MINI-REVIEW

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Aerobic degradation of polychlorinated biphenyls

Abstract The microbial degradation of polychlorinated biphenyls (PCBs) has been extensively studied in recent years. The genetic organization of biphenyl catabolic genes has been elucidated in various groups of microorganisms, their structures have been analyzed with respect to their evolutionary relationships, and new information on mobile elements has become available. Key enzymes, specifically biphenyl 2,3-dioxygenases, have been intensively characterized, structure/sequence relationships have been determined and enzymes optimized for PCB transformation. However, due to the complex metabolic network responsible for PCB degradation, optimizing degradation by single bacterial species is necessarily limited. As PCBs are usually not mineralized by biphenyl-degrading organisms, and cometabolism can result in the formation of toxic metabolites, the degradation of chlorobenzoates has received special attention. A broad set of bacterial strategies to degrade chlorobenzoates has recently been elucidated, including new pathways for the degradation of chlorocatechols as central intermediates of various chloroaromatic catabolic pathways. To optimize PCB degradation in the environment beyond these metabolic limitations, enhancing degradation in the rhizosphere has been suggested, in addition to the application of surfactants to overcome bioavailability barriers. However, further research is necessary to understand the complex interactions between soil/sediment, pollutant, surfactant and microorganisms in different environments.

Introduction

Polychlorinated biphenyls (PCBs) are a class of compounds where the aromatic biphenyl skeleton carries be-

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tween one and ten chlorine atoms and, theoretically, 209 different PCB congeners can thus be produced. Typical commercial PCB mixtures, marketed as Aroclor, Kaneclor, Clophen or Delor, usually contain 20–60 congeners, predominantly tri-hexachlorinated congeners. Due to their non-flammability, chemical stability, high boiling point and electrical insulating properties, PCBs have been used for hundreds of industrial and commercial purposes, such as in electrical applications, heat transfer, and hydraulic equipment; as plasticizers in paints, plastics and rubber products; in pigments, dyes and carbonless copy paper, and many other applications. More than 1.5 million tons of PCBs were manufactured worldwide between 1927 and the early 1980s (Faroon et al. 2003), a significant amount of which has been released to the environment. The first adverse health effects were recorded in the 1930s (Drinker et al. 1937). Since then, PCBs have been shown to cause cancer in animals (Mayes et al. 1998), and also to cause a number of serious effects on the immune, reproductive, nervous and endocrine systems, among others (ATSDR 2000; Aoki 2001; Faroon et al. 2001). Studies in humans provide supportive evidence for the potential carcinogenic and non-carcinogenic effects of PCBs (ATSDR 2000).

Lunt and Evans (1970) and Catelani et al. (1970) were the first to isolate microorganisms capable of growing on biphenyl as sole source of carbon and energy and since then several biphenyl-degrading organisms have been isolated. These organisms belong to both Gram-negative and Gram-positive genera and comprise various *Pseudomonas*, *Burkholderia*, *Achromobacter*, *Comamonas*, *Ralstonia*, *Acinetobacter*, *Rhodococcus* and *Bacillus* isolates.

The degradation of biphenyl by microorganisms isolated thus far is initiated by a biphenyl 2,3-dioxygenase. Biphenyl 2,3-dioxygenases belong to a large family of Rieske non-heme iron oxygenases (Gibson and Parales 2000). They comprise a terminal oxygenase composed of a large α- and a small β-subunit, a ferredoxin and a ferredoxin reductase. The ferredoxin and ferredoxin reductase act as an electron transport system to transfer electrons from NADH to the terminal oxygenase. The *cis*-2,3-dihydro-2,3-dihydroxybiphenyl formed by this reaction is dehydrogenated by a dehydrogenase to give 2,3-di-

hydroxybiphenyl, which is subject to extradiol cleavage. The 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate thereby formed undergoes hydrolysis, yielding benzoate and 2-hydroxypenta-2,4-dienoate as reaction products. This sequence of reactions forms the so-called biphenyl upper pathway (Fig. 1). Ahmed and Focht (1973a,b) were the first to describe that biphenyl-degrading organisms have the capacity to transform several PCB congeners. Since then, a major focus of research on biphenyl-degrading organisms was their capability to transform PCBs, which differs significantly between different isolates.

The biphenyl "upper pathway"

Genetic organization

Genes encoding enzymes of the biphenyl upper pathway (termed *bph*) were first cloned from *Pseudomonas pseudoalcaligenes* KF707 (Furukawa and Miyazaki 1986) and later from *Burkholderia* sp. LB400 (Mondello 1989). In both strains, the genes encoding for biphenyl 2,3-dioxygenase (*bphA1A2A3A4*), *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*) are organized in an operon, with genes encoding a glutathione S-transferase and enzymes involved in the transformation of 2-hydroxypenta-2,4-dienoate released during hydrolysis of HOPDA to form benzoate (2-hydroxypenta-2,4-dienoate hydratase, BphH; acetaldehyde

dehydrogenase, BphJ; 4-hydroxy-2-oxovalerate aldolase, BphI) being localized between *bph*C and *bph*D (Hofer et al. 1994) (Fig. 1). A more detailed analysis revealed that such an organization occurs also in *Ralstonia eutropha* H850 (Bedard et al. 1987) and various other isolates (Bartels et al. 1999). *Pseudomonas putida* KF715 (Hayase et al. 1990) contains a simple *bphABCD* gene cluster (Fig. 1) and sequence comparisons have shown that this gene cluster still contains 106 nucleotides of a 2-hydroxy-2-oxovalerate aldolase-encoding gene, suggesting that this strain used to have the entire *bph* gene cluster as in LB400 (Nishi et al. 2000). The catabolic genes in LB400, KF707 and KF715 are preceded by a so-called *orf0*, and its gene product (Watanabe et al. 2003), belonging to the GntR family, was shown to positively regulate both its own expression and that of *bphKHIJ* in the presence of biphenyl in KF707 (Watanabe et al. 2000). HOPDA was proposed as the inducer. However, whereas *orf0* mutants of KF707 failed to grow on biphenyl, and accumulated high levels of HOPDA due to the failure to express *bphD*, *orf0* mutants of LB400 retained the ability to grow on biphenyl, but produced decreased concentrations of *bphA1A2* RNA (Beltrametti et al. 2001). Thus, *orf0* seems to be involved in expression of *bphA* genes in LB400. Whether *orf0* is also involved in expression of *bphA* in KF707 remains to be elucidated. Recently, a second regulator, BphR2, belonging to the LysR family, was shown to act as a positive regulator activating transcription of *bphA1A2A3A4BC* in KF707 (Watanabe et al. 2003).

A second type of *bph* gene cluster was observed in *Achromobacter georgiopolitanum* (formerly *Pseudomonas*
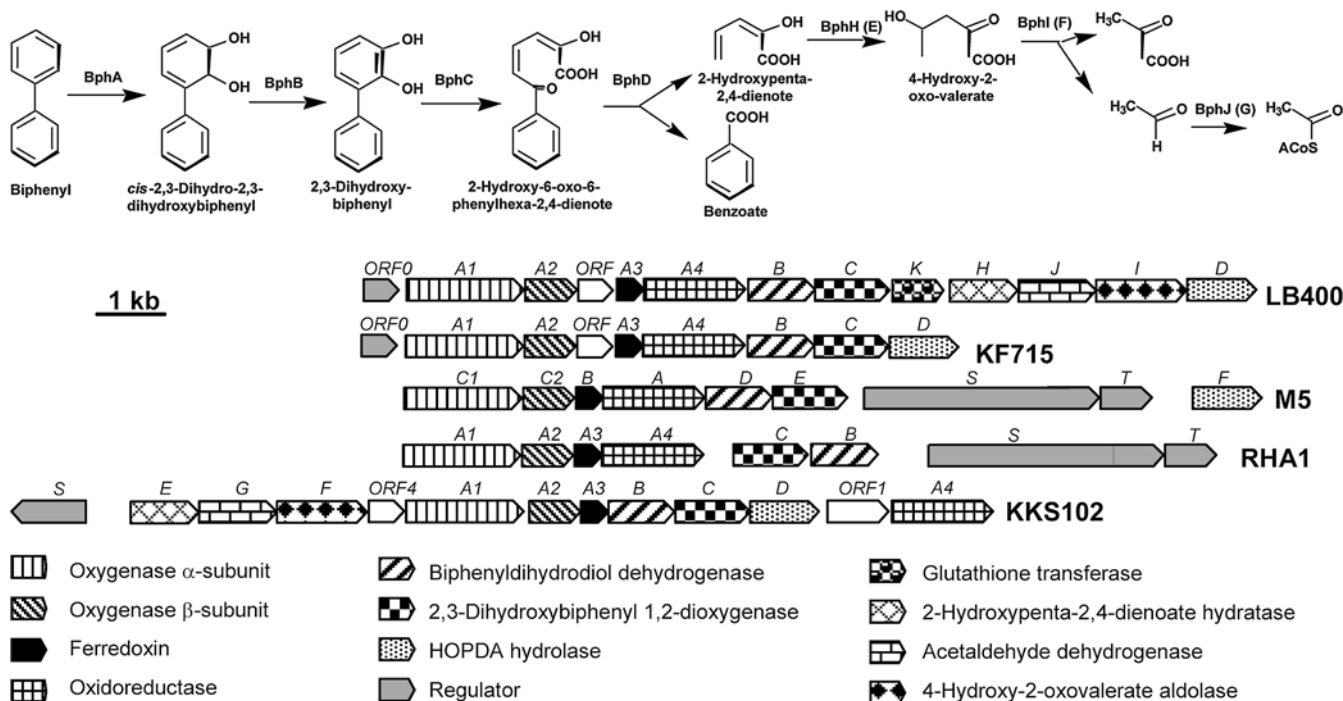

Fig. 1 Pathway for biphenyl degradation and genetic organization of the *bph* gene clusters of *Burkholderia* sp. LB400 (Erickson and Mondello 1992; Hofer et al. 1994), *Pseudomonas putida* KF715 (Hayase et al. 1990; Nishi et al. 2000), *Rhodococcus* sp. strain M5 (Peloquin and Greer 1993; Labbe et al. 1997), *Rhodococcus* sp. strain RHA1 (Masai et al. 1995; Takeda et al. 2004) and *Achromobacter georgiopolitanum* KKS102 (Kimbara et al. 1989; Kikuchi et al. 1994; Ohtsubo et al. 2001)

sp.) strain KKS102 (Kimbara et al. 1989; Kikuchi et al. 1994) and *Wautersia oxalatica* A5 (Springael et al. 1993; Merlin et al. 1997; Mouz et al. 1999) (Fig. 1). In these gene clusters, the order of *bph* genes differs significantly from that found in LB400, with genes encoding 2-hydroxypenta-2,4-dieneoate hydratase, acetaldehyde dehydrogenase, and 4-hydroxy-2-oxovalerate aldolase (designated *bphEGF*) preceding genes encoding upper pathway enzymes. Furthermore, the gene encoding the reductase subunit of biphenyl dioxygenase (*bphA4*) is localized at the end of the gene cluster, separated from *bphD* by an *orf1* of unknown function. Regulation of *bph* genes in strain KKS102 was shown to be dependent on the product of the *bphS* gene located upstream of *bphE*. Its deduced amino acid sequence showed homology with the GntR family of transcriptional repressors. Disruption of *bphS* resulted in constitutive expression of *bph* genes, suggesting that the *bphS* gene product negatively regulated the pE promoter located upstream of the *bphE* gene. Binding of BphS was abolished in the presence of HOPDA (Ohtsubo et al. 2001).

A third type of gene cluster was observed in biphenyl-degrading *Rhodococcus* strains M5 (Peloquin and Greer 1993), RHA1 (Masai et al. 1995) and TA421 (Arai et al. 1998) (Fig. 1). The catabolic gene clusters differ slightly in their arrangements, and whereas the gene encoding dihydrodiol dehydrogenase follows that encoding 2,3-dihydroxybiphenyl dioxygenase in RHA1 (*bphA1A2A3A4CB*), it precedes the 2,3-dihydroxybiphenyl dioxygenase-encoding gene in TA421 (*bphA1A2A3A4BC*) and M5 (termed *bpdC1C2BADE*). However, in all three strains, this catabolic gene cluster is followed by *bphS* and *bphT* genes (termed *bpdS* and *bpdT* in strain M5) (Labbe et al. 1997; Arai et al. 1998; Takeda et al. 2004) that are believed to encode two-component signal transduction systems. BphT has a typical response regulator sequence, whereas BpdS, the predicted sensor kinase, is an unusually large transmembrane protein that contains ATP-binding and leucine-rich repeat motifs and some conserved residues of protein kinases. Expression of *bpdST*, like that of the *bpdC1-C2BADE* degradative operon, is inducible by biphenyl (Labbe et al. 1997). The *bphS* and *bphT* genes promote transcriptional induction by a variety of aromatic compounds in RHA1, including biphenyl, benzene, alkylbenzenes, and chlorinated benzenes (Takeda et al. 2004). However, 4-chlorobiphenyl was only a poor inducer.

Mobile genetic elements

The *bphA1A2A3A4CBST* gene cluster of *Rhodococcus* sp. RHA1 has been localized to the 1,100-kb linear plasmid pRHL1 (Masai et al. 1997; Takeda et al. 2004). Similarly, the *bph* gene cluster of TA421 was shown to be plasmid localized (Kosono et al. 1997). The complete nucleotide sequence of the *Rhodococcus erythropolis* BD2 linear plasmid pBD2, which comprises an *ipbA1A2A3A4CB* gene cluster for isopropylbenzene degradation, has recently been obtained (Stecker et al. 2003). The similarities of the key enzymes and the regulators of the *ipb* pathway genes in BD2 and the linear plasmid-encoded *bph* pathways indicate that the *ipb* and *bph* operons have been distributed among Gram-positive organisms via plasmid-mediated horizontal gene transfer. Moreover, the *ipb* gene cluster was shown to be flanked by a total of 22 ORFs showing significant similarities to insertion sequences, integrases and transposases, indicating that the *ipb* genes (and possibly also the *bph* genes) were acquired via transposition events and subsequently distributed among the rhodococci via horizontal transfer.

In strains KF715 (Hayase et al. 1990; Lee et al. 1995), LB400 (Mondello 1989) and KF707 (Furukawa and Miyazaki 1986) the biphenyl catabolic genes are chromosomally encoded. The presence of similar chromosome-localized genes in different strains implies that even the chromosomal *bph* genes possess, or have used, mechanisms for mobilization in other strains (Nishi et al. 2000). In fact, a 90-kb DNA region, including the *bph* genes, could be transferred by conjugation from KF715 to *P. putida* AC30 (Nishi et al. 2000). Transconjugants gained the ability to grow on biphenyl and the *bph* element was located on the chromosome. The element thus behaves like a conjugative transposon.

Conjugative transposons excise by site-specific recombination, transfer the resulting circular form by conjugation, integrating by recombination between a specific site on this circular form and a site in the host genome (Burrus et al. 2002). As these features are shared with "integrative plasmids" and genomic islands, it was recently proposed to classify them all as "integrative and conjugative elements (ICEs)" and the generic term "genomic island" has been proposed for a wider range of large integrated mobile (or potentially mobile) elements (Toussaint et al. 2003). Thus far, the best characterized genomic island carrying *bph* catabolic genes is Tn4371, a 55-kb element in *Wautersia oxalatica* A5 comprising the *bphEGF(orf4)A1A2A3A4BCD(orf1)A4* gene cluster (Toussaint et al. 2003). Transfer of Tn4371 to other bacteria was initially observed only after insertion of the element into a plasmid with a broad host range, suggesting that the element was a transposon. However, the element was found to integrate easily into the host chromosome at specific locations (Merlin et al. 1999), and the sequence indicates that the element can be considered as a genomic island. 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.

Biphenyl 2,3-dioxygenases

A major interest in the analysis of biphenyl-degrading bacteria is their capacity to transform PCB congeners. Based on the analysis of various biphenyl-degrading isolates, it could be deduced that lower chlorinated congeners are more easily transformed than higher chlorinated con-

geners, and PCB congeners with chlorines on one aromatic ring only are more easily degraded than 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, with some strains having a narrow spectrum and others, notably strain LB400 (Mondello 1989), being able to transform a broad range of congeners.

To a significant extent, the spectrum of PCB congeners that can be transformed by an organism is determined by the specificity of the biphenyl 2,3-dioxygenase, the enzyme that catalyzes the first step in the upper pathway and which belongs to the toluene/biphenyl family of Rieske non-heme iron oxygenases (Gibson and Parales 2000). Studies on various biphenyl 2,3-dioxygenases have revealed considerable differences in their congener selectivity patterns, as well as their preference for the ring attacked (Erickson and Mondello 1993; Kimura et al. 1997; McKay et al. 1997; Seeger et al. 1999; Zielinski et al. 2002). The α-subunit was found to be crucially responsible for recognition and binding of the substrates, and also for substrate specificity (Furukawa et al. 1993; Kimura et al. 1997). As an example, the biphenyl 2,3-dioxygenases of strains LB400 and KF707 differ in only 20 amino acid residues; however, the two enzymes differ dramatically in substrate specificity and regioselectivity. The LB400 dioxygenase shows broad substrate specificity and transforms PCBs with up to six chlorine atoms (Mondello 1989). Congeners with chlorines at the 2- and 5-position on the ring were readily attacked, whereas activity was poor with congeners with chlorines at both *para*-positions (Mondello 1989; Arnett et al. 2000). 2,5,2′,5′-Tetrachlorobiphenyl was subject to 3,4-dioxygenation (Haddock et al. 1995) (Fig. 2). A detailed analysis of the dioxygenation products of several di- to pentachlorinated biphenyls

formed by this enzyme revealed a complex dependence of the regiospecificity and the yield of dioxygenation on the substitution patterns of both the oxidized and the nonoxidized rings (Seeger et al. 1999). Biphenyl-2,3-dioxygenase attack on 2,2′- or 2,4′-dichlorobiphenyl gave rise to virtually quantitative *ortho*-dechlorination of these congeners by hydroxylation at the chlorinated carbon 2 and its unsubstituted neighbor (Fig. 2). Elimination of chloride leads directly to 2,3-dihydroxy-chlorobiphenyls and obviates the need for biphenyl-2,3-dihydrodiol-2,3-dehydrogenase for the catabolism of such congeners (Seeger et al. 1995). *Ortho*-dechlorination was also observed for various other 2-chlorosubstituted biphenyls (Haddock et al. 1995; Seeger et al. 1999). Also 2,2′-difluoro-, 2,2′-dibromo-, 2,2′-dinitro-, and 2,2′-dihydroxybiphenyl were accepted as substrates and transformed to 2′-substituted 2,3-dihydroxybiphenyls with concomitant elimination of the substituent (Seeger et al. 2001). Dibenzofuran and dibenzodioxin, which may be regarded as analogues of doubly *ortho*-substituted biphenyls or diphenylethers, respectively, were attacked at the "quasi *ortho*" carbon (the angular position 4a) and its neighbor (Fig. 2). This shows that LB400 biphenyl dioxygenase can catalyze angular dioxygenation, a reaction previously assumed to be restricted to certain specialized dioxygenases involved in dibenzo-*p*-dioxin and dibenzofuran degradation (Armengaud et al. 1998).

Compared to the LB400-derived biphenyl 2,3-dioxygenase, the enzyme of strain KF707 has a much more restricted substrate range and is not capable of *ortho*-dechlorination, but catalyzes 5,6-dioxygenation of 2,2′-dichlorobiphenyl with low activity (Suenaga et al. 2002) (Fig. 2). However, it is superior in its activity in transforming 4,4′-dichlorobiphenyl (Erickson and Mondello 1993).

Fig. 2 Transformation of selected polychlorinated biphenyl (PCB) congeners and of dibenzo-*p*-dioxin by biphenyl 2,3-dioxygenases of **a** *Burkholderia* sp. LB400 (Haddock et al. 1995; Seeger et al. 1995, 1999), **b** *Pseudomonas pseudoalcaligenes* KF707 (Suenaga et al. 2002) and **c** Phe227Val and Phe377Ala mutants of KF707 dioxygenase (Suenaga et al. 2002). Unstable intermediates are enclosed in brackets
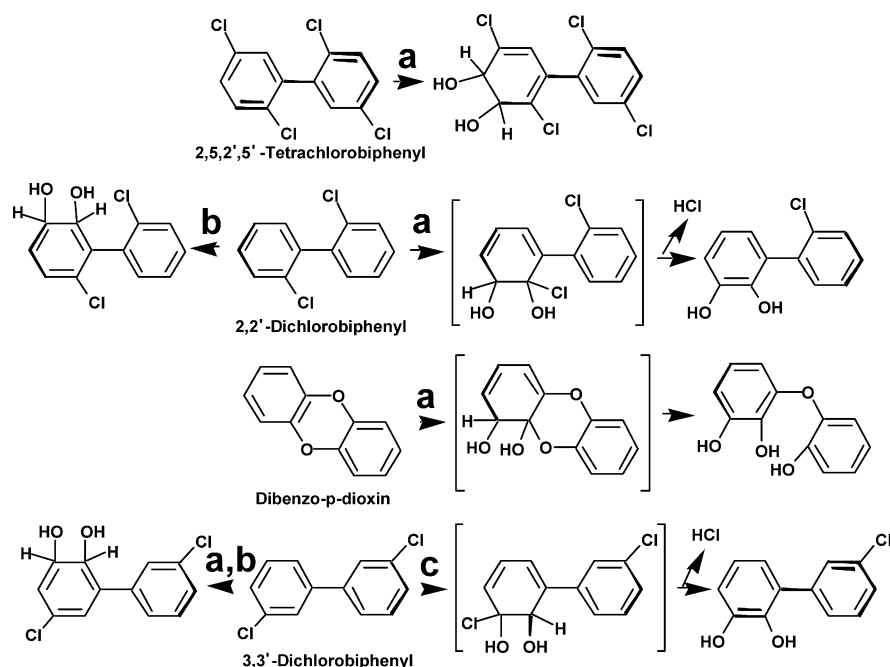
Site-directed mutagenesis and gene shuffling methods were applied to identify biphenyl dioxygenase sequences that determine specificity and to construct enzymes combining the broad congener specificity of strain LB400 with increased activity against congeners oxidized especially by the dioxygenase of strain KF707 (Erickson and Mondello 1993; Kimura et al. 1997; Mondello et al. 1997; Kumamaru et al. 1998; Brühlmann and Chen 1999b; Suenaga et al. 1999, 2002). Positions 335, 376 and 377 (numbering according to KF707 dioxygenase) were identified to be particularly important for specificity. Mutation of Thr376 (KF707) to Asn376 (LB400) in KF707 dioxygenase resulted in an expansion of the range of biotransformable PCB congeners (Kimura et al. 1997). Replacement of Phe 335 by Ile in LB400 dioxygenase improved the capacity to transform 4,4′-dichlorobiphenyl. In further attempts to modify the KF707 enzyme it was shown that mutations in position 335 (Ile335Phe), 376 (Thr376Asn) and 377 (Phe 377Leu) enabled the enzyme to perform *ortho*-dechlorination and to transform 2,5,2′,5′-tetrachlorobiphenyl. Phe 227Val and Phe377Ala mutants resulted in a completely new ability to *meta*-dechlorinate 3,3′-dichlorobiphenyl; those variants, however, completely lost the ability to transform other PCB congeners (Suenaga et al. 2002) (Fig. 2). However, the greatest improvements in activity over wild-type enzymes usually resulted from multiple amino acid modifications, suggesting that the effects of these mutations are cooperative (Mondello et al. 1997; Suenaga et al. 2001a). The availability of an enzyme model based on the available crystal structure of a related enzyme [a three-dimensional structure of naphthalene dioxygenase is available (Kauppi et al. 1998)] has helped significantly in the design of directed mutagenesis studies and in explaining the effects obtained by gene shuffling experiments. However, in various cases, residues that are not in the immediate vicinity of the bound substrate were found to significantly influence the structure of the active site (Zielinski et al. 2003). The medium- or long-range interactions responsible for such indirect effects are currently only poorly predictable. Moreover, the structure of the substrate-binding site is determined by segments that are rather distant in the sequence. Thus, the effect of individual exchanges of such segments on binding site structure will vary depending on the structures forced by other segments, and generalization of conclusions drawn from individual effects in single experiments must be treated with caution (Zielinski et al. 2002).

Efforts to create chimeric dioxygenases with novel specificity/regioselectivity are on the one hand forced by the need to optimize PCB transformation efficacy (Barriault et al. 2002). On the other hand, optimized hybrid biphenyl dioxygenases are useful not only for the transformation of trichloroethylene (Maeda et al. 2001) but also for the introduction of hydroxyl groups into flavonoids to produce antioxidants and cancer chemoprotectants (Chun et al. 2003; Kim et al. 2003; Seeger et al. 2003).

Diversity in "upper pathway" enzymes

Although certain PCBs serve as substrates for biphenyl dioxygenases, PCB-degrading organisms do not usually use PCBs as an energy source, but rather catabolize these substrates cometabolically. Therefore, it is not surprising that metabolites of the upper pathway may be formed as dead-end products (Furukawa et al. 1979; Bedard and Haberl 1990; Seeger et al. 1997).

The second step in the metabolic pathway is catalyzed by *cis*-2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase. *Cis*-dihydrodiol dehydrogenases are involved in various aromatic degradation pathways and are usually members of the family of short-chain alcohol dehydrogenases. Generally, *cis*-dihydrodiol dehydrogenases are broad substrate enzymes, and most dehydrogenases are able to transform several *cis*-dihydrodiol isomers (Patel and Gibson 1974; Rogers and Gibson 1977; Raschke et al. 1999). Even though the specificity of BphB enzymes for differently chlorinated biphenyl dihydrodiols has not yet been rigorously determined, it was shown that BphB of LB400 dehydrogenates all 2,3-biphenyl dihydrodiols chlorinated at the non-oxidized ring except for 2′,6′-substituted compounds (Hülsmeyer et al. 1998). In addition, various dihydrodiols chlorosubstitued at both aromatic rings were dehydrogenated (Hülsmeyer et al. 1998). Even the 2,2′,5, 5′-tetrachlorinated derivative of biphenyl 3,4-dihydrodiol, initially assumed not to be dehydrogenated (Haddock and Gibson 1995), was shown to be transformed by BphB of LB400 as well as by BphB of *Comamonas testosteroni* B-356 (Barriault et al. 1999). Despite the broad substrate range of BphB, this enzyme was determined as a crucial pathway bottleneck in the transformation of PCBs when combined with a highly active dioxygenase (Brühlmann and Chen 1999a).

The third reaction in the *bph* pathway is catalyzed by 2,3-dihydroxybiphenyl 1,2-dioxygenases, which are of particular significance in the degradation of PCBs, as they are subject to various types of inhibition including suicide inactivation (Vaillancourt et al. 2002). 2,3-Dihydroxybiphenyl 1,2-dioxygenases belong to the family of extradiol dioxygenases and use mononuclear Fe^{2+} to cleave dihydroxybiphenyl. Usually, extradiol dioxygenases, which are involved in biphenyl degradation, belong to the I.3.A subfamily of extradiol dioxygenases (Eltis and Bolin 1996) and are specialized for transformation of 2,3-dihydroxybiphenyls. These enzymes differ in their substrate specificity, but seem to be generally capable of transforming various chlorosubstituted derivatives (Hein et al. 1998; Dai et al. 2002). However, both 3,4-dihydroxybiphenyl (Lloyd-Jones et al. 1995; McKay et al. 2003) and 2′-chlorosubstituted 2,3-dihydroxybiphenyls strongly inhibit biphenyl dioxygenases. 2′-Chlorosubstituted 2,3-dihydroxybiphenyls promote suicide inactivation, which involves release of superoxide during catalysis and oxidation of the active site Fe^{2+}, and thus interfere with the degradation of other compounds (Dai et al. 2002). However, a new class of

single domain extradiol dioxygenases, thus far observed only in *Sphingomonas* (Heiss et al. 1995) and *Rhodococcus* strains (Asturias and Timmis 1993; Sakai et al. 2002), has been shown to have adapted to the transformation of 2-chlorosubstituted 2,3-dihydroxybiphenyls (McKay et al. 2003; Vaillancourt et al. 2003).

The fourth step in the bph pathway is catalyzed by HOPDA hydrolase BphD, which hydrolyzes HOPDA at a carbon-carbon bond to yield 2-hydroxypenta-2,4-dienoate and benzoate. Recent studies using LB400 BphD revealed that this enzyme relatively rapidly transformed HOPDAs bearing chlorine substituents at the phenyl moiety, whereas HOPDAs bearing chlorine substituents on the dienoate moiety were poor substrates, although they act as competitive inhibitors of BphD (Seah et al. 2000). Moreover, HOPDAs undergo nonenzymatic transformation to products that included acetophenones (Seah et al. 2000). These results explain the accumulation of HOPDAs and chloroacetophenones in the microbial degradation of certain PCB congeners. More significantly, they indicate that during the degradation of PCB mixtures, BphD would be inhibited, thereby slowing the mineralization of all congeners. BphD is thus a key determinant in the aerobic microbial degradation of PCBs. Even though the *R. globerulus* P6-derived BphD also exhibits significantly higher activity with HOPDAs bearing chlorine substituents at the phenyl moiety compared to HOPDAs bearing chlorine substituents at the dienoate moiety, it was shown that these *bphD* gene products differ significantly in their kinetic properties.

Metabolic complexity in *Rhodococcus*

A simplified assumption in biphenyl degradation and PCB catabolism is that the strains isolated have a single set of biphenyl degradation genes clustered in a single locus, and that only the corresponding gene products are responsible for the PCB-transforming capability of the strain. In recent years, it has become clear that various microorganisms contain multiple metabolic pathways or isoenzymes involved in PCB metabolism. *R. globerulus* P6 was shown early to contain at least three BphC-encoding genes (Asturias and Timmis 1993), with two of them being expressed during growth on biphenyl (McKay et al. 2003). Recent analysis indicated that this strain contains at least four BphC genes (Kosono et al. 1997). Six extradiol dioxygenase genes were identified in strain RHA1 (Sakai et al. 2002), and three are expressed when the organism is grown on biphenyl. A total of seven *bphC* genes were found in strain TA421 (Maeda et al. 1995; Kosono et al. 1997) and eight in *R. rhodochrous* K37 (Taguchi et al. 2004). Thus, the presence of multiple extradiol dioxygenases seems to be common in rhodococcal strains and is thought to contribute to the versatility of this group of bacteria with respect to the degradation of haloaromatic compounds. Later, it was observed that strain RHA1 also harbors multiple aromatic ring hydroxylating dioxygenases

(Kitagawa et al. 2001b) and hydrolases (Yamada et al. 1998), which could function in the transformation of PCB congeners. The complex gene organization (Shimizu et al. 2001; Takeda et al. 2004) resembles slightly that previously observed in a dibenzo-*p*-dioxin degrading *Sphingomonas* strain (Armengaud et al. 1998).

Biphenyl pathways in *Sphingomonas*

Biphenyl-degrading *Sphingomonas* strains such as *Sphingobium yanoikuyae* B1 (Gibson et al. 1973; Zylstra and Kim 1997), *Sphingobium yanoikuyae* Q1 (Furukawa et al. 1983; Taira et al. 1988), *Sphingomonas chungbulensis* DJ77 (Kim et al. 2000) or *Novosphingobium aromaticivorans* F199 (Romine et al. 1999) have also been described, and genetic analysis has revealed a highly complex arrangement of catabolic genes differing immensely in sequence homology and gene order from those reported for other genera (Fig. 3). In strain B1, a *bphB* gene was localized after a transposon mutation rendered the strain incapable of mineralizing biphenyl (Kim and Zylstra 1995). Analysis of a 40 kb DNA fragment comprising this gene revealed the presence of genes for no less than five different oxygenase α subunits (*bphA1a-e*) (Zylstra and Kim 1997), with only two of them (*bphA1a and b*) having adjacent genes for the β subunit. However, deletion or inactivation by insertional mutagenesis of any single α-subunit gene does not impair the ability of B1 to oxidize biphenyl, indicating that the biphenyl oxygenase α-subunit-encoding genes are located in a different gene region. In contrast, single genes for ferredoxin (*bphA3*) and a ferredoxin reductase (*bphA4*) were found, with lack of the ferredoxin resulting in a nonfunctional biphenyl dioxygenase; growth on *m*-xylene, phenanthrene and 3-methylbenzoate was also abolished (Kim and Zylstra 1999), implying that the ferredoxin is associated with multiple terminal oxygenases. Complete sequencing of the 184 kb catabolic plasmid pNL1 from strain F199 showed the presence of a gene region with high homology to that identified in B1. Thirty-five genes (Fig. 3) are arranged in the same order and transcriptional direction (Romine et al. 1999) and there is a mixture of genes probably involved in biphenyl catabolism (*bph*-genes) and those probably involved in xylene/toluene/methylbenzoate (*xyl*-genes) degradation, including xylJQK genes, which probably fulfill a function in 3-methylbenzoate degradation, but could possibly be recruited for 2-hydroxypenta-2,4-dienoate degradation formed by hydrolysis of HOPDA. As well as five genes encoding an α-subunit, a sixth terminal oxygenase (*bphA1fA2f*) was identified and suggested to encode the terminal dioxygenase component of biphenyl/naphthalene dioxygenase. However, no evidence for this function, nor the function of predicted *bphDEF* genes have been proposed thus far. For a more detailed review on aromatic catabolic genes in Sphingomonads, see Pinyakong et al. (2003).
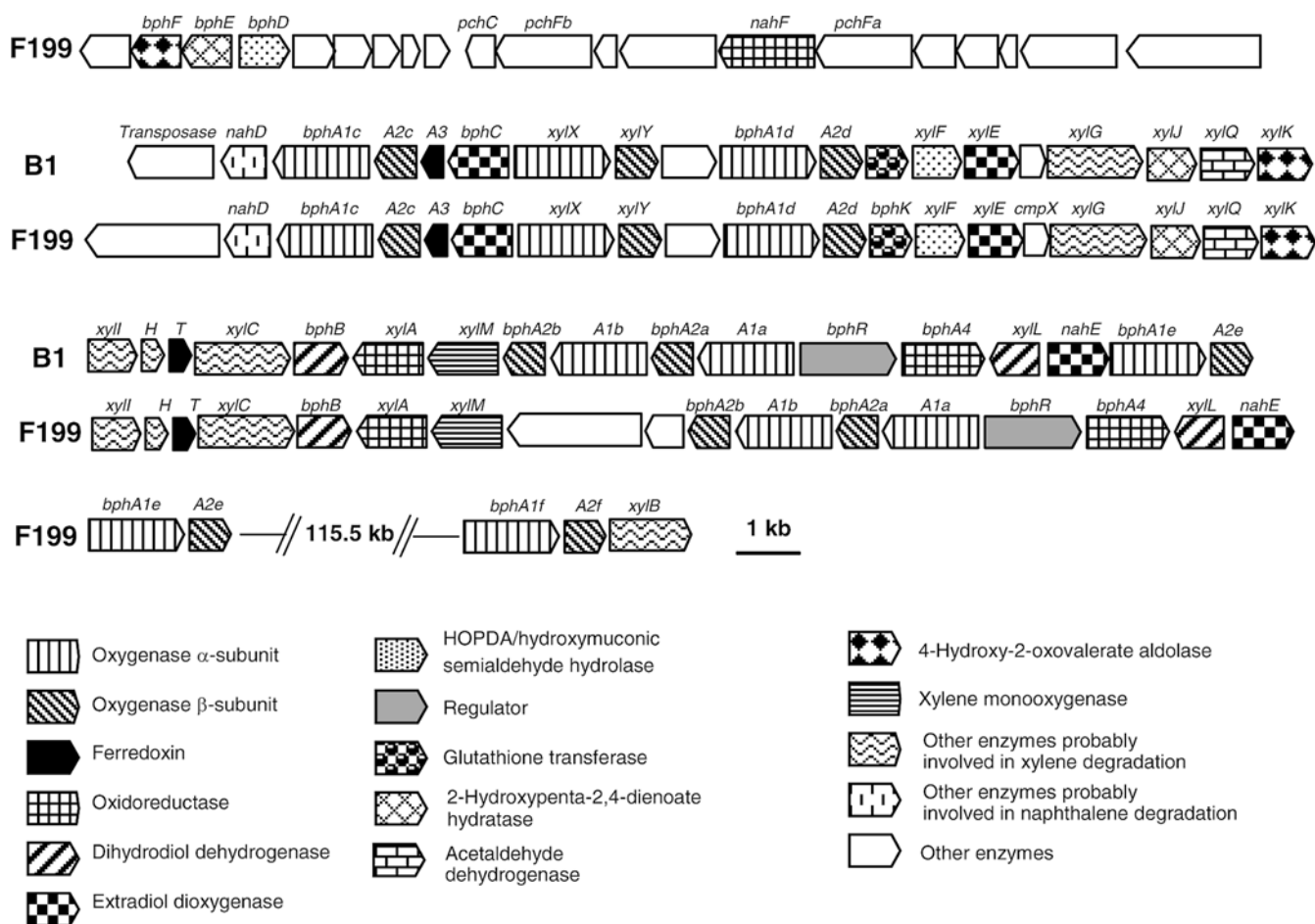
Fig. 3 Organization of aromatic hydrocarbon degradation genes in *Sphingobium yanoikyae* B1 (Zylstra and Kim 1997; Kim and Zylstra 1999; Pinyakong et al. 2003) and *Novosphingobium aromaticivorans* F199 (Romine et al. 1999)

Transformation of biphenyls by enzymes distinct from those of the biphenyl upper pathway

Despite differences in genetic organization, the initial enzymatic steps in the aerobic degradation of many aromatic compounds in different bacterial genera are very similar, and enzymes homologous to BphA, B and C are found in pathways responsible for the degradation of naphthalene, benzoate and benzene (Fig. 4). Despite the evolutionary adaptation of enzymes to specific substrates, the enzymes of a particular pathway usually catalyze the transformation of a range of substrate analogues. Hence, enzymes in the PCB pathways may transform not only PCBs and their metabolites, but also other related compounds such as monocyclic aromatics (Suenaga et al. 2001b), and vice versa. This substrate overlap means that other pollutants in a site may act as co-substrates that can influence the composition and activity of biphenyl-metabolizing bacteria. As an example, chlorobenzene dioxygenase of *Pseudomonas* sp. P51 (which belongs, like biphenyl dioxygenases, to the toluene/biphenyl family of Rieske non-heme iron oxygenases) (Fig. 4) was shown to be capable of efficiently transforming monochlorinated biphenyls (Raschke et al. 1999, 2001); however, higher substituted biphenyls were not attacked. Even naphthalene dioxygenase of *P. putida* G7 (belonging to the naphthalene family of Rieske non-heme iron oxygenases) (Fig. 4) can efficiently transform chlorinated biphenyls (Barriault and Sylvestre 1999b). However, this enzyme differed in regioselectivity and a significant amount of biphenyl was subject to 3,4-dioxygenation (Barriault and Sylvestre 1999a; Parales et al. 2000). Because 3,4-dihydroxylated biphenyls were cleaved efficiently by the subfamily I.3.E extradiol dioxygenase DoxG of *Pseudomonas* sp. strain C18 (Eltis and Bolin 1996), it was suggested to use naphthalene catabolic enzymes to expand the capabilities of the *bph* pathway to degrade PCBs (Barriault et al. 1998).

Interestingly, not all aromatic ring-hydroxylating dioxygenases are capable of biphenyl transformation and whereas phenanthrene dioxygenase PhnA1A2A3A4 of *Cytoclasticus* sp. strain A5 exhibited such activity (Kasai et al. 2003), the phenanthrene dioxygenase PhdABCD of *Nocardioides* sp. strain KP7 (Fig. 4) was not active against biphenyl (Shindo et al. 2001). However, a systematic evaluation of different groups of aromatic ring hydroxylating dioxygenases for their capability to transform PCBs is still lacking. Nevertheless, the capacity to transform biphenyls is evidently not restricted to biphenyl-degrad-
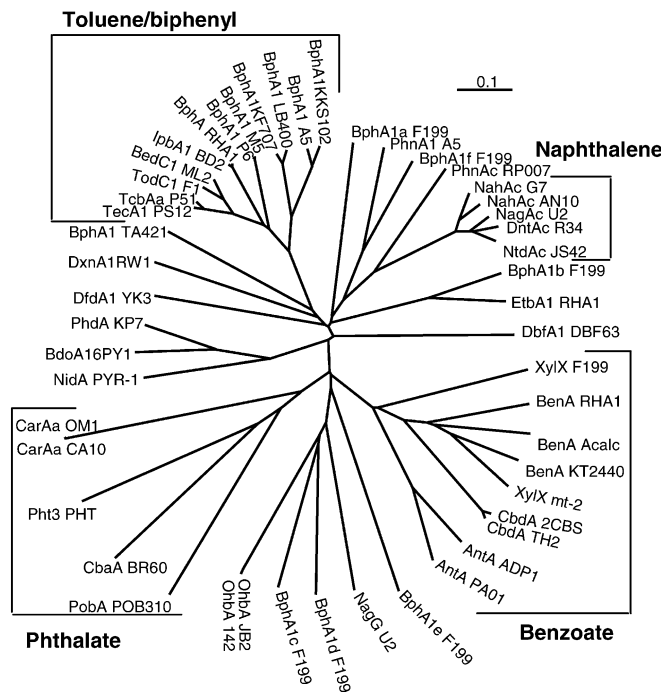
Fig. 4 Phylogenetic tree of the α-subunits of selected Rieske non-heme iron oxygenases. Families identified by Gibson and Parales (2000) are indicated. For clarity, only protein and strain designations are indicated

ing organisms. Growth on biphenyl even in the absence of a biphenyl pathway has been reported. As an example, introduction of the capacity to mineralize toluene via the *tod* pathway into *P. putida* F1 (Zylstra et al. 1988) allowed the strain to mineralize biphenyl (Furukawa et al. 1993), showing that toluene dioxygenase (TodC1C2BA), toluene dihydrodiol dehydrogenase (TodD) and the *meta*-cleavage enzyme TodE have significant cross-reactivity with biphenyl or metabolites produced during biphenyl degradation, but that TodF 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase cannot deal with HOPDA. Recently, *P. putida* strain CE2010 was reported to mineralize biphenyl by a similar mosaic of *tod* (toluene) and *cmt* (cumate) pathways in the absence of an authentic biphenyl pathway (Ohta et al. 2001). It is possible that such mosaic routes, both within individual strains and within communities, are important for the metabolism of complex pollutant mixtures.

Metabolic pathways for chlorobenzoate degradation

The *bph*-encoded biphenyl pathway enzymes present in a variety of native bacteria transform many PCBs. However, most of those bacteria only cometabolize chlorinated congeners, and thus, concomitant with the transformation of PCBs, different dead-end metabolites accumulate, including dihydrodiols (Brühlmann and Chen 1999a), dihydroxybiphenyls (including 3,4-dihydroxylated derivatives) (Triska et al. 2004), or chlorinated HOPDAs (Furukawa et al. 1979; Seeger et al. 1997). It was recently shown that dihydrodiols and dihydroxybiphenyls are very toxic me-

tabolites for bacteria, affecting cell viability—and thus bacterial performance—much more than (chloro)biphenyls (Cámara et al. 2004). It will be of critical importance to overcome dead-end reactions in the catabolic process and balance the activities of enzymes involved in the degradation to avoid accumulation of toxic metabolites. However, as different pathway bottlenecks for different PCB congeners operate in different organisms, is it expected that only diverse communities with complementary activities will to able to deal with complex PCB mixtures.

The complexity of PCB degradation is enhanced by the fact that most PCB degraders at best transform chlorinated biphenyls into chlorinated benzoates and thus live at the expense of the 2-hydroxypenta-2,4-dienoate generated during hydrolysis of HOPDAs. Even though chlorobenzoates themselves are obviously non-toxic to bacteria, the negative effects of chlorobenzoate metabolism on chlorobiphenyl degradation have already been reported (Havel and Reineke 1992; Sondossi et al. 1992; Stratford et al. 1996) and thus the metabolic fate of chlorobenzoates must be understood when analyzing PCB metabolism.

Transformation of chlorobenzoates by benzoate degradative pathways

Benzoate 1,2-dioxygenase genes are widespread and have been identified in a wide variety of microorganisms and localized in diverse genome sequencing projects. The substrate specificity of benzoate dioxygenases is usually restricted to benzoate and 3-chloro-/3-methylbenzoate, as shown for chromosomally encoded enzymes from strain *Pseudomonas* sp. B13 (Reineke and Knackmuss 1978a), and *Wautersia eutropha* JMP134 (Pieper et al. 1993), as well as for the distantly related enzyme from *Rhodococcus* sp. strain RHA1 (Kitagawa et al. 2001a). Enzymes from both B13 and JMP134 form 3-chloro- and 5-chlorodihydro-dihydroxybenzoate in a 2:1 ratio (Reineke and Knackmuss 1978a), which are dehydrogenated to 3-chloro- and 4-chlorocatechol, respectively (Reineke and Knackmuss 1978b). Chlorocatechols can be regarded as environmentally important intermediates. Their metabolic fate, when processed by enzymes of widespread pathways for catechol metabolism, can be of environmental significance.

The 3-oxoadipate pathway

The chromosomally encoded 3-oxoadipate pathway is widely distributed in soil bacteria and fungi (Harwood and Parales 1996). One branch of this pathway converts protocatechuate, whereas the other converts catechol. Two additional steps, a 3-oxoadipate:succinyl-CoA transferase and a 3-oxoadipyl-CoA thiolase, accomplish the conversion of 3-oxoadipate to tricarboxylic acid cycle intermediates (Stanier and Ornston 1973). Enzyme studies and amino acid sequence data indicate that the pathway is highly conserved in diverse bacteria. In the catechol branch, catechol is cleaved by catechol 1,2-dioxygenases giving

rise to muconate. Usually, catechol 1,2-dioxygenase transforms 4-chlorocatechol with relatively high activity (Dorn and Knackmuss 1978a; Schmidt et al. 1980; Blasco et al. 1997). Muconate cycloisomerases catalyze dehalogenation and decarboxylation of 3-chloromuconate, the product of 4-chlorocatechol ring-cleavage, to form protoanemonin (Blasco et al. 1995) (Fig. 5), a compound of high toxicity (Seegal and Holden 1945). Protoanemonin formation in turn was assumed to be the reason for the poor survival of polychlorobiphenyl cometabolizing organisms in soil microcosms due to channeling of intermediary 4-chlorocatechol into the 3-oxoadipate pathway (Blasco et al. 1997). Chloroprotoanemonin was even reported to be formed from 2,4-dichloromuconate by muconate cycloisomerase (Kaulmann et al. 2001). In the case of 2-chloromuconate turnover, muconate cycloisomerases catalyze cycloisomerization only, to form both 2-chloro- and 5-chloromuconolactone as stable products (Vollmer et al. 1994) (Fig. 5). Both chloromuconolactones are substrates of proteobacterial muconolactone isomerases (Prucha et al. 1996a,b; Skiba et al. 2002) of the 3-oxoadipate pathway. Muconolactone isomerase catalyzes dehalogenation of 5-chloromuconolactone to form predominantly cis-dienelactone (Fig. 5). The proposed mechanism acts via abstraction of the C4 proton followed by spontaneous chloride elimination. 2-Chloromuconolactone was transformed into protoanemonin (Fig. 5), probably by elimination of CO_2 and chloride from chlorosubstituted 3-oxoadipate enol-lactone (Skiba et al. 2002). Thus, the presence of a 3-oxoadipate

pathway will severely interfere with the degradation of PCBs via chlorobenzoates as intermediates.

The meta-cleavage pathways

Catechol meta-cleavage routes are also widespread and are usually involved in the degradation of methylsubstituted compounds such as toluene or methylphenols (Bayly et al. 1966; Worsey and Williams 1975; Zylstra et al. 1988; Shingler et al. 1992). For a long time the presence of a catechol meta-cleavage pathway was generally assumed to severely interfere with the degradation of chloroaromatics. One of the reasons for this interference was assumed to be the formation of a suicide product, reactive acyl chloride from 3-chlorocatechol (Fig. 5), resulting in irreversible inactivation of the catechol 2,3-dioxygenase of P. putida mt-2 (Bartels et al. 1984). However, in other cases reversible inactivation was shown to be due to rapid oxidation of the active site ferrous iron into its ferric form with concomitant loss of activity (Vaillancourt et al. 2002), and a general mechanism for the inactivation of extradiol dioxygenases during catalytic turnover involving the dissociation of superoxide from the enzyme-catecholic-dioxygen ternary complex was suggested. However, independent of the inactivation mechanism involved, a general inhibitory effect of 3-chlorocatechol formed during the metabolism of 3-chlorobiphenyl on biphenyl metabolism was observed, at least in C. testosteroni B-356 (Sondossi
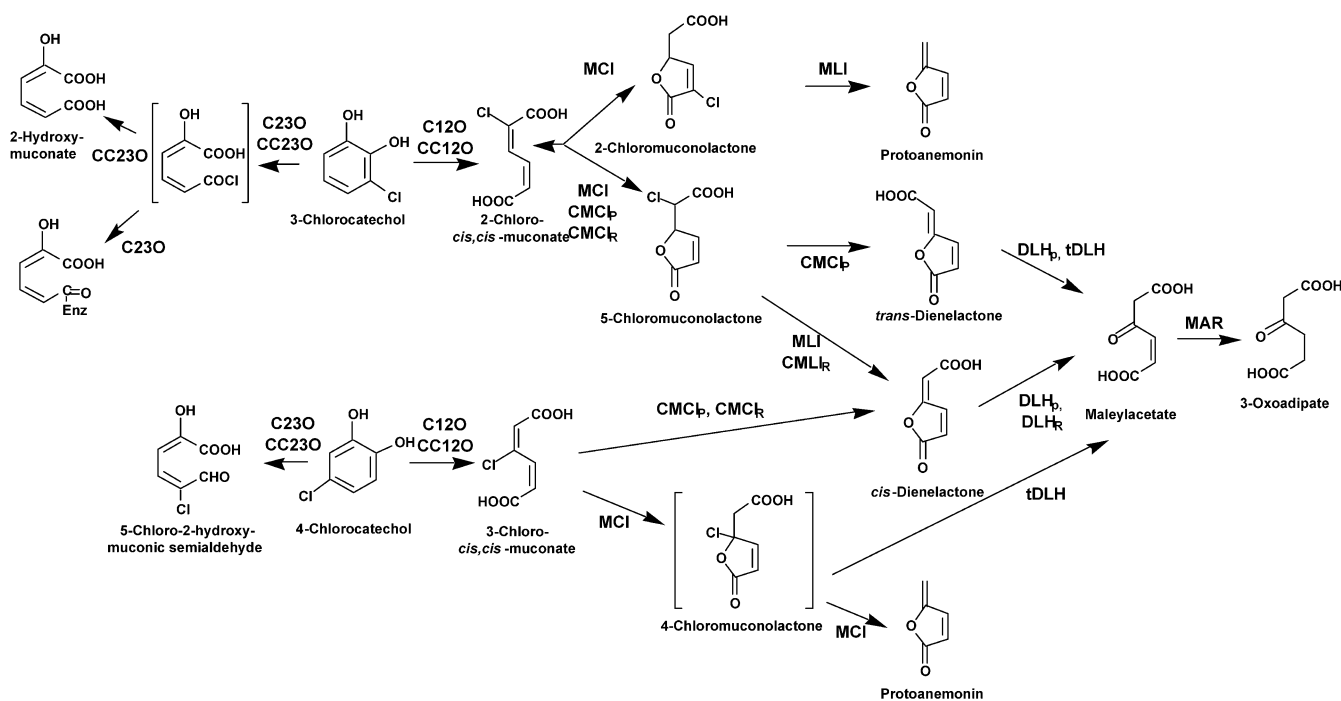

Fig. 5 Diversity of pathways for the degradation/transformation of chlorocatechols. *C23O* Catechol 2,3-dioxygenase, *CC23O* chlorocatechol 2,3-dioxygenase, *C12O* catechol 1,2-dioxygenase, *CC12O* chlorocatechol 1,2-dioxygenase, *MCI* muconate cycloisomerase, *CMCI*$_P$ proteobacterial chloromuconate cycloisomerase, *CMCI*$_R$ chloromuconate cycloisomerase of *R. opacus* 1CP, *MLI* muconolactone isomerase, *CMLI*$_R$ chloromuconolactone isomerase of *R. opacus* 1CP, *DLH*$_P$ proteobacterial dienelactone hydrolase, *DLH*$_R$ dienelactone hydrolase of *R. opacus* 1CP, *tDLH* trans-dienelactone hydrolase, *MAR* maleylacetate reductase. Unstable intermediates are enclosed in brackets

et al. 1992), most probably caused by inactivation of BphC.

In contrast to 3-chlorocatechol, 4-chlorocatechol is a moderate substrate for various catechol 2,3-dioxygenases (Murray et al. 1972; Bartels et al. 1984; Kitayama et al. 1996), among them catechol 2,3-dioxygenases of family I.2.A (Eltis and Bolin 1996). However, despite high sequence identity, members of this subfamily exhibit very different substrate specificities (Kitayama et al. 1996). Some authors postulated that compounds degraded via 4-chlorocatechol might be mineralized via a catechol-degrading *meta*-cleavage pathway (Janke and Fritsche 1979; Higson and Focht 1992; Arensdorf and Focht 1994; Hollender et al. 1994, 1997; McCullar et al. 1994; Arensdorf and Focht 1995). *B. cepacia* P166 was shown to mineralize 4-chlorobiphenyl via 4-chlorobenzoate, 4-chlorocatechol followed by a *meta*-cleavage route (Arensdorf and Focht 1995). 3-Chlorocatechol metabolism, in contrast, was toxic to the strain (Arensdorf and Focht 1994).

Mineralization of chlorobenzoates

In recent years, it has become more and more evident that bacteria have evolved various strategies to degrade chlorobenzoates, and degradation can occur via chlorocatechol (*clc* pathway and others) (Dorn and Knackmuss 1978b), via hydrolytic dehalogenation of 4-chlorobenzoate to give 4-hydroxybenzoate [*fcb* pathway (Klages and Lingens 1979)], via 4,5-dioxygenation of 3-chlorobenzoate and 3,4-dichlorobenzoate to give 5-chloroprotocatechuate (Nakatsu and Wyndham 1993), and probably via a fourth pathway with gentisate as intermediate [gp-pathway (Krooneman et al. 1996, 1998)].

The chlorocatechol pathway

In most organisms isolated thus far, chlorocatechols are degraded by a chlorocatechol *ortho*-cleavage pathway (Dorn and Knackmuss 1978a,b; Schmidt and Knackmuss 1980; Schmidt et al. 1980; Kaschabek and Reineke 1992), where degradation is initiated by a broad specificity chlorocatechol 1,2-dioxygenase to produce the corresponding chloro-*cis,cis*-muconates (Dorn and Knackmuss 1978a,b; Schmidt et al. 1980; Broderick and O'Halloran 1991) (Fig. 5). As chlorocatechols are highly toxic (Schweigert et al. 2001), microorganisms grow on chloroaromatics only when they possess an activity equilibrium between chlorocatechol-producing and -consuming activities (Klemba et al. 2000; Perez-Pantoja et al. 2003).

A key enzyme in the pathway is chloromuconate cycloisomerase, which differs in various aspects from muconate cycloisomerases (Fig. 5). Proteobacterial chloromuconate cycloisomerases catalyze a specific cycloisomerization of 2-chloromuconates to form preferentially 5-chloromuconolactone, which is dehalogenated to *trans*-dienelactone (Vollmer and Schlömann 1995). Thus, proteobacterial chloromuconate cycloisomerases are specific dehalogenases.

The formation of *trans*-dienelactone is due to the fact that, after cycloisomerization, the lactone ring has to rotate in the catalytic center to achieve proton abstraction and thus dehalogenation (Schell et al. 1999). In contrast, chloride from the 3-position of chloromuconate seems to be directly eliminated during cycloisomerization (Kaulmann et al. 2001) to form *cis*-dienelactone. Evidently, for the degradation of various chloroaromatics, an important prerequisite is that the dienelactone hydrolase involved in the pathway is of relaxed substrate specificity and accepts both *cis*- and *trans*-isomers of dienelactone as substrates (Schlömann et al. 1990). The maleylacetates formed are then transformed by a maleylacetate reductase, which catalyzes the reduction of the double bond to form 3-oxoadipate (Kaschabek and Reineke 1992). Maleylacetates with chlorine substituents in the 2-position, such as 2-chloromaleylacetate (formed from 3,5-dichloro-, and 3,6-dichlorocatechol), are reduced in a first step to give maleylacetate. Obviously, enzymatic attack on the C2-carbon results in an intermediate that spontaneously eliminates chloride.

The genes encoding chlorocatechol pathway enzymes form clusters, and the structures of the corresponding operons are highly conserved, in spite of the geographically distinct origins of the bacteria or differences in their phylogenetic position (Fig. 6). In *P. putida* AC858, *Pseudomonas* sp. strain P51 (van der Meer et al. 1991) and others, the gene cluster encoding chlorocatechol dioxygenase, chloromuconate cycloisomerase, dienelactone hydrolase and maleylacetate contain an ORF of unknown function following the chloromuconate cycloisomerase gene. Such an ORF is absent in the otherwise identically structured chlorocatechol gene clusters *tfdI* of *Wautersia eutropha* JMP134 (Don and Pemberton 1981, 1985), and *tfd* of *Burkholderia* sp. strain NK8 (Liu et al. 2001). Regulatory genes orientated in the opposite direction precede the catabolic genes; their gene products, members of the LysR family, act as positive regulators (Coco et al. 1993) with chloromuconates as effectors (McFall et al. 1997; Ogawa et al. 1999). 2-Chloro- and 3-chlorobenzoate, as well as 3-chloro- and 4-chlorocatechol, were shown to act as effectors of the TfdT regulator of *Burkholderia* sp. strain NK8 (Liu et al. 2001). In contrast to organisms isolated based on their capacity to degrade chlorobenzoates or chlorobenzenes, organisms capable of degrading 2,4-dichlorophenoxyacetate usually contain a *tfdRCEBKA* gene cluster, comprising a chlorocatechol dioxygenase and dienelactone hydrolase-encoding gene together with those encoding a chlorophenol hydroxylase, a transport protein and a 2,4-D/α-ketoglutarate dioxygenase, but lack the *tfdD* and *tfdF* genes encoding chloromuconate cycloisomerases and maleylacetate reductase encoding genes (Vedler et al. 2000; Poh et al. 2002; Hoffmann et al. 2003) (Fig. 6). But not only the structures of the chlorocatechol gene are conserved: isofunctional enzymes of the chlorocatechol pathway were shown to be homologous and more similar to each other than to the corresponding enzymes of catechol pathways, indicating a common origin of the proteobacterial chlorocatechol pathways (Schlömann 1994). In contrast, the enzymes of the Gram-positive organism
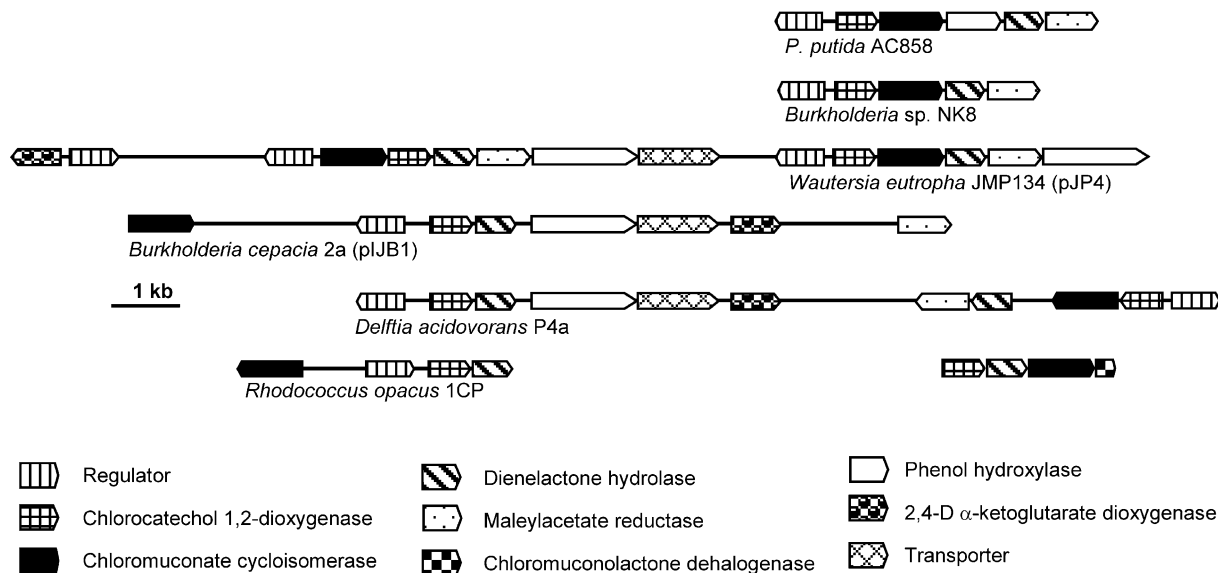
Fig. 6 Genetic organization of chlorocatechol gene clusters *clcR-ABCDE* in *P. putida* AC858 (Chatterjee et al. 1981b) [similar gene clusters were observed in *P. aeruginosa* JB2, *clcR-ABCDE* (Hickey et al. 2001); *Pseudomonas* sp. P51, *tcbR-CD* ORF *EF* (van der Meer et al. 1991); *P. chlororaphis RW71, tetR-CD* ORF *EF* (Potrawfke et al. 2001) and *W. eutropha* NH9, *cbnR-ABCD*, (Ogawa and Miyashita 1999)]; *tfdT-CDEF* in *Burkholderia* sp. *NK8* (Liu et al. 2001), *tfdA-S—R-D$_{II}$C$_{II}$E$_{II}$F$_{II}$B$_{II}$K— T-C$_I$D$_I$E$_I$F$_I$ B$_I$* in *W. eutropha* JMP134 (Trefault et al. 2004); *tfdD—R-CEBKA—F* in *Burkholderia cepacia* 2a (pIJB1) (Poh et al. 2002) [similar gene clusters were observed in *Variovorax paradoxus* TV1 (pTV1) (AF028643) and *Achromobacter xylosooxidans* subsp. *denitrificans* EST4002 (pEST 4011) (Vedler et al. 2000)], *tfdR-C$_{II}$E$_{II}$BKA—FE-DC-R* in *Deftia acidovorans* P4a (Hoffmann et al. 2003), and *clcB—R-AD and clcA2D2B2F* in *Rhodococcus opacus* 1CP (Moiseeva et al. 2002)

Rhodococcus opacus 1CP turned out to have unusual biochemical properties and sequences with relatively little similarity to proteobacterial chlorocatechol gene sequences, thus indicating an independent origin (Eulberg et al. 1998; Moiseeva et al. 2002; Solyanikova et al. 1995).

Mating methods mimicking natural gene transfer were used to transfer genes for chlorocatechol degradation (*clc*) from strain B13 to various recipients (Reineke and Knackmuss 1979; Reineke 1998). It had been suspected that the genes were transferred by means of a conjugative plasmid, pB13 (Chatterjee and Chakrabarty 1983). However, in contrast to various other chlorocatechol genes, which are obviously localized on plasmids (Chatterjee et al. 1981a; van der Meer et al. 1991; Trefault et al. 2004), the clc genes of B13 reside on a 105 kb conjugative genomic island (the *clc* element) in two copies on the chromosome (Ravatn et al. 1998a,b). In contrast to many pathogenicity islands, the *clc* element is self-transmissible to other recipient bacteria (Ravatn et al. 1998c; Springael et al. 2002). With a certain frequency the element excises, resulting in a circular intermediate that can be transferred to a new recipient. It integrates into the 3′-end of a target glycine tRNA gene and employs a phage-type integrase, IntB13, to achieve site-specific integration (Ravatn et al. 1998b; van der Meer et al. 2001). Integrase expression was significantly stimulated by growing cultures on 3-chlorobenzoate (Sentchilo et al. 2003), indicating that this chloroaromatic stimulates transfer of the genes encoding its own metabolism.

Alternative pathway of 3-chlorocatechol degradation in *Rhodococcus opacus* 1CP

Among Gram-positive organisms, chlorocatechol catabolism has been investigated mainly in *R. opacus* 1CP. 4-Chloro- and 3,5-dichlorocatechol are degraded by an enzymatic sequence similar to that in proteobacteria (Solyanikova et al. 1995; Eulberg et al. 1998); however, the enzymes involved differ in various aspects from those described in proteobacteria (Maltseva et al. 1994a). Pronounced differences were observed between proteobacterial chloromuconate cycloisomerase and the *Rhodococcus* enzyme, as the latter showed high specificity constants for 3-chloro- and 2,4-dichloromuconate (Maltseva et al. 1994a), but negligible activity with 2-chloromuconate. Moreover, 2-chloromuconate is not dehalogenated but transformed to 5-chloromuconolactone only, whereas 3-chloro- and 2,4-dichloro-*cis,cis*-muconate are converted to the expected (2-chloro-)*cis*-dienelactone metabolites (Fig. 5). The *Rhodococcus* dienelactone hydrolase is special as this enzyme is practically inactive against *trans*-dienelactone (Maltseva et al. 1994b). The sequence of the *Rhodococcus* 4-chlorocatechol/3,5-dichlorocatechol catabolic gene cluster reveals a low sequence similarity to the corresponding proteobacterial genes and, together with biochemical evidence, suggests that the chlorocatechol pathway of strain 1CP should have evolved to be functionally convergent to the pathway in proteobacteria (Maltseva et al. 1994b). After adaptation, the strain grew on chloroaromatics, which are metabolized via 3-chlorocatechol (Moiseeva et al. 1999), and a second chloro-

catechol 1,2-dioxygenase of broad substrate specificity, a second chloromuconate cycloisomerase, and a second dienelactone hydrolase were identified (Moiseeva et al. 2001). The second chloromuconate cycloisomerase is specific for 2-chloro-*cis,cis*-muconate; however, in contrast to proteobacterial chloromuconate cycloisomerases, it cannot catalyze dehalogenation, and 5-chloromuconolactone was observed as product (Moiseeva et al. 2001, 2002). Dehalogenation is achieved by an enzyme with high sequence similarity to the muconolactone isomerases of the 3-oxoadipate pathway (Fig. 5), which in proteobacteria have been shown to be capable of dehalogenating 5-chloromuconolactone to *cis*-dienelactone. Transformation of *cis*-dienelactone in the pathway is achieved by a second dienelactone hydrolase, which, like its counterpart in 4-chlorocatechol degradation, is specific for *cis*-dienelactone. A major difference between chlorocatechol degradation in proteobacteria and in *Rhodococcus* is based on the fact that *cis*-dienelactone is the intermediate of both 4-chloro- and 3-chlorocatechol degradation in *Rhodococcus*, whereas proteobacteria form *cis*- and *trans*-dienelactone, respectively, thus requiring a broad substrate specificity dienelactone hydrolase.

Alternative *ortho*-cleavage pathway of 4-halocatechol degradation

As muconate cycloisomerases form protoanemonin as a toxic dead-end product (Blasco et al. 1995), transformation of 4-chlorocatechol by enzymes of the 3-oxoadipate pathway constitutes a problematic catabolic misrouting in bacterial communities (Blasco et al. 1997). A new strategy for avoiding protoanemonin formation has been elucidated in *Pseudomonas* sp. strain MT1 (Pelz et al. 1999). Strain MT1 degrades 4-chlorocatechol without having a chlorocatechol pathway and a *trans*-dienelactone hydrolase was shown to be the key enzyme of a novel metabolic route (Nikodem et al. 2003). 4-Chlorocatechol is subject to ring-cleavage by a catechol 1,2-dioxygenase to yield 3-chloromuconate. 3-Chloromuconate is transformed into protoanemonin as predominant reaction product by muconate cycloisomerase of strain MT1, as previously shown for other muconate cycloisomerases (Fig. 5). Protoanemonin is obviously a dead-end product of the pathway. However, a *trans*-dienelactone hydrolase acts on 4-chloromuconolactone as an intermediate in the muconate cycloisomerase-catalyzed transformation of 3-chloromuconate, thus preventing protoanemonin formation in favor of maleylacetate formation (Fig. 5). The maleylacetate thereby formed is reduced by maleylacetate reductase. Chlorocatechol degradation in strain MT1 thus occurs via a new pathway consisting of a patchwork of reactions known from the 3-oxoadipate pathway (catechol 1,2-dioxygenase, muconate cycloisomerase), the chlorocatechol pathway (maleylacetate reductase) and a *trans*-dienelactone hydrolase.

Metabolism of 3-chlorocatechol by the chlorocatechol *meta*-cleavage pathway

Recently, *P. putida* GJ31 was found to degrade chlorobenzene rapidly via 3-chlorocatechol using a *meta*-cleavage pathway (Mars et al. 1997). In contrast to other catechol 2,3-dioxygenases, which are subject to rapid inactivation, the chlorocatechol 2,3-dioxygenase of strain GJ31 productively converts 3-chlorocatechol (Kaschabek et al. 1998; Mars et al. 1999). Stoichiometric displacement of chloride leads to the production of 2-hydroxymuconate (Fig. 5), which is further converted through the *meta*-cleavage pathway. Additional pseudomonads using a *meta*-cleavage route for 3-chlorocatechol degradation were recently isolated from various contaminated environments (Göbel et al. 2004) suggesting that productive *meta*-cleavage of 3-chlorocatechol is not atypical for chloroaromatic degradation. The new isolates, *Pseudomonas fluorescens* SK1, *Pseudomonas veronii* 16-6A and *Pseudomonas* sp. strain MG61, harbor chlorocatechol 2,3-dioxygenases, highly resistant to inactivation during 3-chlorocatechol turnover, sharing 97 % identical amino acids with the enzyme from strain GJ31, thus forming a distinct subfamily of catechol 2,3-dioxygenases.

Microbial degradation of 2-chlorobenzoates

Since the 1980s, different groups have succeeded in isolating bacteria capable of degrading 2-chlorobenzoate. The isolates could be grouped into those capable of degrading 2-chlorobenzoate (Zaitsev and Karasevich 1984; Engesser and Schulte 1989; Fetzner et al. 1989; Higson and Focht 1990; Suzuki et al. 2001) and those also capable of degrading either 2,4-dichloro- or 2,5-dichlorobenzoate (Hickey and Focht 1990; Hernandez et al. 1991; Kozlovsky et al. 1993; Romanov et al. 1993; Pavlu et al. 1999). All these organisms catalyze a 1,2-dioxygenation of 2-chlorobenzoate such that one of the *vic*-hydroxyl groups in the *cis*-dihydrodiol is bound to the same carbon as the chloro-substituent (Fig. 7). From such an unstable *vic*-dihydrodiol, the chloro-substituent will be spontaneously eliminated to form catechol (from 2-chlorobenzoate) or 4-chlorocatechol (from 2,4-dichloro- and 2,5-dichlorobenzoate), respectively. As the degradation of 4-chlorocatechol requires the presence of a chlorocatechol degradative pathway, a preliminary assumption is that organisms capable of degrading 2-chlorobenzoate only, and those capable of also degrading 2,4-dichloro- or 2,5-dichlorobenzoate, differ by the presence of a chlorocatechol pathway. However, two distinct 2-chlorobenzoate-degrading dioxygenase systems have been described. The plasmid-borne two-component 2-halobenzoate 1,2-dioxygenases (oxygenase consisting of α- and β-subunits and a reductase) from strain 2CBS (Fetzner et al. 1992; Haak et al. 1995) and TH2 (Suzuki et al. 2001), are similar to two component toluate (Harayama et al. 1991) and benzoate 1,2-dioxygenases (Cowles et al. 2000) of the family of benzoate dioxygenases (Gibson and Parales 2000) (Fig. 4).
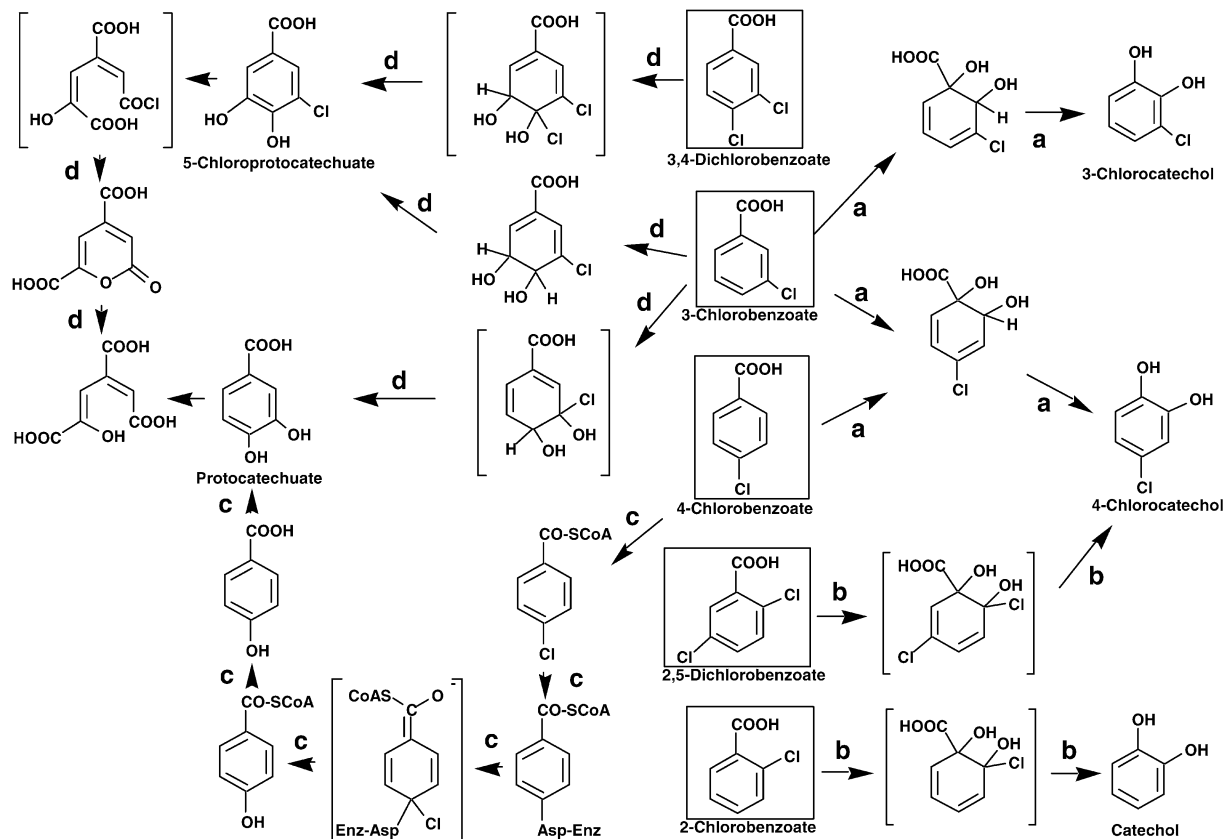
Fig. 7a–d Pathways for the degradation of chlorobenzoates. Initial metabolic steps of **a** 3- and 4-chlorobenzoate degradation involving benzoate or toluate 1,2-dioxygenase, **b** 2-chloro- and 2,5-dichlorobenzoate involving 2-halobenzoate 1,2-dioxygenase, **c** 4-chlorobenzoate involving hydrolytic dehalogenation, and **d** 3-chloro- and 3,4-dichlorobenzoate involving 4,5-dioxygenation are shown. Unstable intermediates are enclosed in brackets

These 2-halobenzoate dioxygenases are characterized by their high activity against 2-halosubstituted benzoates (Fetzner et al. 1992; Suzuki et al. 2001) but have negligible activity with 4-chloro-, 2,4-dichloro- or 2,5-dichlorobenzoate. In contrast, the 2-chlorobenzoate dioxygenase system of *P. aeruginosa* strain 142 is a three-component dioxygenase system (oxygenase consisting of α- and β-subunits, ferredoxin and reductase) (Romanov and Hausinger 1994). Moreover, the α-subunits of 2-chlorobenzoate dioxygenases of strains JB2 (Hickey and Sabat 2001) and 142 (Tsoi et al. 1999) exhibit only 22% sequence identity with that of strain 2CBS, but a significant similarity (42%) to salicylate 5-hydroxylase NagG from *Pseudomonas* sp. strain U2 (Fuenmayor et al. 1998) (Fig. 4) and putative biphenyl dioxygenase α-subunits from *S. aromaticivorans* F199 (Romine et al. 1999). Thus, 2-chlorobenzoate dioxygenases are functionally similar, but represent two different lineages with distinct activities.

The 4-chlorobenzoate hydrolytic pathway

The degradation of 4-chlorobenzoate by a pathway involving an early dehalogenation event, was indicated as early as 1976 (Ruisinger et al. 1976). Since then, various strains of different genera degrading 4-chlorobenzoate by

a similar mechanism have been isolated (Klages and Lingens 1979, 1980). The 4-chlorobenzoate dehalogenase system has been shown to consist of three distinct enzymes (Elsner et al. 1991) (Fig. 7). The activation of the substrate is carried out by an ATP-dependent 4-halobenzoate-coenzyme A ligase (Löffler and Müller 1991; Chang et al. 1992; Löffler et al. 1992), which shares significant sequence similarity with proteins catalyzing similar chemistry in the β-oxidation pathway (Babbitt et al. 1992). Dehalogenation is catalyzed by a 4-halobenzoyl-CoA dehalogenase, forming 4-hydroxybenzoyl-CoA (Liang et al. 1993; Löffler et al. 1995) in a hydrolytic substitution reaction. Substrate binding to the dehalogenase active site is followed by nucleophilic attack of the carboxylate side chain of Asp145 at the C4 of the benzoyl group (Yang et al. 1994; Crooks et al. 1995; Liu et al. 1995) to form a Meisenheimer intermediate (Dong et al. 2002). The chloride is displaced from this intermediate because of rearomatization of the benzoyl-group to form an enzyme-ester; hydrolysis of the ester generates the 4-hydroxybenzoyl-CoA product. The last step in the reaction to form 4-hydroxybenzoate is carried out by 4-hydroxybenzoate:coenzyme A thioesterase. The absence of serine, cysteine, or histidine catalytic residues distinguish this protein from all other thioesterases characterized to date (Babbitt et al. 1992; Benning et al. 1998). However,

crystallographic investigation revealed a high similarity of its three-dimensional structure to that of β-hydroxydecanoyl thiol ester dehydrase from *Escherichia coli* (Leesong et al. 1996) and, by superimposing the two structures, it has been possible to identify the putative active site region and to propose a catalytic mechanism whereby the carboxylate side chain of Asp17 activates a water molecule for subsequent nucleophilic attack at the thioester carbonyl carbon of the substrate (Benning et al. 1998).

The 3-chlorobenzoate 4,5-dioxygenase pathway

Another chlorobenzoate transforming enzyme system has been described in *Comamonas testosteroni* strain BR60 (Providenti and Wyndham 2001). 3-Chlorobenzoate is predominantly subject to 4,5-dioxygenation by CbaAB (3-chlorobenzoate 4,5-dioxygenase and reductase) (Nakatsu et al. 1995) to give 5-chloroprotocatechuate after CbaC-mediated dehydrogenation (Fig. 7). A minor amount of 3-chlorobenzoate is 3,4-dioxygenated, resulting in an unstable dihydrodiol, which spontaneously eliminates chloride to form protocatechuate (Nakatsu et al. 1997). 3,4-Dichlorobenzoate is exclusively dechlorinated to give 5-chloroprotocatechuate (Nakatsu and Wyndham 1993; Nakatsu et al. 1997). The oxygenase belongs to the phthalate family of oxygenases (Gibson and Parales 2000) (Fig. 4), comprising both mono- and dioxygenases, where the oxygenase subunit has an α_n subunit configuration (in contrast to other oxygenase systems, which consist of α- and β-subunits). 5-Chloroprotocatechuate serves as substrate for the protocatechuate 4,5-dioxygenase pathway in BR60. As described by Kersten et al. (1982, 1985), 2-pyrone-4,6-dicarboxylate is formed after ring-cleavage by nucleophilic displacement of a halide ion from 5-halosubstituted protocatechuates. Hydrolysis of the pyrone is followed by degradation through this *meta*-cleavage pathway.

Optimizing PCB degradation under environmental conditions

To overcome limitations due to poisoning caused by the production of toxic metabolites, the application of co-cultures and the construction of hybrid strains have been considered. Microorganisms capable of mineralizing some lower chlorinated biphenyls have been obtained by judicious combination of pathway segments comprising the biphenyl upper pathway, a benzoate/toluate or 2-chlorobenzoate dioxygenase system and a chlorocatechol pathway (Mokross et al. 1990; Havel and Reineke 1991; Hickey et al. 1992; McCullar et al. 1994), usually by in vivo conjugative mating of appropriate strains. Among these, the hybrids *B. cepacia* JHR22 and *Pseudomonas* sp. UCR2 have been reported to grow on the environmentally important 2-, 2,4- and 2,5-substituted PCB congeners (Havel and Reineke 1991; Hickey et al. 1992). Use of *Pseudomonas*-derived chlorobenzoate dehalogenase genes

like 4-chlorobenzoate dehalogenase or 2-chlorobenzoate dioxygenase resulted in highly efficient mineralization pathways, due primarily to circumvention of the formation of toxic chlorocatechols (Hrywna et al. 1999; Rodrigues et al. 2001). Actually, the use of hybrid organisms (Havel and Reineke 1993), or coinoculation with chlorobenzoate-degrading organisms could, to a certain extent, enhance PCB mineralization in environmental model systems (Hickey et al. 1993; Blasco et al. 1997).

Degradation in the rhizosphere

Microbial degradation of PCBs in the environment is influenced by various biological, chemical and physical factors as well as the survival of microorganisms in cases where bioaugmentation is the application of choice (Blumenroth and Wagner-Döbler 1998; Barriault et al. 1999; Ahn et al. 2001), and by the fact that higher chlorinated PCBs provide the organisms neither with energy nor with carbon to support the degradation process. The challenge for successful bioremediation of PCB-contaminated soil is to devise methods to encourage the growth of selected microorganisms that are capable of transforming PCBs.

It was speculated that certain plant-derived compounds, such as flavonoids, may serve as growth substrates for PCB-degrading bacteria and may induce *bph* genes. Donelly et al. (1994) showed a high PCB turnover capacity of biphenyl-degrading organisms after growth with certain flavonoids, suggesting that plant roots could serve as a natural injection system capable of dispensing cometabolites into the rhizosphere and inducing PCB degradation in indigenous microorganisms over long periods. Focht (1995) proposed that plant terpenes might stimulate PCB oxidation and, out of several terpenoid compounds, carvone, limonene and *p*-cymene induce PCB transformation activity in a biphenyl-degrading *Arthrobacter* strain (Gilbert and Crowley 1997), explaining why growth in a medium containing *Mentha spicata* (spearmint containing significant amounts of carvone) resulted in enhanced PCB metabolizing activity of that strain. Similar results were obtained with a PCB-transforming *P. stutzeri* isolate (Tandlich et al. 2001). Interestingly, the addition of orange peel, ivy leaves, pine needles or eucalyptus leaves to PCB-contaminated soil resulted not only in an enhanced PCB transformation activity, but also in significantly enhanced levels of biphenyl degraders compared to unamended soil (Hernandez et al. 1997), and biphenyl degraders isolated during such experiments could grow on terpenes. However, a clear relationship between terpene degradation and the ability to transform PCBs remains to be elucidated. Nevertheless, root exudates may maintain or induce expression of genes involved in PCB transformation.

As the majority of the microbial population found in the soil is associated with plant roots, the rhizosphere is the ideal site in which to modify microbial populations. *Pseudomonas fluorescens* F113, an important biocontrol strain for sugar beet, was chosen as host strain for *bph* genes

(Brazil et al. 1995). The modified organism colonized roots as effectively as the wild-type and *bph* genes were expressed in situ, indicating considerable potential for the manipulation of the rhizosphere as a useful strategy for bioremediation. In a complementary approach, it was shown that phenylpropanoids constitute 84% of the secondary metabolites exuded by *Arabidopsis* roots (Narasimhan et al. 2003). Comparison in a soil/plant system of a *Pseudomonas* strain that could efficiently colonize *Arabidopsis* roots, uses phenylpropanoids and is capable of transforming PCBs, with a mutant incapable of degrading phenylpropanoids showed significantly enhanced PCB degradation by the wild type.

Influence of surfactants on PCB degradation

PCBs are superhydrophobic, have extremely poor solubility in water and are poorly bioavailable. Microbes that utilize hydrophobic substrates often produce surface active substances, and the natural roles of biosurfactants have been claimed to increase the surface area of hydrophobic, water-insoluble growth substrates, increasing their bioavailability by increasing the apparent solubility or desorbing them from surfaces and regulating attachment and detachment of microorganisms to and from surfaces (Ron and Rosenberg 2001). A number of studies on the influence of surfactants on the biodegradation efficacy of PCBs have been made, including chemically produced surfactants (Billingsley et al. 1999), humic substances (Fava and Piccolo 2002) and biosurfactants such lipopeptides (Golyshin et al. 1999) or maltotriose esters (Ferrer et al. 2003). Whereas in some cases an increase in degradation rate was observed after addition of surfactants, in others, surfactants decreased degradation rates. On the one hand, addition of surfactants can provoke changes in community composition resulting in a decrease in the degrader population (Colores et al. 2000). On the other hand, as the addition of surfactants reduces bacterial adhesion to surfaces (Stelmack et al. 1999), the net effect of a surfactant on biodegradation depends on the benefits that result from enhanced solubility of target compounds versus the reduction in direct adhesion of bacteria to those compounds. However, how PCBs are degraded by microbial communities under environmental conditions is still poorly understood.

In attempts to study de novo development of PCB-degrading microbial communities, the formation of composite biofilms consisting of bacteria and clay minerals, in which the clay leaflets were arranged around the bacteria to form hutch-like structures, was observed (Lünsdorf et al. 2000). Obviously, the clay minerals performed an essential role in biofilm formation, probably acting as a nutrient shuttle, mediating transfer of PCBs in a palatable form to the bacteria, and it was suggested that clay minerals might fulfil a similar function in soil. Thus, the contrasting effects of surfactant application are a result of the poorly understood complexity of interactions between soil/sediment, pollutant, surfactant and microorganisms in different environments.

Concluding remarks

Significant advances have been made in recent years in the elucidation of the genetic and biochemical basis of aerobic degradation of PCBs, and detailed knowledge of the enzymatic steps and substrate specificity determinants of key enzymes as well as of different catabolic pathway segments necessary for mineralization is available. Although various approaches have been followed to optimize PCB degradation, e.g., by optimizing key enzymes or combination of pathway segments, it is obvious that such approaches are necessarily limited. Given the facts that (1) PCBs in the environment are complex congener mixtures, (2) different catabolic key enzymes have been identified as bottlenecks for each congener, and (3) optimization of a biocatalyst for a single congener often results in decreasing its performance on other congeners, it becomes increasingly evident that micobial communities have to become the focus of research. In contrast to single bacterial species, which are very unlikely to be able to deal with complex congener mixtures, microbial communities can be more versatile, and carbon sharing through complex metabolic interaction can increase PCB degradation. Gaining an understanding of PCB degradation will require integration of single organism studies with efforts to understand functioning of complex communities.

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