which was suggested to have evolved from a LB400-type gene cluster [Nishi et al., 2000].

In the strain LB400, the biphenyl catabolic genes are located on a genomic island on the mega plasmid [Chain et al., 2006]. The presence of bph genes on a genomic island implies that they have been acquired by HGT from other bacteria. The chromosomal bph genes in strains KF715 [Hayase et al., 1990; Lee et al., 1995] and in strain KF707 [Furukawa and Miyazaki, 1986] are able to move to other strains [Nishi et al., 2000]. The presence of bph genes on mobile genetic elements such as genomic islands, plasmids or (conjugative) transposons indicate that these genes are able to move between genomes, thus allowing adaptation of microbial communities to PCBs.

Another type of bph gene cluster (bphAaAbAcAdCB) was observed in Rhodococcus RHA1 [Masai et al., 1995] (fig. 3) and localized on the linear plasmid pRHL1 [Takeda et al., 2004]. This catabolic gene cluster is followed by bphS and bphT genes which encode a two-component signal transduction system composed of a BphT response

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**Archetype bph Gene Clusters**

Most of the information on biphenyl degradation and PCB metabolism comes from bacterial isolates, among them strains LB400 and RHA1, whose genomes have recently been elucidated. Strain LB400 [Mondello, 1989], similar to P. pseudoalcaligenes KF707 [Furukawa and Miyazaki, 1986], C. necator H850 [Bedard et al., 1987] and others [Bartels et al., 1999], harbor an operon comprising genes encoding biphenyl 2,3-dioxygenase (bphA1A2A3-A4), cis-2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase (dihydrodiol dehydrogenase, bphB), 2,3-dihydroxybiphenyl 1,2-dioxygenase (bphC) and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (HOPDA hydrolase, bphD). The operon contains genes encoding a glutathione S-transferase (bphK) and genes encoding enzymes involved in the transformation of 2-hydroxyphenylaceta-2,4-dienoate (which is released during hydrolysis of HOPDA to form benzoate) that are localized between bphC and bphD [Hofer et al., 1994] (fig. 3). Regulation of these clusters is assumed to be mediated by the orf0 gene product, which belongs to the GntR family of transcriptional regulators [Beltrametti et al., 2001; Watanabe et al., 2000]. P. putida KF715 contains a bphABCD gene cluster [Hayase et al., 1990] (fig. 4) which was suggested to have evolved from a LB400-type gene cluster [Nishi et al., 2000].

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A regulator and a BpdS sensor kinase and promote transcriptional induction by a variety of aromatic compounds, including biphenyl, benzene, alkylbenzenes, and chlorinated benzenes [Takeda et al., 2004]. Importantly, this gene cluster is devoid of a bphD gene. A nearly identical gene cluster termed ipbA1A2A3A4CB involved in isopropylbenzene degradation has been localized on the linear plasmid pBD2 of R. erythropolis BD2 [Stecker et al., 2003]. This indicates that such gene clusters are involved in the degradation of differently substituted aromatics and that their designation as bph or ipb genes is rather historical than based on specificity. Moreover, their plasmid localization indicates that the ipb and bph operons have been distributed among Gram-positive organisms via plasmid-mediated HGT. Similar bph(ipb) gene clusters, which differ slightly in arrangement were observed in Rhodococcus strains M5 [Peloquin and Greer, 1993] and TA421 [Arai et al., 1998].

Another type of differently structured bph gene cluster was observed in Acidovorax sp. strain KKS102 [Kikuchi et al., 1994] and Cupriavidus oxalaticus A5 [Merlin et al., 1997; Mouz et al., 1999; Springael et al., 1993] (bphSEGF(orf4)A1A2A3A4BCD(orf1)A4) (fig. 3). In these clusters, genes encoding for a 2-hydroxypenta-2,4-dieneoate hydratase, acetaldehyde dehydrogenase, and 4-hydroxy-2-oxovalerate aldolase (designated bphEGF) are preceding genes encoding upper pathway enzymes. The gene encoding the reductase subunit of biphenyl dioxygenase...
Toluene/biphenyl/isopropylbenzene branch

Naphthalene branch

Benzoate/anthranilate branch

Archetype HOPDA hydrolases

Archetype HOHDA hydrolases

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(bphA4) is localized at the end of the gene cluster. Regulation in strain KKS102 was shown to be dependent on the bphS gene product, which showed homology with the GntR family of transcriptional regulators [Mouz et al., 1999; Ohtsubo et al., 2001]. In Cupriavidus oxalaticus A5, the bph genes are located on Tn4371, a 55-kb mobile genomic island [Toussaint et al., 2003] which was found to integrate easily into the host chromosome at specific locations [Merlin et al., 1999]. Similar to other genomic islands, Tn4371 shows a mosaic structure of several building blocks including a tyrosine recombinase that is related to phage integrases [Merlin et al., 1999] and transfer most likely involves a site-specific excision/integration process.

Metabolic Versatility

The abundant information on PCB metabolism suggests that these archetype strains and catabolic gene clusters are those of major importance for PCB metabolism in the environment. However, enzymes homologous to those involved in biphenyl degradation could be found in pathways responsible for the degradation of other aromatic pollutants such as naphthalene, isopropylbenzene or benzene (fig. 4). Despite the evolutionary adaptation of enzymes for specific substrates, the enzymes of a particular pathway catalyze usually the transformation of a range of substrate analogues. On the other hand, the annotation of a certain gene/enzyme is usually based on a phenotype the host was selected for and does not necessarily reflect the detailed enzyme function. As an example, bphAa of strain RHA1 and ipbA1 of strain BD2 were initially described for their involvement in biphenyl and isopropylbenzene degradation, respectively [Dabrock et al., 1994; Masai et al., 1995]. However, their high sequence identity (99%) and functional analyses [Goncalves et al., 2006; Iwasaki et al., 2006; Kesseler et al., 1996] indicate that in both strains their function is to activate a diverse range of aromatic pollutants. In addition, other studies indicate that gene abbreviations given based on some ‘archetype’ isolates could be misleading. Studies on a benzene polluted site revealed an abundance of genes, which based on their function were termed isopropylbenzene dioxygenases, rather then those encoding the expected benzene/toluene dioxygenases [Witzig et al., 2006]. Evidence was given that the selected isopropylbenzene dioxygenases differed from previously described enzymes by a distinct active site architecture which prevent the transformation of even toluene.

It is well documented that various Rieske type non-heme iron oxygenases have the capability to transform biphenyl. As an example, chlorobenzene dioxygenase of Pseudomonas sp. P51 (belonging, like biphenyl dioxygenases, to the toluene/biphenyl branch of Rieske non-heme iron oxygenases) was shown to be capable of efficiently transforming monochlorinated biphenyls [Raschke et al., 2001]. On the other hand, enzymes outside of the toluene/biphenyl branch of Rieske non-heme iron oxygenases were capable of transforming biphenyls, such as naphthalene dioxygenase of P. putida G7 (belonging to the naphthalene family of Rieske non-heme iron oxygenases) (fig. 4) [Barriault and Sylvestre, 1999], phenanthrene dioxygenase of Cycloclasticus sp. strain A5 [Kasai et al., 2003] and carbazole 1,9a-dioxygenase from Pseudomonas resinovorans CA10 [Nojiri et al., 1999]. It should be also noted that culture independent studies revealed the abundance of novel branches of Rieske-type non-heme iron oxygenases in contaminated sites. The importance and environmental function of these oxygenases still remains to be elucidated [Taylor and Janssen, 2005; Taylor et al., 2002; Witzig et al., 2006]. Overall, information on the sequence diversity of Rieske-type non-heme iron oxygenases has dramatically increased in recent years, also due to information available from genome projects, however, in most cases, a clear functional assignment could not be made.

Information is accumulating that Rieske-type non-heme iron oxygenases outside of the archetype tolune/biphenyl branch are important for biphenyl degradation. The bph operon of Bacillus sp. JF8 harbors a novel bphRDA1A2BC cluster [Mukerjee-Dhar et al., 2005] (fig. 3) encoding enzymes only distantly related to enzymes previously described to be important for biphenyl degradation (fig. 4). BphA1 showed only 31% identity with BphA1 of LB400 and is more closely related to naphthalene dioxygenases NidA from Rhodococcus sp. strain I24 [Larkin et al., 1999] or NidA from Mycobacterium vanbaalenii strain PYR-1 [Khan et al., 2001]. Also, the Mn(II)-dependent BphC and BphD (fig. 4) evidently belong to new subfamilies in the phylogeny of extradiol dioxygenases and hydrocases acting on extradiol cleavage products [Hatta et al., 2003; Mukerjee-Dhar et al., 2005].

The genetic analysis of biphenyl degrading Sphingomonas strains such as Sphingobium yanoikuyae BI [Zylstra and Kim, 1997] or Novosphingobium aromaticivorans F199 [Romine et al., 1999] revealed a highly complex arrangement of catabolic genes differing immensely in sequence homology and gene order from those reported for other genera. The presence of various genes encoding oxygenase α subunits located in a large complex chromosomal gene cluster [Kim and Zylstra, 1999; Zylstra and
Kim, 1997] significantly complicated the elucidation of their functions. Analysis revealed that a single ferredoxin and a single ferredoxin reductase, encoded by bphA3 and bphA4, respectively, are shared by multiple oxygenase systems [Baeyens and Kim, 2000]. Recently, the correct biphenyl dioxygenase genes (bphA1fA2f) were identified [Yu et al., 2007] which obviously receive electrons also from BphA3 and BphA4. In a phylogenetic analysis, BphA1f does not cluster with known BphAs, but is more related with the dioxygenase PhnI from Sphingomonas sp. strain CHY-1, which was shown to be able to oxidize at least 8 PAHs made of 2–5 aromatic rings [Demeneche et al., 2004; Jakoncic et al., 2007] and phenanthrene dioxygenase PhnA1 of Cycloclasticus sp. strain A5 [Kasai et al., 2003] (fig. 4).

These studies on Sphingomonas showed that different metabolic pathways are important, even within individual strains, for biphenyl degradation. However, this net of metabolic pathways is not exclusive of Sphingomonas sp. As shown in figure 4, the bph cluster of Rhodococcus sp. strain RHA1 does not comprise a bphD gene, and such activity has to be recruited from elsewhere in the genome. In fact, three hydrolases were shown to be upregulated during growth of RHA1 on biphenyl [Gonzáles et al., 2006] with one of them, termed BphD previously shown to be capable to attack HOPDA [Yamada et al., 1998]. Notably, the bphD gene is located in a gene cluster termed etbAa1A2BcphDE2F2 (fig. 3) in a gene region comprising an additional gene cluster (etbAa2Ab2AcD2) and various other genes involved in ethylbenzene/biphenyl degradation [bphF4, bphG4, bphE4, bphT2, bphS2, etbAd and bphB2] an ‘astonishing’ mix of genes being involved in ethylbenzene (etb) and biphenyl degradation (bph) (fig. 3). Although the etbA1 encoded oxygenase α-subunit is only distantly related to previously characterized BphA1 proteins (fig. 4), it is more active on highly chlorinated congeners than the bphAa encoded one [Iwasaki et al., 2006] and obviously appropriate for both biphenyl and ethylbenzene transformation. Furthermore, other subfamilies of biphenyl oxygenase α subunits are currently been discovered (e.g. BphA1 of Rhodococcus sp. strain K37, fig. 4), evidencing that diversity of oxygenases involved in biphenyl degradation is underestimated.

We can conclude that the metabolism of biphenyl and PCBs should not be regarded as a simple linear pathway. In fact, the different catabolic gene modules interact in a complex metabolic network. This is also supported by the observation that P. putida strain CE2010 mineralizes biphenyl by a mosaic of tod (toluene) and cmt (cumate) pathways in the absence of an ‘authentic’ biphenyl pathway [Ohta et al., 2001]. As previously reported, toluene dioxygenase (TodCIC2BA), toluene dihydrodiol dehydrogenase (TodD) and the meta-cleavage enzyme TodE have a significant cross-reactivity with biphenyl or metabolites produced during biphenyl degradation [Furu-kawa et al., 1993], whereas TodD 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase cannot cope with HOPDA. Recruitment of a hydrolase active with HOPDA, such as in RHA1 then allows CE21010 to mineralize biphenyl. As shown in figure 4, various different types of hydrolases active with HOPDA have been described and the recruitment of any of those can, at least in theory, complement bph gene modules. The same holds for extradiol dioxygenases, and especially in Rhodococcus, where the presence of multiple extradiol dioxygenase encoding genes has been reported [McLeod et al., 2006; Taguchi et al., 2004]. The metabolic versatility of catabolic enzymes and pathways is an indication of the ongoing evolution of bacterial metabolism, thus endowing environmental microbes with the capabilities to deal with a broad range of pollutants.

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