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Genetic construction of PCB degraders

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Abstract

Genetic construction of recombinant strains with expanded degradative abilities may be useful for bioremedation of recalcitrant compounds, such as polychlorinated biphenyls (PCBs). Some degradative genes have been found either on conjugative plasmids or on transposons, which would facilitate their genetic transfer. The catabolic pathway for the total degradation of PCBs is encoded by two different sets of genes that are not normally found in the same organism. The *bphABCD* genes normally reside on the chromosome and encode for the four enzymes involved in the production of benzoate and chlorobenzoates from the respective catabolism of biphenyl and chlorobiphenyls. The genes encoding for chlorobenzoate catabolism have been found on both plasmids and the chromosome, often in association with transposable elements. Ring fission of chlorobiphenyls and chlorobenzoates involves the *meta*-fission pathway (3-phenylcatechol 2,3-dioxygenase) and the *ortho*-fission pathway (chlorocatechol 1,2-dioxygenase), respectively. As the catecholic intermediates of both pathways are frequently inhibitory to each other, incompatibilities result. Presently, all hybrid strains constructed by in vivo matings metabolize simple chlorobiphenyls through complementary pathways by comprising the *bph*, benzoate, and chlorocatechol genes of parental strains. No strains have yet been verified which are able to utilize PCBs having at least one chlorine on each ring as growth substrates. The possible incompatibilities of hybrid pathways are evaluated with respect to product toxicity, and the efficiency of both in vivo and in vitro genetic methods for the construction of recombinant strains able to degrade PCBs is discussed.

Introduction

Environmental pollution from xenobiotic compounds, i.e. substrates of a nonbiological origin, has become an increasing public issue. Polychlorinated biphenyls (PCBs) comprise a group of especially persistent compounds that continue to be a major environmental problem, despite laws banning or curtailing their use and production. The biphenyl ring may be chlorinated at any or all of ten available sites varying in the number and position of the attached chlorines. The individual isomers and homologs are referred to as congeners. Their persistence in the environment is due to extreme chemical inertness, which is enhanced with increased chlorine substitution.

The complete destruction of extremely recalcitrant compounds, such as PCBs is brought about not by single microbial species, but by the action of consortia. Thus, it seems that bacteria able to utilize PCBs as sole carbon sources for growth do not exist in nature. Many of the earlier claims attesting to the isolation of PCB-utilizing microorganisms must be viewed as equivocal in light of the inability to meet all of the five criteria raised in a review by Focht (1993):

- the culture must be available,
- the growth media must be adequately described,
- utilization of the substrate must be verified beyond that of an exogenous substrate (e.g. yeast extract),
- mineralization (chloride release or $^{14}CO_2$ production) must be demonstrated, and
- composition of PCBs must be known (e.g. biphenyl is an occasional contaminant).

Natural isolates which grow on monochlorobiphenyls generally do not produce chloride as they utilize

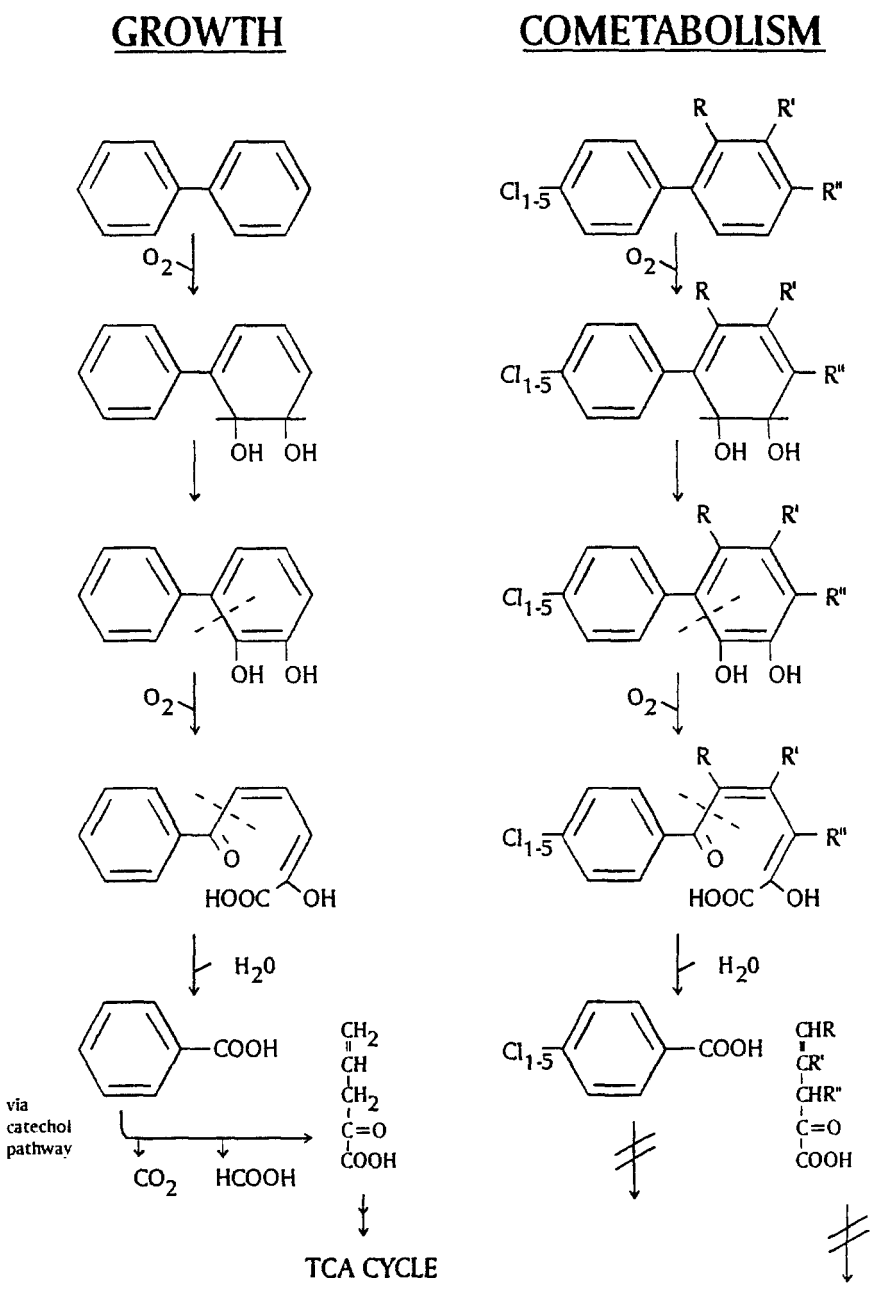

Fig. 1. Bacterial metabolism of biphenyl and cometabolism of PCBs. For brevity, the Cl1-5 designation refers to any number of chlorines that can be substituted on the ring not attacked by a dioxygenase. The ring being attacked by the dioxygenase is presumed to occur only where free 2- and 3- positions occur. R,R′,R″ = H or Cl. Redrawn from Focht (1987).

the carbon in the unsubstituted ring and produce chlorobenzoates as final products (Ahmed & Focht 1973). The exception to this rule has been reported by Barton & Crawford (1988) with *Pseudomonas* sp. strain MB86 that was isolated on 4-chlorobenzoate and that grew poorly on 4-chlorobiphenyl. However, 4-chloroacetophenone, which is formed as an intermediate from 4-chlorobiphenyl, was shown to be toxic. Other products generated from the degradation of PCBs are presumably chlorinated unsaturated aliphatic acids (Fig.1), based on analogy to the biphenyl pathway (Catelani et al. 1971; Omori et al. 1986). As these compounds have not been characterized, their fate is

unknown. Thus, complete catabolism of PCBs involves the action of pathways from three types of bacteria:

- biphenyl-utilizers, which cometabolize PCBs to chlorobenzoates and 5-C chloroaliphatic acids,
- chlorobenzoate-utilizers,
- those that dehalogenate 5-C chloroaliphatic acids.

Dechlorination of highly chlorinated congeners to lesser chlorinated products by anaerobic bacteria has been recently documented (Brown et al. 1989; Morris et al. 1992; Nies & Vogel 1990; Quensen et al. 1988; Quensen et al. 1990; van Dort & Bedard 1991). As these lesser chlorinated products are substrates for aerobic bacteria, the potential for complete mineralization of PCBs by sequential anaerobic-aerobic conditions is a possibility that has been reviewed by others (Abramowicz 1990; Boyle et al. 1992 & Focht 1993). In the review herein, we focus on genetic approaches leading to the construction of aerobic recombinant bacteria which can utilize chlorobiphenyls and their metabolic products (chlorobenzoates and chloroaliphatics) as sole carbon sources.

Construction of novel catabolic pathways

The construction of a new catabolic pathway involves:

- the combination of complementary pathways,
- changes in regulation, activity, or specificity of critical enzymes, and
- inactivation of unproductive branches in the new complementary pathway.

Aside from the first criterion, the precise nature of events leading to the creation of a new genotype is difficult to determine, particularly for pathways involving as many enzymatic steps as in PCB degradation (Fig.1). The advantages of construction of complementary catabolic pathways into a single strain, as opposed to microbial consortium, may be summarized by four points:

- no cosubstrate analog (e.g.biphenyl) is required for growth and enzyme induction,
- there is no competition for the growth substrate because indigenous bacteria cannot grow on PCBs,
- availability of cometabolic products (chlorobenzoates, chloroaliphatic acids) is not limited by diffusion, and
- growth of a consortium is more difficult to control than the growth of a single strain (Focht 1988).

Two strategies have been used for the construction of catabolic pathways by means of genetic manipulation in vitro. The first is based on the recruitment of isofunctional enzymes from other pathways to horizontally expand the substrate profile of an existing pathway (Lehrbach et al. 1984) or on the mutational alteration of the substrate specificities of existing key enzymes (Ramos et al. 1987). Recent work by Furukawa et al (1993) suggests that substrate specificity may be horizontally expanded by replacing only one subunit of a multi-enzyme complex. When the gene for the large subunit (*bphA1*) of biphenyl dioxygenase (*bphA1A2A3A4*) in *Pseudomonas pseudoalcaligenes* KF707 was replaced by the complementary gene from toluene dioxygenase (*todC1* from *Pseudomonas putida* F1), the resultant recombinant enzyme was able to attack toluene. Furthermore, this one discrete alteration conferred upon strain KF707 the ability to metabolize and grow on toluene. It is conceivable that a similar approach could be used to expand pathway specificities to include chlorinated substrates. The second strategy involves the vertical expansion of the pathway by incorporating genes coding for additional enzymes (Timmis et al. 1985). Once a catabolic pathway for a xenobiotic compound can be assembled, based on knowledge of related pathways or of the catabolic steps effected by all strains, the corresponding enzymes can be conceptually recruited into a single bacterium. This can be accomplished in some cases by simple mating (Reineke and Knackmuss 1979) or by cloning of the structural and regulatory genes for their insertion into the appropriate host organism (Timmis et al. 1988).

Catabolic plasmids and their stability

Conjugative catabolic plasmids have been isolated from a wide variety of environments, although the principal source has been soil (Sayler et al.1990). The process and effect of plasmid transfer is regulated by several factors:

- the frequency of transmissibility between two species,
- its stability and the maintenance in a new host,
- the expression level of genes introduced on a foreign plasmid into a new host, and
- the fitness of the host as a result of the new plasmid.

The frequency of plasmid transfer is generally higher on solid surfaces than in liquids, and more likely to occur between closely related isolates. The host range of catabolic plasmids is dependent upon their incompatibility group. Thus, plasmid pJP4, coding genes for the degradation of 2,4-dichlorophenoxyacetic acid, belongs to group Inc P-1 and can be transferred into

a large number of Gram-negative bacteria, including *Pseudomonas*, *Acinetobacter*, *Enterobacteriaceae*, and even into some Gram-positive bacteria (Thomas & Smith 1987). In contrast, catabolic plasmids belonging to groups Inc P-7 or Inc P-9 have a narrow host range and reside mainly in *Pseudomonas* (Jacoby 1986).

Plasmid stability and maintenance depends on the factors affecting plasmid replication and segregation (Nordstrom 1983). The regulation of plasmid partitioning provided by *par* loci was first identified by Meacock and Cohen (1980), and its importance for the stability of catabolic plasmids was emphasized by Keshavarz et al. (1985). The stability of catabolic plasmids was addressed by Ensley (1985) and Boronin et al. (1985), who concluded that plasmids would be a burden for the host cell unless they encoded environmentally advantageous functions. In some cases, mutational changes, gene rearrangements, and deletions in catabolic plasmids are observed in response to changed environmental conditions, such as growth substrates (Chatterjee & Chakrabarty 1982, Wyndham et al. 1988, Brenner et al. 1993). Mutations are often necessary when transconjugants contain incompatibilities between the complementary catabolic pathways contributed by the two parental strains (Jeenes et al. 1982, Reineke et al. 1982, Hartmann et al. 1989, Havel & Reineke 1991). This point is discussed in more detail later.

The development of novel transposon and plasmid-based broad-host-range expression systems has facilitated analysis of gene products and construction of new activities in Gram-negative bacteria (de Lorenzo et al. 1993). Moreover, a large number of cloning vectors, derived mainly from broad-host-range plasmids belonging to incompatibility groups P-1 (RK2, RP1, R68), Q (RSF1010, R1162), and W (pSa), have been constructed (Bagdasarian & Timmis 1982, Friedmann et al. 1982, Mermod et al. 1986, Itoh & Haas 1985, Barry 1988, Rothmel et al. 1991). In vitro construction of new hybrid pathways has some advantages over conventional mating methods. First, specific and well-characterized cloned DNA fragments are used, which enables the investigator to assess the contribution of individual genes, or combinations of genes. Second, genetic changes for which there is no obvious selection procedure can be effected in a single step. Third, the application of sophisticated expression vectors with regulated strong promoters may enhance the production of desired enzymes by degradative bacteria.

Standard mating procedures

The combination of conjugation and spontaneous mutation on agar plates has been used by Reineke & Knackmuss (1979, 1980) to prepare strains utilizing various chlorobenzoates for growth. The TOL plasmid from *Pseudomonas putida* mt-2 was transferred into a 3-chlorobenzoate degrader, *Pseudomonas sp.* B13, which acquired the ability to utilize 4-chlorobenzoate and 3,5-dichlorobenzoate. The initial oxidation of the chlorinated benzoates via the chlorodihydrodihydroxybenzoate to chlorocatechol intermediates was effected by enzymes encoded by the TOL plasmid, whereas total degradation was accomplished by the *clc* genes encoding chlorocatechol metabolism in strain B13 (Reineke et al. 1982, Jeenes et al. 1982). In addition, exconjugants were observed, in which *P. putida* mt-2 acquired the genes coding for the enzymes of chlorocatechol metabolism. Ramos et al. (1986) suggested that the substrate specificity of the positive regulatory gene *xylS* from the plasmid pWWO was responsible for the inability of 4-chlorobenzoate utilizer *Pseudomonas* sp. WR216 to use 3,5-dichlorobenzoate. 3,5-Dichlorobenzoate was unable to activate the non-mutated *xylS* gene, and thus did not induce synthesis of the TOL plasmid-encoded toluate 1,2-dioxygenase. The authors were able to confirm their theory by introducing the mutated *xylS*352 allele with altered effector specificity by conjugation with strain WR216. Thus, all transconjugants carrying plasmid pERD352 with the mutant allele were able to grow on 3,5-dichlorobenzoate.

The introduction of plasmid-encoded catabolic pathways by conjugation has become a favored method in the construction of pathways for the catabolism of many other chlorinated aromatic compounds, such as chlorophenols (Schwien & Schmidt 1982), chloroanilines (Latorre et al. 1984), chlorobenzenes (Oltmanns et al. 1988), chloronaphthalenes (Weightmann et al. 1984), chlorotoluenes (Brinkmann & Reineke 1992), and chlorobiphenyls (Mokross et al. 1990, Havel & Reineke 1991).

In another study, Springael et al. (1993a) used intraspecific conjugation in *Alcaligenes eutrophus* for the construction of hybrids which degraded various PCB congeners in the presence of heavy metals. This topic is further discussed in another chapter of this issue (Springael et al. 1994).

Selection in the chemostat

Although the chemostat has classically been used to study the kinetics of bacterial growth and metabolism, it has been a very useful tool in isolation of xenobiotic-degrading bacteria. In some cases, the selection process leads to the establishment of stable consortia (Senior et al. 1976; Slater 1985), while in others it may lead to the isolation of pure cultures. The latter example is characteristic of more biodegradable compounds, and presumably forms the basis for the enrichment culture procedure by the competitive exclusion principle (Focht 1993). If no organism able to utilize a recalcitrant compound can be isolated, the chemostat may be run initially with a metabolizable analogue, which is progressively replaced by the recalcitrant compound, in the hope of selecting spontaneous mutants containing an enzyme of broader substrate specificity. Horizontal expansion of a catabolic pathway by use of the chemostat was first demonstrated by Dorn et al. (1974): the gradual replacement of benzoate, in the nutrient influx, by 3-chlorobenzoate led to the eventual selection and isolation of the strain *Pseudomonas* sp. B13, which utilized 3-chlorobenzoate as a growth substrate.

Another application of the chemostat came about by the discovery that catabolic genes were transmissible. Hartmann et al. (1979) mixed cultures of *P. putida* mt-2, a toluate-degrader, with *Pseudomonas* sp. B13, a 3-chlorobenzoate-degrader, in a chemostat that was initially fed with 3-chlorobenzoate and 4-toluate. The concentrations of these two substrates were progressively diminished while the concentration of 4-chlorobenzoate (during the first 2 months) and 3,5-dichlorobenzoate (during the subsequent 3 months) was progressively increased. Within 6 months of continuous culture, *Pseudomonas* sp. WR912 was isolated and able to utilize 3-chlorobenzoate, 4-chlorobenzoate, and 3,5-dichlorobenzoate. The genetic mechanism was elucidated in independent studies by Chatterjee et al (1981) and Chatterjee & Chakrabarty (1982) in which similar variants were obtained from matings with a 3-chlorobenzoate utilizer, harboring plasmid pAC25, and an undescribed pseudomonad, harboring a TOL plasmid. *Eco*RI restriction digests revealed that transconjugants utilizing 4-chlorobenzoate contained plasmid pAC27, derived from plasmid pAC25 by a deletion of a 11kb. However, the ability to utilize 4-chlorobenzoate was due to transposition of part of the TOL plasmid, which conferred broader benzoate dioxygenase activity, from the donor to the chromosome of the recipient. The possibility that the 11kb fragment was associated with the transposon and not the structural genes was evident upon further enrichment and selection of the 4-chlorobenzoate utilizing cells in the presence of 3,5-dichlorobenzoate. A variant which grew on 3,5-dichlorobenzoate contained plasmid pAC31, which was identical in size and *Eco*RI restriction profile as plasmid pAC27. The authors suggested that plasmid pAC31 had been generated by a homologous recombination of the plasmid encoded segment coding the modified enzymes dienelactone hydrolase and maleylacetate reductase, which are specific only for the degradation of 3,5-dichlorobenzoate as neither of the two chlorinated metabolites are formed from monochlorocatechol degradation.

A similar conversion to a new phenotype was recently reported by continuous subculture of the chlorobenzoate-degrader *Pseudomonas putida* P111 on progressively lower and higher concentrations of 3-chlorobenzoate and 3,5-dichlorobenzoate, respectively (Brenner et al. 1993). The original strain contained plasmid pPB111, which coded for an *ortho*-chlorobenzoate dioxygenase, while the genes for the benzoate and 3-chlorobenzoate pathways were chromosomally coded. A new phenotypic variant, designated P111D, grew on 3,5-dichlorobenzoate as a sole carbon source and contained a new plasmid pPH111, which was derived from the wild-type plasmid pPB111 by a set of rearrangements, including deletion, and transposition. However, this organism still retained the ability to utilize 3-chlorobenzoate despite the transposition of the *clc* operon from the chromosome of the strain P111 to the plasmid pPH111.

One important aspect of a chemostat is that the organism within it represents an evolving system. Dykhuizen & Hartl (1983) have summarized the effects that such events as periodic selection, wall growth, and recombinations between variants have on the evolution of chemostat-derived strains. The most important implication is that mutation or selection rates can not be assumed to be constant for more than the first 100 to 200 hours of chemostat culture. Thus, the possibility of evolutionary divergence among replicate chemostats is extremely high, especially if the preadaptation conditions of strains, before placing them in the chemostat, are not identical.

Continuously amalgamated culture (CAC)

As a result of sexual incompatibility or of insufficient cell density of the requisite organisms, the frequency

of genetic exchange in the chemostat may be too low and the selection pressure inadequate to bring about the creation of the new progeny in a reasonable time period. Levin et al. (1979) found the rates of plasmid transfer in the chemostat to be 100-fold smaller than in exponential-phase batch cultures. The isolation of recombinant strains from the chemostat usually requires long time periods (7–13 months), as noted in all cases involving the selection of chlorobenzene-degraders (Reineke & Knackmuss 1984, De Bont et al. 1986), probably because of the low frequency of genetic exchange. Kröckel & Focht (1987) introduced an enhanced mating system, in which chlorobenzene-degraders were isolated within 3 weeks. The system consisted of two chemostats, in which each parental strain was grown separately, and where the effluent from each was amalgamated onto a column containing ceramic beads. The importance of the ceramic beads and column is most likely related to the following characteristics:

- cell density is increased on surfaces, which increases the frequency of cell-to-cell contact,
- washout of parental and recombinant strains is decreased,
- as growth substrate becomes depleted down the column selection pressure of recombinants is enhanced by introduction of chlorobenzene from the bottom of the column.

During the process of CAC, plasmid and chromosomal DNA released from the cells which did not adapt to the novel substrate is bound to the surfaces of ceramic beads in the column. This DNA is resistant to nucleolytic degradation (Lorenz & Wackernagel, 1987), and therefore is available for the transformation of a naturally competent Gram-negative bacteria (Chamier et al.1993). Thus, the reaction bed column in the CAC serves as an enhancer of genetic exchange for conjugation and transformation.

Genes involved in chlorobenzoate metabolism

The aerobic catabolism of chlorobenzoates can be grouped into three major pathways, which all converge at some point at the TCA cycle. The most studied pathway involves the metabolism of 3-chlorobenzoate, which is also relevant to 4-chlorobenzoate and some isomers of di- and trichlorobenzoates and proceeds through chlorocatechols. The pathway involving the hydrolytic dehalogenation of 4-chlorobenzoate to 4-hydroxybenzoate is distinctly different from the one

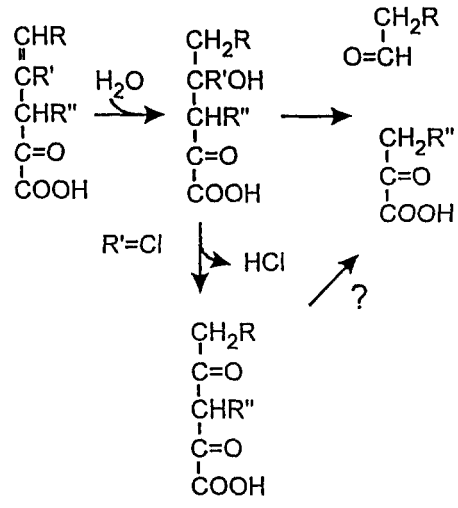

Fig. 2. Hypothetical metabolic pathway for chlorinated 5-C aliphatic acids produced from cometabolism of PCBs (Fig. 1). See text for an explanation of the two possibilities regarding the 4-chloroaliphatic acid. R,R′,R″ = H or Cl

proceeding through 4-chlorocatechol, and is covered in another chapter of this volume (Dunaway-Mariano & Babbitt 1994). Finally, the pathway involving the dioxygenolytic removal of chlorine from 2-chlorobenzoates also shares some commonality with the 3-chlorobenzoate pathway.

In order to fully appreciate the nomenclature of genes involved in chlorobenzoate catabolism, it is necessary to consider for each characterized operon the growth substrate on which the respective organism was isolated. The *clc* genes, which code for the 3-chlorocatechol pathway, were first described for bacteria growing on 3-chlorobenzoate (Chatterjee & Chakrabarty 1984). Note that the genes coding for conversion of 3-chlorobenzoate to catechol were not considered in the initial description. Thus, A, B, C, and D refer specifically to the *clc* genes which code for metabolism of 3-chlorocatechol, 2-chloromuconate, unidentified function, and carboxymethylenebutenolide (Fig. 3). In contrast, the *tfd* genes, which code for 2,4-D catabolism (Don et al. 1985), were characterized with A and B representing the genes coding for the ether hydrolase and the 3,5-dichlorophenol monooxygenase, respectively. The genes coding for metabolism of 3,5-dichlorocatechol, 2,4-dichloromuconate, and 2-chloro-4-carboxymethylene-butenolide were designated as C, D, and E, respectively. The *tcb* genes (van der Meer et al. 1991a,b) were characterized from *Pseudomonas* sp. P51, which was grown on 1,2,4-trichlorobenzene. Thus, comparison between the *clc*

and *tfd* (or *tcb*) genes coding for the three enzymes in the catechol pathway would be A vs C, B vs D, and D vs E, respectively.

3-Chlorobenzoate metabolism

The genes coding for chlorocatechol degradation (*clc*) have been cloned by Chatterjee & Chakrabarty (1984) from plasmid pAC27 (*Pseudomonas putida* AC866) into a broad host-range vector pLAFRI . The recombination of the chlorocatechol degradative genes on the chromosome of a*rec A* mutant occurred after the introduction of an incompatible plasmid pAC8, which suggests that these genes are transposable. Ghosal et al. (1985) subcloned the *clc* genes from plasmid pAC27 on a 4.2kb fragment, which was homologous to a 10kb DNA segment containing *clc* genes from plasmid pJP4, isolated from *A. eutrophus*. In spite of the detectable sequence homology in the structural genes on both plasmids, the regulation of their expression was different. The *clc* genes in plasmid pAC27 were readily expressed when transferred to other *Pseudomonas* species, while the same set of genes on pJP4 behaved like a cryptic operon that was activated occasionally through the mechanism of complex sequence rearrangements such as insertion, deletion-fusion, and other types of illegitimate recombination. The authors suggested that stem-loop structures present on plasmid pJP4 were most likely the starting points for such rearrangements.

Much of the genetic data on 3,5-dichlorobenzoate catabolism and its similarity to 3-chlorobenzoate catabolism originates from studies on 3,5-dichlorocatechol, where both the 2,4-D and 3,5-dichlorobenzoate pathways converge. The degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) in *A. eutrophus* JMP134 is coded on plasmid pJP4 (Don et al. 1985). When a cloned fragment containing the first three genes of chlorocatechol degradation (*tfdC, tfdD, tfdE*) was added to a strain cured of plasmid pJP4, growth on 3-chlorobenzoate occurred, no growth occurred on 2,4-D, and a new smaller plasmid was evident as a result of the transfer. Growth of the strain was unaffected by the transposon-mediated inactivation of the fourth gene (*tfdF*), adjacent to the gene encoding dienelactone hydrolase (*tfdE*), as the gene product 2-chloromaleylacetic acid dehydrogenase is irrelevant to 3-chlorocatechol degradation. Presumably, the recombinant strain would be unable to mineralize 3,5-dichlorocatechol. Genes specifying the utilization of 3-chlorobenzoate in *Pseudomonas* sp. strain B13 WR1 were cloned on a fragment less than 11 Kb by Weisshaar et al. (1987) . A number of clones were identified which enabled both *Escherichia coli* and *A. eutrophus* to grow on 3-chlorobenzoate, albeit poorer than strain WR1. The authors concluded that poor expression of genes in the host or the absence of the regulatory region on the cloned fragment retarded growth of these recombinants.

The organization and the complete nucleotide sequence of the *clc* gene cluster was first reported by Frantz & Chakrabarty (1987). They found three structural genes and a ribosomal binding site on a 4.2kb fragment, while the promoter for the structural genes was detected on an adjoining 385bp segment. The nucleotide sequence of both fragments cloned from plasmid pAC27 revealed four major open reading frames (ORFs). The first ORF revealed a nucleotide sequence identical to the total amino acid composition of purified chlorocatechol 1,2-dioxygenase (Ngai & Ornston 1988), which acts on 3-chlorocatechol. The termination codon for the *clcA* gene overlapped the initiation codon of a second major ORF, which by agreement with N-terminal amino acid (AA) sequence analysis and the total AA composition of the purified protein, was designated as the *clcB* gene, encoding chloromuconate cycloisomerase. A third major ORF did not produce a polypeptide in *E. coli*, nor was any enzyme function identified. The N-terminal amino acid sequence of the translated fourth ORF matched the N-terminal sequence of purified dienelactone hydrolase from *Pseudomonas* sp. B13, and therefore this ORF was designated as the *clcD* gene.

The regulatory gene *clcR* is divergently oriented on the opposite strand upstream of the *clc* structural genes. The transcription initiation site showed considerable similarity to promoters of the *nah*, *sal*, and *xyl* operons in the region −10. The promoter of the *clcABD* cluster was also shown to contain the consensus proposed for positively regulated operons (Sangodkar et al. 1989). In other studies, Ghosal & You (1988, 1989) sequenced chlorocatechol genes *tfdCDE*, originating from the 2,4-D degradative plasmid pJP4, and showed an overall homology of 63% between proximal genes (*clcA* vs *tfdC*; *clcB* vs *tfdD*) and 50% homology between the sequences of the genes *clcD* vs *tfdE*. Their results were confirmed in a separate study by Perkins et al. (1990), who described yet another sequence of a fourth gene *tfdF*, which they postulated as encoding *trans*-chlorodienelactone isomerase activity. As the *cis*-chlorodienelactone (Fig. 3) is formed directly from 2,4-dichloro-*cis,cis*-muconate (Schlomann et al.

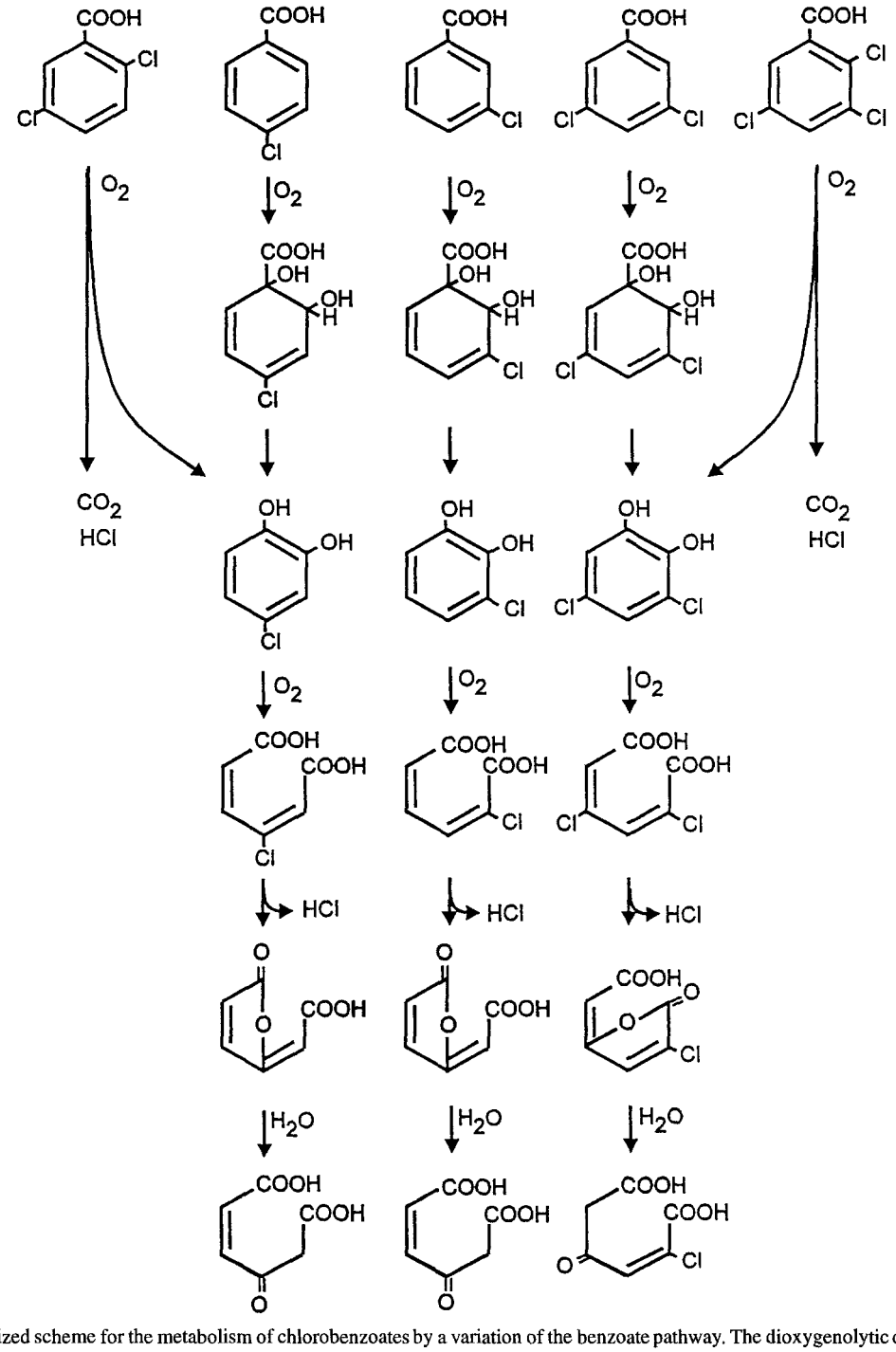

Fig. 3. Generalized scheme for the metabolism of chlorobenzoates by a variation of the benzoate pathway. The dioxygenolytic dehalogenation of *ortho* chlorobenzoates, which spontaneously forms the corresponding catechol is carried out by a different enzyme than the benzoate dioxygenase, which results in the creation of unstable carboxydiols. Chlorocatechol portions of the pathway are redrawn from Reineke (1984).

1990), it appears more likely that *tfdF* encodes a protein with 2-chloromaleylacetic acid reductase activity.

The organization and the nucleotide sequence of the chlorocatechol gene cluster *tcbCDEF* from *Pseudomonas* sp. P51 has been compared with both *clcABD* genes from pAC27 and *tfdCDEF* genes from pJP4 by van der Meer et al. (1991a, 1991c). They found the gene *tcbF* 55% homologous to the *tfdF* gene but

detected no *trans*-chlorodienelactone isomerase activity. The regulatory genes *clcR* and *tcbR* were both positively regulated and transcribed divergently from the operons they control in such a way that the promoters overlap (van der Meer et al. 1991b). In contrast, the *tfdCDEF* operon is controlled by a negative regulatory gene *tfdR*, which acts as an activator in the presence of inducers such as 2,4-D, 2,4-dichlorophenol, and 4-chlorocatechol.

The transposable nature of *clc* operons has been recognized in several independent studies by Chatterjee & Chakrabarty (1984), Wyndham et al. (1987), Weisshaar et al. (1987), and Ghosal &You (1989). Nakatsu et al. (1991) have shown that genes allowing strain *Alcaligenes* sp. BR60 to grow on 3-chlorobenzoate and 4-chlorobenzoate are located on a class II 17kb transposon Tn*5271* that resides in the plasmid or chromosome of this strain. The transposon was flanked by a directly repeated 3.2kb sequence which was in addition flanked by 110bp of inverted repeats. The 3.2kb sequence, designated IS*1071*, was found in multiple copies in the genome of *Alcaligenes* sp. BR60 and was shown to be involved in the recombination of the catabolic genes into the chromosome of this strain. The transfer of the *clc* operon from the chromosome into the plasmid was also observed during continuous culture of variants of *P. putida* P111, which had different abilities for utilization of chlorobenzoate congeners (Brenner et al. 1993).

4-Chlorobenzoate catabolism

Metabolism of 4-chlorobenzoate by dioxygenation (Fig. 3) to the corresponding dihydrodiol and to 4-chlorocatechol is governed by the same genes involved in the metabolism of 3-chlorobenzoate. The other pathway via the hydrolytic dehalogenation to 4-hydroxybenzoate is covered in detail in another chapter of this issue . However, it is pertinent to note that 3,4-dichlorobenzoate, which has been identified as a cometabolic product of 3,3′, 4,4′-tetrachlorobiphenyl (Adriaens & Focht 1990), the most carcinogenic PCB congener, is hydrolytically dehalogenated in two steps by *Acinetobacter* sp. 4-CB1 to 3-chloro-4-hydroxybenzoate and 4-carboxy-1,2-benzoquinone (Adriaens et al 1989, Adriaens & Focht 1991a). The organism grows on 4-chlorobenzoate and 3-chloro-4-hydroxybenzoate, but can not grow on 3,4-dichlorobenzoate because neither it nor its product induce for its catalysis (Adriaens & Focht 1991b). The dehalogenase is induced only in the presence of 4-chlorobenzoate.

2-Chlorobenzoate catabolism

The molecular genetics of 2-chlorobenzoate degraders is not clearly understood as the gene involved in the initial dioxygenation has not been cloned yet. Generally, the enzyme involved in this reaction preferentially attacks 2-substituted benzoates (Fetzner et al. 1989, Hartmann et al. 1989, Hickey & Focht 1990, Sylvestre et al. 1989, Zaitsev & Karasevich 1984). Fetzner et al. (1992) recently isolated a two-component dioxygenase which exhibited broad substrate specificity with preference for benzoate analogs with electron-withdrawing substituents at the *ortho* position. Hernandez et al. (1991) proposed that *P. putida* P111 metabolized all *ortho*-chlorobenzoates to the corresponding catechols by dioxygenation and spontaneous liberation of CO_2 and HCl through an unstable chloro-carboxydiol. The existence of two separate benzoate dioxygenases in strain P111 could be observed by the inhibitory effect that 3,5-dichlorobenzoate had upon growing cells and resting cells in the presence of *ortho*-chlorobenzoates: in the latter case, cells grown on 2,5-dichlorobenzoate metabolized 3,5-dichlorobenzoate to completion prior to metabolizing 2,5-dichlorobenzoate. The existence of the two separate benzoate dioxygenases was confirmed in a later study with mutants (Brenner et al. 1993). The benzoate dioxygenase, that converts 3-chlorobenzoate, 4-chlorobenzoate and benzoate to the corresponding catechols via reduction of a dihydrodiol, was shown to be chromosomally coded, whereas the chlorobenzoate 1,2-dioxygenase, that converts *ortho*-chlorobenzoates to the corresponding catechols without the need of a functional dioldehydrogenase, was shown to be encoded on plasmid pPB111.

The biphenyl pathway

The pathway for the oxidative catabolism of biphenyl has been well studied (Catelani et al. 1971). Biphenyl is converted to benzoate by the action of four enzymes encoded by the *bphABCD* operon. The reaction sequence (Fig. 1) proceeds from biphenyl to 1-phenyl-2,3-dihydrodiol, 2,3-dihydroxybiphenyl (also referred to as 3-phenylcatechol), 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, and finally to benzoate + 5-C aliphatic acid. (A more detailed account of these genes can be found in another chapter of this

volume by K. Furukawa 1994). The fate of benzoate and the aliphatic acid produced through this pathway has not been well studied. In related pathways, benzoate is generally converted to catechol, which is then oxidized via the *ortho* or *meta* fission pathways. *Acinetobacter* sp. strain P6 oxidizes catechol by *meta* pyrocatechase, although it also contains *ortho* pyrocatechase (Furukawa & Chakrabarty 1982). Catechol 2,3-dioxygenases, which act on catechol, have been characterized and cloned from biphenyl-degraders (Selifonov & Starozoitov 1990; Chang et al. 1992), but the importance of this enzyme to biphenyl/PCB metabolism (i.e. 3-phenylcatechol dioxygenase) and to the lower pathway of benzoate metabolism is unclear.

In natural isolates that have been characterized, expression of the *bph* operon requires induction by growth on biphenyl. In contrast, most recombinants constitutively express the *bph* operon without the need for biphenyl induction (Furukawa & Miyazaki 1986; Kimbara et al. 1989; Mondello 1989; Kahn & Walia 1989). However, recombinant pseudomonads containing *bph* genes from *P. testosteroni* B-356 that were constitutively expressed, showed higher activity with 4-CBP than in its absence (Ahmad et al. 1990).

The genetic diversity of biphenyl-degraders appears to be comprised of at least two characterized groups, which also have distinct differences with respect to preferential oxidation of PCB congeners (Yates & Mondello 1989).

Genetic recombination of the BP and chlorobenzoate pathways

The starting point for the construction of a PCB-degrader is the isolation and characterization of genes which can effect transformation of PCB congeners. Many biphenyl-degrading isolates can transform PCBs (Ahmed & Focht 1973; Furukawa et al. 1978; Bedard et al 1986), utilize monochlorobiphenyls as sole carbon sources, yet cannot mineralize chlorobenzoates. Cocultures of biphenyl and chlorobenzoate-degraders can mineralize some PCB congeners (Adriaens et al. 1989). Thus, a single organism capable of mineralizing PCBs might be constructed by pooling biphenyl and chlorobenzoate genes, a concept first advanced by Furukawa and Chakrabarty (1982). Presently, the in vitro combination of biphenyl and chlorobenzoate genes for construction of PCB-utilizing strains has not been reported. However, the cloned *bphABCD* operon from *P. putida* KF715 was placed in a broad-host-range vector, introduced into benzoate-utilizers (Hayase et al. 1990), and the resulting recombinants, *P. aeruginosa*, *P. putida*, and *Achromobacter xerosis*, were able to utilize biphenyl. Thus, the introduction of the *bphABCD* operon into chlorobenzoate-utilizers would be the next logical step. There may be reasons, however, why this may be problematical, as all chlorobiphenyl-utilizing recombinants constructed from mating experiments with biphenyl and chlorobenzoate-utilizing parental strains, can not utilize biphenyls containing a chlorine substituent on both rings.

Hybrid chlorobiphenyl degraders

Simple mating procedures between a biphenyl-degrader, *P. putida* BN10, and a 3-chlorobenzoate-degrader, *Pseudomonas* sp. B13, were used to construct strains growing on 3-chlorobiphenyl and releasing 90% inorganic chloride (Mokross et al. 1990). The direction of genetic transfer was variable as some hybrid strains were more closely related to the biphenyl-utilizer, while others were more related to the chlorobenzoate-utilizer. Havel & Reineke (1991) combined mating on millipore filters with a 2-chlorobenzoate-utilizer, *P. cepacia* JH230, and a biphenyl-degrader, *P. putida* JHR to obtain the recombinant strain JHR2 which grew on all monochlorobenzoates, and 3- and 4-chlorobiphenyl, yet not on 2-chlorobiphenyl. Similarly, the hybrid strain utilized 2,4- and 3,5-dichlorobenzoate, yet would not grow on either 2,4- or 3,5-dichlorobiphenyl. However, growth on 4-chlorobiphenyl induced for catabolism of 2-chlorobiphenyl as noted by greater cell yields than when the latter compound was absent. Moreover, successive transfers into fresh media containing only 2-chlorobiphenyl eventually resulted in rapid growth and utilization of that substrate and also 2,4- and 3,5-dichlorobiphenyl.

Hickey et al. (1992) reported the mineralization of 2-chlorobiphenyl and 2,5-dichlorobiphenyl by *Pseudomonas* sp. UCR2, which was formed (via the CAC apparatus) by transfer of biphenyl genes from *Arthrobacter* sp. B1Barc into the 2,5-dichlorobenzoate utilizer *Pseudomonas aeruginosa* JB2. The hybrid strain also cometabolized 2,2′- and 2,3′-dichlorobiphenyl, but the rates were 10% of that observed for 2,5-dichlorobiphenyl. The CAC apparatus was also used in an intergeneric mating between a biphenyl-utilizer *Acinetobacter* sp. P6 and the 3-chlorobenzoate utilizer *Pseudomonas sp.* HF1

to produce a hybrid strain able to mineralize 3-chlorobiphenyl (Adams et al. 1992). The hybrid strain *Pseudomonas* sp. CB15 was genetically and phenotypically more similar to the chlorobenzoate-utilizing parent and grew on both 3-chlorobenzoate and 3-chlorobiphenyl. However, it would not grow on 3,3′-dichlorobiphenyl.

Incompatibilities of pathways

The basic problem in the construction of a microorganism with the complete chlorobiphenyl pathway is the incompatibility of the *meta* and *ortho* cleavage pathways, the simultaneous functioning of which usually creates suicidal products (Reineke et al.1982). Specifically, *meta*-fission of 3-chlorocatechol produces an acylchloride, which irreversibly inactivates the catechol 2,3-dioxygenase (Bartels et al. 1984). Naturally occurring catabolic plasmids which encode for genes participating in the degradation of PCBs (Table 1) possess either the *meta* or *ortho* cleavage part of the pathway, but never both. In the case of 3-CBP, there are two benzene rings which are ruptured chronologically by *meta* cleavage (3-phenylcatechol) and by *ortho* cleavage (chlorobenzoate). Not surprisingly, inhibition of both *ortho* and *meta* fission reactions was noted during growth of hybrid strain CB15 on 3-CBP (Fig.4). 3-Chlorocatechol inhibited *meta* fission of 3-phenylcatechol, and 3-phenylcatechol inhibited *ortho* fission of 3-chlorocatechol (Adams et al. 1992). Moreover, the accumulation of 3-chlorocatechol led to the formation of black-colored polymeric products. However, the stoichiometry of chloride release was 87% of the substrate. Other hybrid strains which grow on chlorobiphenyls release close to stoichiometric equivalents of chloride, and simultaneously induce *meta* and *ortho* cleavage enzymes (Mokross et al. 1991, Havel & Reineke 1991, Adams et al. 1992), so it would appear *prima facie* that the possession of both *ortho* and *meta* fission activity is not problematic.

The greatest deficiency of knowledge relevant to PCB degradation and the construction of PCB-degraders is the fate of the 5-carbon chlorinated fragment that is produced by the conversion of PCBs to the corresponding chlorobenzoates. The hypothetical pathway of the 5-C fragment (Fig.2) is based on the classic *meta* fission pathway (Dagley et al 1964), which may not always be applicable. The only reported case of a productive *meta* fission pathway with a chlorinated substrate was reported in *Pseudomonas cepacia* MB2: 3-chloro-2-methylbenzoate is converted via the carboxyhydrodiol to 4-chloro-3-methylcatechol, which is processed through the *meta* fission pathway to pyruvate, acetate, and chloroacetaldehyde (Higson & Focht 1992). The latter compound is apparently converted to chloroacetate, which also serves as a growth substrate. Thus, the fate of 2-oxo-3-chloropenta-4,5-dienoate (Fig. 2) via the *meta* fission pathway would eventually generate chloroacetaldehyde and/or chloropyruvate, depending on the PCB congener. In the case of a 4-chloropentadienoate (originating from oxidation of 3,3′-CBP), the instability of two electron withdrawing groups would cause instantaneous dehalogenation to give the hypothetical pentanoic acid shown in Fig. 2.

In fact, any 5-C chlorinated acid having a 3- or 5-Cl substitution would eventually lead to chloroacetate, as shown in Fig. 2, and dehalogenation of this compound would be essential before entering the TCA cycle or fatty acid biosynthesis. Not surprisingly, the only documented case of a productive *meta* fission pathway for a chlorocatechol occurs with an organism able to utilize chloroacetate as a growth substrate (Higson & Focht 1992). Moreover, the ability of biphenyl-utilizers to utilize chloroacetate is rare (1 in 44 strains, representing 11 genera; B. Hernandez, unpublished data) in contrast to growth on other chloroaliphatic acids. Thus, chloroacetate-degradation may be central to the fate of chlorinated *meta* ring fission products.

In recognition of possible problems regarding toxicity or inertness of chlorinated aliphatic acids, we recently combined the genes of the hybrid strain CB15 (a 3-CBP-utilizer) with those from *Pseudomonas acidovorans* CC1 (formerly described as *Alcaligenes* sp. CC1 by Kohler & Kohler-Staub (1990), which utilizes *cis*- and *trans*-3-chlorocrotonate. The original rationale was based on the premise that this compound was the only one commercially available that resembled the hypothetical intermediate shown in Fig. 2. The resulting recombinant *P. acidovorans* M3GY grew on 3,4′-DCBP. Hybrid strains cured of the plasmid encoding for chlorocrotonate degradation were able to utilize 3,4′-DCBP, and released over 75% as chloride. As the mutants were able to utilize chloroacetate but not chlorocrotonate, it would appear that the critical genes obtained from the CC1 parental strain were those encoding for chloroacetate dehalogenation.

Metabolic interferences to PCB mineralization

The assumption that the four gene products of the biphenyl pathway will fortuitously catalyze the same

Table 1. Plasmids relevant to chlorobiphenyl degradation*

Substrate	Plasmid	kb	Bacterial strain	Reference
2-CB	pPB111	75	*P. putida* P111	Brenner et al. (1993)
3-CB	pAC25	117	*P. putida* AC858	Chatterjee & Chakrabarty (1983)
3-CB	pB13	111	*Pseudomonas* sp. B13	Chatterjee & Chakrabarty (1983)
3-CB	pBR60	85	*Alcaligenes* sp. BR60	Wyndham et al. (1988)
3-CB	pJP4	78	*A. eutrophus* JMP134	Don & Pemberton (1987)
biphenyl	pBS241	195	*P. putida* BS893	Kochetkov et al. (1982)
4-CBP	pKF1	78	*Acinetobacter* sp. P6**	Furukawa & Chakrabarty(1982)
4-CBP	pAC21	98	*K. pneumoniae*	Kamp & Chakrabarty (1979)
4-CBP	pSS50	53	*Alcaligenes* A5	Shields et al. (1985)
4-CBP	pSS60	63	*Alcaligenes* 1C1	Layton et al. (1992)
4-CBP	pSS70	66	*Alcaligenes* ALP83	Layton et al. (1992)

* Abbreviations CB and CBP refer to chlorobenzoate and chlorobiphenyl, respectively.

** Two strains were reported to be isolated as contaminants of *Acinetobacter* sp. strain P6 : *Arthrobacter* M5 (Furukawa & Chakrabarty 1982) and *Corynebacterium* sp. strain MB1 (Bedard et al. 1987). Strain P6 has recently been reclassified as a *Rhodococcus* (Asturias & Timmis 1993), thus it cannot be excluded that strains P6, M5, and MB1 are all the same isolate.

reaction with all chlorinated analogs has limits, and must be carefully evaluated with regard to construction of PCB-degrading recombinants. Different metabolites will arise from respective congeners, and some of them may be inhibitors, be inert to the enzymes for the biphenyl pathway, or be acted upon by an unknown suite of enzymes from an unrelated pathway. For example, not all PCB congeners that are attacked by the biphenyl dioxygenase are converted to the corresponding chlorobenzoates. Furukawa et al. (1979) noted that many congeners gave rise to chlorinated dihydrodiols and phenylcatechols, that were not metabolized further. The accumulation of chlorinated 2,3-dihydroxybiphenyl might lead to inactivation of 3-phenylcatechol 2,3-dioxygenase, which exhibits substrate inhibition in some strains (Adams et al. 1992; Eltis et al. 1993) although not in others (Furukawa & Arimura 1987). The accumulation of chlorinated metabolites may also lead to unproductive and potentially toxic transformations by enzymes unrelated to biphenyl catabolism. Transformation of chlorinated 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (the ring-fission product) to acidic metabolites such as chlorocinnamic acid, has been reported (Ahmad et al. 1991b). The production of these metabolites, originally thought to be formed from an alternate biphenyl pathway, was mediated by unknown enzymes distinct from those encoded by the *bph* operon. As cinnamic acid was found to be a precursor of acetophenone by Hilton & Cain (1990), it is possible that chloroacetophenone may be produced from chlorocinnamic acid.

There is evidence for overlapping substrate specificity between biphenyl and catechol degradation enzymes which could potentially route metabolites along unproductive pathways. Some 3-phenylcatechol 2,3-dioxygenases attack catechol (Eltis et al. 1993), and some catechol 2,3-dioxygenases attack 3-phenylcatechol (Selifonov & Starozoitov 1990). The production of trace amounts of 4-chlorobenzoate from 4-chlorobiphenyl by *P. testosteroni* B-356 recombinants (*bphABC*) lacking the hydrolase gene (*bphD*) might have been the result of nonspecific action on 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid by the hydrolase of the catechol pathway (Ahmad et al. 1990). In addition, multiple metabolites may be produced from the action of a single enzyme on a single substrate. For example, biphenyl 2,3-dioxygenase from strain B-356 attacks either ring of some PCB congeners (Ahmad et al. 1991a) leading to different *meta*-cleavage products if the chlorine substitution pattern is different on the two rings (Sondossi et al. 1992). In some cases both rings were attacked to form tetrahydroxybiphenyls.

Although most PCB-degraders accumulate chlorobenzoates when transforming PCBs, there is evidence that some strains can cometabolize chlorobenzoates to chlorocatechols and chlorinated ring-cleavage prod-

ucts (Parsons et al. 1988; Bedard & Haberl 1990; Sondossi et al. 1992). In *P. testosteroni* B-356, chlorobenzoates were converted to chlorocatechols and chlorinated muconic semialdehydes, both of which inhibited 3-phenylcatechol 2,3-dioxygenase (Sondossi et al. 1992). However, biphenyl was required to induce the oxidation of chlorobenzoates to the inhibitory products. Whether the conversion of chlorobenzoates to dead-end products is mediated by a second suite of enzymes (e.g. the benzoate pathway) or by the *bph* genes themselves is unclear.

When the production of toxic or unproductive metabolites by enzymes unrelated to the main degradation pathway is a barrier to strain construction, the genes coding for the undesirable activity might be eliminated by mutation. A spontaneous mutation of *meta* pyrocatechase allowed Reineke et al. (1982) to construct a productive chlorobenzoate-degrader. More direct approaches to gene inactivation include transposon mutagenesis or in vitro mutagenesis of cloned copies and subsequent reinsertion into the chromosome. For example, inactivation of specific *bph* genes on the chromosome was demonstrated in *P. alcaligenes* KF707 (Furukawa et al. 1991). Cloned *bph* genes were mutated by transposon insertion and reintroduced on a suicide plasmid back into the parental strain. Double crossover homologous recombination led to the elimination of the functional gene and the establishment of the mutated copy in the chromosome. In this way, each of the few genes in the biphenyl pathway could be selectively turned off. In another case, the *bphC* gene of *Pseudomonas* sp. LB400 was placed under the control of a *tac* promoter in a broad host-range expression plasmid which also carried *lacIq* (de Lorenzo et al. 1993). When placed in various pseudomonads, the *bphC* gene could be conditionally hyper-expressed by addition of IPTG.

Cometabolism and constitutive expression of biphenyl genes

A different strategy for the construction of recombinants capable of transforming PCBs relies on cometabolism, rather than mineralization, and thus circumvents completely the requirement for chlorobenzoate genes. In this strategy *bph* genes are introduced into a strain which can grow on some unique but inexpensive carbon source. Since cloned *bph* genes are expressed constitutively, they will cometabolize PCBs while the strain grows on the inexpensive carbon source. This obviates the addition of biphenyl, which is itself a priority pollutant and is utilized by indigenous as well as inoculant bacteria. This strategy was used in a construction where the *bphABC* genes were incorporated from *Pseudomonas* sp. strain ENV307 into *P. paucimobilis* 1IGP4, an isolate which can grow on the surfactant Igepal CO-720 (Lajoie et al. 1993). Treatment of contaminated soil with the surfactant and the bacteria resulted in significant loss of Aroclor 1242 congeners by cometabolism. The recombinant, because of its ability to grow on the surfactant, facilitated the expression of the *bph* genes in a competitive natural environment where that expression offered no selective advantage. In addition, the surfactant may have aided in solubilizing PCBs. The stability of the recombinant plasmid was not high, but chromosomal integration of the *bph* genes could produce constructs stable enough for environmental use. As the authors measured only the disappearance of PCBs, it was not possible to verify if the cometabolic products were further mineralized by the indigenous soil microflora.

Gene transfer in the environment

A number of recent studies have addressed processes for gene transfer under environmental conditions (van der Meer et al. 1992; Fulthorpe & Wyndham 1992; Hickey et al. 1993; Chamier et al. 1993). From these experiments, it is evident that the horizontal transfer of genes within a natural system is very frequent, and may lead to the creation of novel strains when coupled with unique selection pressure from a xenobiotic chemical. There are three possible mechanisms for the exchange of genetic material between microorganisms: transduction, transformation, and conjugation via plasmid. Transduction, particularly in lysogenic strains, has been recently demonstrated with *P. aeruginosa* phages in a freshwater habitat during in situ incubation (Saye et al. 1990). However, as a mechanism of gene dispersal in nature, transduction has not been well characterized, and its application for genetic construction has been limited by a lack of suitable phages. The role of transformation in nature was emphasized in studies by Romanowski et al. (1991), Lorenz & Wackernagel (1992), Stewart et al. (1991), Khanna & Stotzky (1992), and Chamier et al. (1993). They suggest that the direct uptake of plasmid and chromosomal DNA from mineral surfaces is common in nature and that extracellular DNA adsorbed to minerals or clay particles is highly resistant to nucleolytic degradation. Direct uptake of DNA on the surface of ceramic beads probably enhanced genetic exchange during continu-

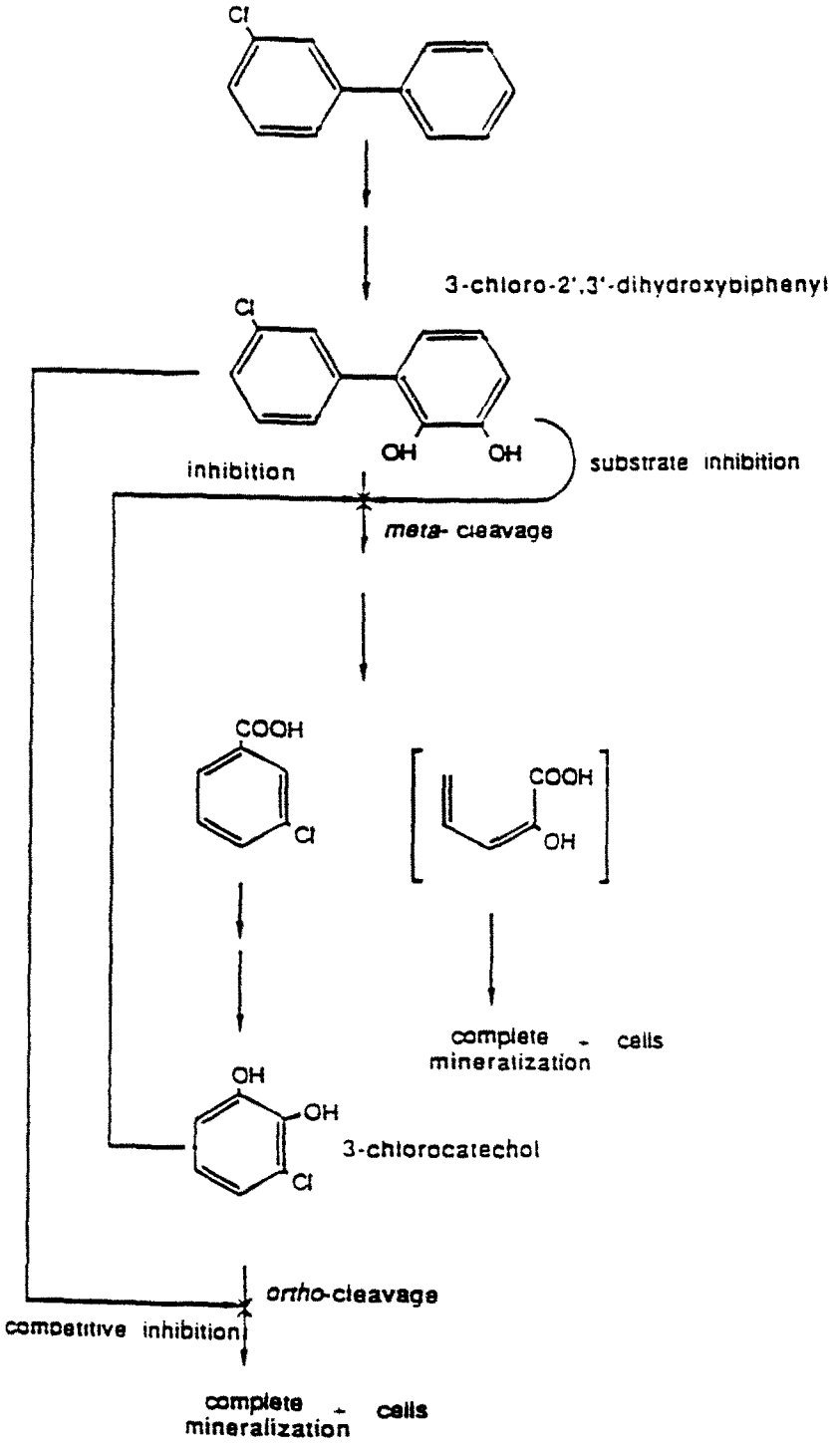

Fig. 4. Inhibitory effects of catecholic products on the catechol dioxygenases involved in mineralization of 3-chlorobiphenyl by hybrid strain *Pseudomonas* sp. CB15. From Adams et al. (1992).

ous amalgamated culture developed by Kröckel and Focht (1987).

The catabolic transposon Tn5271, which codes for chlorocatechol degradation, played a significant role in the degradation of 3-chlorobenzoate and 4-chloroaniline in freshwater microcosms (Fulthorpe and Wyndham 1992). During community adaptation to 4-chloroaniline, insertion sequence IS1071, which brackets Tn5271, was mobilized into alternative hosts. Organisms other than the donor, *Alcaligenes* sp. strain BR60, mediated these processes of community change, thereby emphasizing the key importance of horizontal genetic transfer. Similarly, *Alcaligenes eutrophus* A5 was able to horizontally transfer a chromosomally located biphenyl operon (*bph*) to other strains of *A. eutrophus* (Springael et al. 1993). The operon was found to be located on the transposon Tn4371, which was mobilized by plasmid conjugation.

Conclusion

Theoretically, it should be possible to construct an efficient PCB-utilizer by simply assembling all of the genes responsible for the mineralization of these compounds. However, the complexity of inhibitory effects of diphenolic intermediates on catechol dioxygenases during the mineralization of a simple congener (3-chlorobiphenyl) by *in vivo* constructed strain *Pseudomonas* sp. CB15 (Fig.4) suggests that multiple genetic events have occured during the selection of this hybrid. The degradation of highly chlorinated congeners results in the production of chlorinated benzoates and also chlorinated aliphatic acids, the fate of which has not been elucidated . In addition, no strains have yet been reported which can utilize any chlorobenzoate having a chloro-substitution at the 2,6- positions, although anaerobic consortia which reductively dehalogenate 2,3,6-trichlorobenzoate to 2,5-dichlorobenzoate have been reported (Gerritse et al. 1992).

Recently, Erickson & Mondello (1993) have reported the modification of the PCB congener specificity by site-directed mutagenesis of the *bphA* subunit of the biphenyl dioxygenase in *P. putida* LB400. The introduction of chlorobenzoate genes into such biphenyl degraders or the transfer of biphenyl genes into a versatile chlorobenzoate utilizer as *P. putida* P111 may result in the construction of an efficient PCB-utilizer if the molecular mechanisms for inhibitory effects between chlorobiphenyl and chlorobenzoate pathways are understood. However, the experiments in soil and wastewater systems indicated a dependence of genetically engineered strains on a specific substrates (Dwyer et al. 1988; Pipke et al. 1992; Wagner-Dobler 1992). Thus, these organisms in general may not survive in a multisubstrate, multicomponent environment without specific selection pressure. Hickey et al. (1993) reported the enhanced mineralization of PCBs in soil after inoculation with chlorobenzoate-degrading bacteria and suggested the possibility of genetic exchange between the inoculants and the indigenous bacteria. In a later study, Searles et al. (1993) demonstrated that 2-chlorobenzoate degraders were not indigenous to that soil. More significantly, several recombinant strains, which contained genes for biphenyl and chlorobenzoate utilization, were isolated, as a result of genetic exchange between the chlorobenzoate degrading inoculant *Pseudomonas aeruginosa* JB2 and the indigenous biphenyl-utilizers.

Population densities of the requisite parental strains of a complementary catabolic pathway are probably too low in uninoculated soil to effect genetic exchange. Thus, the biodegradation of a recalcitrant compound may be significantly enhanced by the addition of a nonindigenous microorganism which harbors the missing part of a catabolic pathway (e.g. 2-chlorobenzoate catabolism). Hence, such 'genetic engineering' performed in situ by soil bacteria might offer an alternative approach to the construction of novel catabolic pathways.

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References

Abramowicz DA (1990) Aerobic and anaerobic degradation of PCBs: a review. CRC Crit. Rev. Biotechnol. 10: 241–251

Adams RH, Huang CM, Higson FK, Brenner V & Focht DD (1992) Construction of a 3-chlorobiphenyl-utilizing recombinant from an intergeneric mating. Appl. Environ. Microbiol. 58: 647–654

Adriaens P, Kohler H-PE, Kohler-Staub D & Focht DD (1989) Bacterial dehalogenation of chlorobenzoates and coculture biodegradation of 4,4′-dichlorobiphenyl. Appl. Environ. Microbiol. 55: 887–892

Adriaens P & Focht DD (1991a) Cometabolism of 3,4-dichlorobenzoate by *Acinetobacter* sp. strain 4CB1. Appl. Environ. Microbiol. 57: 173–179

Adriaens P & Focht DD (1991b) Evidence for inhibitory substrate interactions of 3,4-dichlorobenzoate by *Acinetobacter* sp. strain 4CB1. FEMS Microbiol. Ecol. 85: 293–300

Ahmad D, Masse R & Sylvestre M (1990) Cloning and expression of genes involved in 4-chlorobiphenyl transformation by *Pseudomonas testosteroni*: homology to polychlorobiphenyl-degrading genes in other bacteria. Gene 86: 53–61

Ahmad D, Sylvestre M & Sondossi M (1991a) Subcloning of *bph* genes from *Pseudomonas testosteroni* B-356 in *Pseudomonas putida* and *Escherichia coli*: evidence for dehalogenation during initial attack on chlorobiphenyls. Appl. Environ. Microbiol. 57: 2880–2887

Ahmad D, Sylvestre M, Sondossi M & Masse R (1991b) Bioconversion of 2-hydroxy-6-oxo-6-(4′-chlorophenyl)hexa-2,4-dienoic acid, the *meta*-cleavage product of 4-chlorobiphenyl. J. Gen. Microbiol. 137: 1375–1385

Ahmed M & Focht DD (1973) Degradation of polychlorinated biphenyls by two species of *Achromobacter*. Can. J. Microbiol. 19: 47–52

Asturias JA & Timmis KN (1993) Three different 2,3-dihydroxybiphenyl-1,2-dioxygenase genes in the gram-positive polychlorinated biphenyl-degrading bacterium *Rhodococcus globerulus* P6. J. Bacteriol. 175: 4631–4640

Bagdasarian M & Timmis KN (1982) Host vector systems for gene cloning in *Pseudomonas*. Curr. Top. Microbiol. Immunol. 96: 47–67

Barry GF (1988) A broad-host-range shuttle system for gene insertion into the chromosomes of Gram-negative bacteria. Gene 71: 75–84

Bartels I, Knackmuss HJ & Reineke W (1984) Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols. Appl. Environ. Microbiol. 47: 500–505

Barton MR & Crawford RL (1988) Novel biotransformations of 4-chlorobiphenyl by a *Pseudomonas sp.* Appl. Environ. Microbiol. 54: 594–595

Bedard DL, Unterman R, Bopp LH, Brennan MJ, Haberl ML & Johnson C (1986) Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. 51: 761–768

Bedard DL, Wagner RE, Brennan MJ, Haberl ML & Brown Jr JF (1987) Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. 53: 1094–1102

Bedard DL & Haberl ML (1990) Influence of chlorine substitution pattern on the degradation of polychlorinated biphenyls by eight bacterial strains. Microb. Ecol. 20: 87–102

Boronin AM, Filonov AE, Balkshina VV & Kulakova AN (1985) Stability of naphtalene biodegradation plasmids NPL-1 and NPL-41 in populations of *Pseudomonas putida* under conditions of continuous culture. Mikrobiologiya 54: 610–615

Boyle AW, Silvin JC, Hassett JP, Nakas JP & Tannenbaum SW (1992) Bacterial PCB degradation. Biodegradation 3: 285–298

Brenner V, Hernandez BS & Focht DD (1993) Variation in chlorobenzoate catabolism by *Pseudomonas putida* P111 as a consequence of genetic alterations. Appl. Environ. Microbiol. 59: 2790–2794

Brinkmann U & Reineke W (1992) Degradation of chlorotoluenes by in vivoconstructed hybrid strains : problems of enzyme specificity, induction and prevention of *meta*-pathway. FEMS Microbiol. Lett. 96: 81–88

Brown JF, Bedard DL, Brennan MJ, Carnahan JC, Feng H & Wagner RE (1989) Polychlorinated biphenyl dechlorination in aquatic sediments. Science 236: 709–712

Catelani D, Sorlini C & Treccani V (1971) The metabolism of biphenyl by *Pseudomonas putida*. Experientia 27: 1173–1174

Chamier B, Lorenz MG & Wackernagel W (1993) Natural transformation of *Acinetobacter calcoaceticus* by plasmid DNA adsorbed on sand and groundwater aquifer material. Appl. Environ. Microbiol. 59: 1662–1667

Chang H, Lee J, Roh S, Kim SR, Min KR, Kim CK, Kim EG & Kim Y (1992) Molecular cloning and characterization of catechol 2,3-dioxygenases from biphenyl/polychlorinated biphenyls-degrading bacteria. Biochem. Biophys. Res. Comm. 187: 609–614

Chatterjee DK & Chakrabarty AM (1982) Genetic rearrangements in plasmids specifying total degradation of chlorinated benzoic acids. Mol. Gen. Genet. 188: 279–285

Chatterjee DK & Chakrabarty AM (1983) Genetic homology between independently isolated chlorobenzoate-degradative plasmids. J.Bacteriol.153: 532–534

Chatterjee DK & Chakrabarty AM (1984) Restriction mapping of a chlorobenzoate degradative plasmid and molecular cloning of the degradative genes. Gene 27: 173–181

Chatterjee DK , Kellogg ST, Hanada S & Chakrabarty AM (1981a) Plasmid specifying total degradation of 3-chlorobenzoate by a modified *ortho* pathway. J.Bacteriol. 146: 639–646

Chatterjee DK, Kellogg ST, Watkins DR & Chakrabarty AM (1981b) Plasmids in the biodegradation of chlorinated aromatic compounds. In: Levy S, Clowes RC, Koenig E (Eds.), Molecular biology, pathogenicity and ecology of bacterial plasmids, p.519–528, Plenum Press, New York

Dagley S, Chapman PJ, Gibson DT & Wood JM (1964) Degradation of the benzene nucleus by bacteria. Nature (London) 202: 775–778

de Bont JAM, Vorage MJAW, Hartmans S & van den Tweel WJJ (1986) Microbial degradation of 1,3-dichlorobenzene. Appl. Environ. Microbiol. 52: 677–680

de Lorenzo V, Eltis L, Kessler B & Timmis KN (1993) Analysis of *Pseudomonas* gene products using *lacI*^{q}/ P*trp-lac* plasmids and transposons that confer conditional phenotypes. Gene 123: 17–24

Don RH, Weightman AJ, Knackmuss HJ & Timmis KN (1985) Transposon mutagenesis and cloning analysis of the pathways for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134 (pJP4). J. Bacteriol. 161: 85–90

Dunaway-Mariano D & Babbitt PC (1994) On the origins and functions of the enzymes of the 4-chlorobenzoate to 4-hydroxybenzoate converting pathway. Biodegradation 5: 259–276 (this issue)

Dwyer DF, Rojo F & Timmis KN (1988) Fate and behaviour in an activated sludge microcosm of a genetically-engineered micro-organism designed to degrade substituted aromatic compounds, p. 77–88. In: Sussmann M, Collins CH, Skinner FA & Stewart-Tull (Eds) The release of genetically-engineered micro-organisms, Academic Press, Ltd., London

Dykhuizen DE & Hartl DL (1983) Selection in chemostats. Microbiol. Rev. 47: 150–168

Ensley BD (1985) Stability of recombinant plasmids in industrial microorganisms. CRC Crit. Rev. Biotechnol. 4: 263–283

Eltis LD, Hofmann B, Hecht HJ, Lunsdorf H & Timmis KN (1993) Purification and crystallization of 2,3-dihydroxybiphenyl 1,2-dioxygenase. J. Biol. Chem. 268: 2727–2732

Fetzner S, Müller R & Lingens F (1989) Degradation of 2-chlorobenzoate by *Pseudomonas cepacia* 2CBS. Biol. Chem. Hoppe-Seyler 370: 1173–1182

Fetzner S, Müller R & Lingens F (1992) Purification and some properties of 2-halobenzoate 1,2-dioxygenase, a two component enzyme system from *Pseudomonas cepacia* 2CBS. J. Bacteriol. 174: 279–290

Focht D.D. (1987) Ecological and evolutionary considerations on the metabolism of xenobiotic chemicals in soil. p. 157–167. In: L. Boersma (ed.), Future Developments in Soil Science Research. American Society of Agronomy, Madison, WI.

Focht DD (1988) Performance of biodegradative microrganisms in soil: xenobiotic chemicals as unexploited metabolic niches, p.15–29. In: Omen GS (Ed), Environmental biotechnology, Plenum Publishing, New York

Focht DD (1993) Microbial degradation of chlorinated biphenyls, p.341–407. In: Bollag JM & Stotzky G (Ed) Soil Biochemistry, volume 8, Marcel Dekker Inc.,New York

Frantz B. & Chakrabarty AM (1987) Organization and nucleotide sequence determination of a gene cluster in 3-chlorocatechol degradation. Proc. Natl. Acad. Sci. USA 84: 4460–4464

Friedmann AM, Long SR, Brown SE, Buikema WJ & Ausubel FM (1982) Construction of a broad host range cosmid cloning vector and its use in genetic analysis of *Rhizobium* mutants. Gene 18: 289–296

Fulthorpe RR & Wyndham RC (1992) Involvement of a chlorobenzoate-catabolic transposon, Tn*5271*, in community adaptation to chlorobiphenyl, chloroaniline, and 2,4-dichlorophenoxyacetic acid in a freshwater ecosystem. Appl. Environ. Microbiol. 58: 314–325

Furukawa K, Tonomura K & Kamibayashi A (1978) Effect of chlorine substitution on the biodegradability of polychlorinated biphenyls. Appl. Environ. Microbiol. 35: 223–227

Furukawa K (1994) Molecular genetics and evolutionary relationship of PCB-degrading bacteria. Biodegradation 5: 289–300 (this issue)

Furukawa, K, Tonomura, K & Kamibayashi A (1979) Effect of chlorine substitution of the bacterial metabolism of various polychlorinated biphenyls. Appl. Environ. Microbiol. 38: 301–310

Furukawa K & Chakrabarty AM (1982) Involvement of plasmids in total degradation of chlorinated biphenyls. Appl. Environ. Microbiol. 44: 619–626

Furukawa K & Miyazaki T (1986) Cloning of a gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes*. J. Bacteriol. 166: 392–398

Furukawa K & Arimura N (1987) Purification and properties of 2,3-dihydroxybiphenyl dioxygenase from polychlorinated biphenyl-degrading *Pseudomonas pseudoalcaligenes* and *Pseudomonas aeruginosa* carrying the cloned *bphC* gene. J. Bacteriol. 169: 924–927

Furukawa K, Hayashida S & Taira K (1991) Gene-specific transposon mutagenesis of the biphenyl/polychlorinated biphenyl-degradation-controlling *bph* operon in soil bacteria. Gene 98: 21–28

Furukawa K , Hirose J, Suyama A, Zaiki T & Hayashida S (1993) Gene components responsible for discrete substrate specificity in the metabolism of biphenyl (*bph* operon) and toluene (*tod* operon). J. Bacteriol. 175: 5224–5232.

Gerritse J, van der Woude BJ & Gottschal JC (1992) Specific removal of chlorine from the *ortho*-position of halogenated benzoic acids by reductive dechlorination in anaerobic enrichment cultures. FEMS Microbiol. Lett. 100: 273-280

Ghosal D & You IS (1988) Nucleotide homology and organization of chlorocatechol oxidation genes of plasmids pJP4 and pAC27. Mol.Gen.Genet. 211: 113–120

Ghosal D & You IS (1989) Operon structure and nucleotide homology of the chlorocatechol genes of plasmids pJP4 and pAC27. Gene 83: 225–232

Ghosal D, You IS, Chatterjee DK & Chakrabarty AM (1985) Genes specifying degradation of 3-chlorobenzoic acid in plasmids pAC27 and pJP4. Proc. Natl. Acad. Sci. USA 82: 1638–1642

Hartmann J, Engelberts K, Nordhaus K, Schmidt E & Reineke W (1989) Degradation of 2-chlorobenzoate by in vivo constructed hybrid pseudomonads. FEMS Microbiol. Lett. 61: 17–22

Hartmann J, Reineke W & Knackmuss HJ (1979) Metabolism of 3-chloro, 4-chloro-, and 3,5-dichlorobenzoate by a *pseudomonad.* Appl. Environ. Microbiol. 37: 421–428

Havel J. & Reineke W (1991) Total degradation of various chlorobiphenyls by cocultures and in vivo constructed hybrid pseudomonads. FEMS Microbiol. Lett. 78: 163–170

Hayase N, Taira K & Furukawa K (1990) *Pseudomonas putida* KF715 *bphABCD* operon encoding biphenyl and polychlorinated biphenyl degradation: cloning, analysis, and expression in soil bacteria. J. Bacteriol. 172: 1160–1164

Hickey WJ, Brenner V. & Focht DD (1992) Mineralization of 2-chloro- and 2,5-dichlorobiphenyl by *Pseudomonas* sp. strain UCR2. FEMS Microbiol. Lett. 98: 175–180

Hickey WJ, Searles DB & Focht DD (1993) Enhanced mineralization of polychlorinated biphenyls in soil inoculated with chlorobenzoate-degrading bacteria. Appl. Environ. Microbiol. 59: 1194–1200

Hilton MD & Cain WJ (1990) Bioconversion of cinnamic acid to acetophenone by a pseudomonad : microbial production of a natural flavor compound. Appl. Environ. Microbiol. 56: 623–627

Higson, FK & Focht, DD (1992) Utilization of 3-chloro 2-methylbenzoic acid by *Pseudomonas cepacia* MB2 through the *meta* fission pathway. Appl. Environ. Microbiol. 58: 2501–2504

Huang C-M (1988) Strain construction strategies for chlorinated aromatic hydrocarbon-utilizers by multiple chemostat. PhD Thesis, University of California, Riverside.

Itoh Y & Haas D (1985) Cloning vectors derived from the *Pseudomonas* plasmid pVS1. Gene 36: 27–36

Jacoby GA (1986) Resistence plasmids of *Pseudomonas*. In: Sokatch JR (ed) The bacteria, Vol. X, p.265–293, Academic Press, Orlando, FL

Jeenes DJ, Reineke W, Knackmuss HJ & Williams PA (1982) TOL plasmid pWWO in constructed halobenzoate-degrading *Pseudomonas* strains: enzyme regulation and DNA structure. J. Bacteriol. 150: 180–187

Kamp PF & Chakrabarty AM (1979) Plasmids specifying *p*-chlorobiphenyl degradation in enteric bacteria. In: Timmis KN & Pühler A (eds) Plasmids of medical, environmental, and commercial importance, p.275–285, Elsevier, North Holland Biomedical Press, Amsterdam

Keshavarz T, Lilly MD & Clarke PH (1985) Stability of a catabolic plasmid in continuous culture. J. Gen. Microbiol. 131: 1193–1203

Khan A & Walia S (1989) Cloning of bacterial genes specifying degradation of 4-chlorobiphenyl from *Pseudomonas putida* OU83. Appl. Environ. Microbiol. 55: 798–805

Khanna M & Stotzky G (1992) Transformation of *Bacillus subtilis* by DNA bound on montmorillonite and effect of DNase on the transforming ability of bound DNA. Appl. Environ. Microbiol. 58: 1930–1939

Kimbara K, Hashimoto T, Fukuda M, Koana T, Takagi M, Oishi M & Yano K (1989) Cloning and sequencing of two tandem genes

involved in degradation of 2,3-dihydroxybiphenyl to benzoic acid in the polychlorinated biphenyl-degrading soil bacterium *Pseudomonas* sp. strain KKS102. J. Bacteriol. 171: 2740–2747

Kochetkov VV, Stasovoitov II, Boronin AM & Skryabin GK (1982) *Pseudomonas putida* pBS241: Plasmid mediated biphenyl degradation. Dokl. Akad. Nauk SSSR 226: 241–243

Kohler-Staub D & Kohler H-PE (1989) Microbial degradation of ß-chlorinated four-carbon aliphatic acids. J. Bacteriol. 171: 1428–1434.

Kröckel L & Focht DD (1987) Construction of chlorobenzene-utilizing recombinants by progenitive manifestation of a rare event. Appl. Environ. Microbiol. 53: 2470–2475

Lajoie CA, Zylstra GJ, DeFlaun MF & Strom PF (1993) Development of field application vectors for bioremedation of soils contaminated with polychlorinated biphenyls. Appl. Environ. Microbiol. 59: 1735–1741

Latorre J, Reineke W. & Knackmuss HJ (1984) Microbial metabolism of chloroanilines: enhanced evolution by natural genetic exchange. Arch. Microbiol. 140: 159–165

Layton AC, Sanseverino J, Wallace W, Corcoran C & Sayler GS (1992) Evidence for 4-chlorobenzoic acid dehalogenation mediated by plasmids related to pSS50. Appl. Environ. Microbiol. 58: 399–402

Lehrbach PR, Zeyer J, Reineke W, Knackmuss HJ & Timmis KN (1984) Enzyme recruitment in vitro: Use of cloned genes to extend the range of haloaromatics degraded by *Pseudomonas sp.* strain B13. J. Bacteriol. 158: 1025–1032

Levin BR, Stewart FM & Rice VA (1979) The kinetics of conjugative plasmid transmission: fit of a simple mass action model. Plasmid 2: 247–260

Lorenz MG & Wackernagel W (1992) DNA binding to various clay minerals and retarded enzymatic degradation of DNA in a sand/clay microcosm, p.103–113. In: Gauthier MJ (Ed.), Gene transfers and environment. Springer-Verlag KG, Berlin

Meacock PA & Cohen SN (1980) Partitioning of bacterial plasmids during cell division: a *cis* - acting locus that accomplishes stable plasmid inheritance. Cell 20: 529–542

Mermod N, Ramos JL, Lehrbach PR & Timmis KN (1986) Vector for regulated expression of cloned genes in a wide range of Gram-negative bacteria. J. Bacteriol. 167: 447–454

Mokross H, Schmidt E & Reineke W (1990) Degradation of 3-chlorobiphenyl by in vivo constructed hybrid pseudomonads. FEMS Microbiol. Lett. 71:179–185

Mondello FJ (1989) Cloning and expression in *Escherichia coli* of *Pseudomonas* strain LB400 genes encoding polychlorinated biphenyl degradation. J. Bacteriol. 171: 1725–1732

Morris PJ, Mohn WW, Quensen JF III, Tiedje JM & Boyd SA (1992) Establishment of polychlorinated biphenyl-degrading enrichment culture predominantly meta dechlorination. Appl. Environ. Microbiol. 58: 3088–3094

Nakatsu C, Ng J, Singh R, Straus N & Wyndham C (1991) Chlorobenzoate catabolic transposon Tn5271 is a composite class I element with flanking class II insertion sequences. Proc. Natl. Acad. Sci. USA 88: 8312–8316

Ngai K & Ornston LN (1988) Abundant expression of *Pseudomonas* genes for chlorocatechol metabolism. J. Bacteriol. 170: 2412–2413

Nies L & Vogel TM (1990) Effects of organic substrates on dechlorination of Aroclor 1242 in anaerobic sediment. Appl. Environ. Microbiol. 56: 2612–2617

Nordström, K (1983) Control of plasmid replication. Plasmid 9: 1–7

Oltmanns RH, Rast HG & Reineke W (1988) Degradation of 1,4-dichlorobenzene by enriched and constructed bacteria. Appl. Microbiol. Biotechnol. 28: 609–616

Omori T, Sugimura K, Ishigooka H & Minoda Y (1986) Purification and some properties of a 2-hydroxy-6-oxo-6-phenyl-2,4-dienoic acid hydrolyzing enzyme from *Pseudomonas cruciviae* S93B1 involved in the degradation of biphenyl. Agric. Biol. Chem. 50: 1513–1518.

Parsons JR, Sijm DTHM, van Laar A & Hutzinger O (1988) Biodegradation of chlorinated biphenyls and benzoic acids by a *Pseudomonas* species. Appl. Microbiol. Biotechnol. 29: 82–84

Perkins EJ, Gordon MP, Caceres O. & Lurquin PF (1990) Organization and sequence analysis of the 2,4-dichlorophenol oxidative operons of plasmid pJP4. J. Bacteriol. 172: 2351–2359

Pipke R, Wagner-Dbler I, Timmis KN & Dwyer DF (1992) Survival and function of a genetically engineered pseudomonad in aquatic sediment microcosms. Appl. Environ. Microbiol. 58: 1259–1265

Quensen JF III, Boyd SA & Tiedje JM (1990) Dechlorination of 4 commercial polychlorinated biphenyl mixtures (Aroclors) by anaerobic microorganisms from sediments. Appl. Environ. Microbiol. 56: 2360–2369

Quensen JF III, Tiedje JM & Boyd SA (1988) Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediments. Science 242: 752–754

Ramos JL, Stolz A, Reineke W & Timmis KN (1986) Altered effector specificities in regulators of gene expression: TOL plasmid *xylS* mutants and their use to engineer expansion of the range of aromatics degraded by bacteria. Proc. Natl. Acad. Sci. USA 83: 8467–8471

Ramos JL, Wasserfallen A, Rose K & Timmis KN (1987) Redesigning metabolic routes: Manipulation of TOL plasmid pathway for catabolism of alkylbenzoates. Science 235: 593–596

Reineke W (1984) Microbial degradation of halogenated aromatic compounds p. 319–360. In: D.T. Gibson (ed), Microbial degradation of organic compounds. Marcel Dekker Inc. New York
Reineke W & Knackmuss HJ (1979) Construction of haloaromatics utilising bacteria. Nature 277: 385–386

Reineke W & Knackmuss HJ (1980) Hybrid pathway for chlorobenzoate metabolism in *Pseudomonas* sp. B13 derivatives. J. Bacteriol. 142: 467–473

Reineke W & Knackmuss HJ (1984) Microbial metabolism of haloaromatics: isolation and properties of a chlorobenzene-degrading bacterium. Appl. Environ. Microbiol. 47: 395–402

Reineke W & Knackmuss HJ (1988) Microbial degradation of haloaromatics. Annu. Rev. Microbiol. 42: 263–287

Reineke W, Jeenes DJ, Williams PA & Knackmuss HJ (1982) TOL plasmid pWWO in constructed halobenzoate degrading *Pseudomonas* strains: prevention of *meta* pathway. J. Bacteriol. 150: 195–201

Romanowski G, Lorenz MG & Wackernagel W (1991) Adsorption of plasmid DNA to mineral surfaces and protection against DNase I. Appl. Environ. Microbiol. 57: 1057–1061

Rothmel R.K., Chakrabarty A.M., Berry A & Darzins A (1991) Genetic systems in *Pseudomonas*. Methods Enzymol. 204 : 485–514

Sangodkar UMX, Aldrich TL, Haugland RA, Johnson J, Rothmel RK, Chapman PJ, Chakrabarty AM (1989) Molecular basis of biodegradation of chloroaromatic compounds. Acta Biotechnol. 9: 301–316

Saye DJ, Ogunseitan OA, Sayler GS & Miller RV (1990) Transduction of linked chromosomal genes between *Pseudomonas aeruginosa* strains during incubation in situ in a freshwater habitat. Appl. Environ. Microbiol. 56: 140–145

Sayler GS, Hooper SW, Layton AC & King JMH (1990) Catabolic plasmids of environmental significance. Microb. Ecol. 19: 1–20

Schwien U & Schmidt E (1982) Improved degradation of monochlorophenols by a constructed strain. Appl. Environ. Microbiol. 44: 33–39

Searles D, Brenner V & Focht DD (1993) Genetic exchange in soil among indigenous biphenyl-utilizers and a dichlorobenzoate-utilizing inoculant. Microbiol. Proc. Abstract Q301, ASM Meeting, Atlanta

Selifonov SA & Starozoitov II (1990) Comparative study of aromatic ring *meta*-cleavage enzymes in *Pseudomonas* strains with plasmid and chromosomal genetic control of the catabolism of biphenyl and *m*-toluate. Biokhimiya 55: 2171–81

Senior ET, Bull AT, & Slater JH (1976) Enzyme evolution in a microbial community growing on the herbicide Dalapon. Nature (London) 263: 476–479

Shields MS, Hooper SW & Sayler GS (1985) Plasmid-mediated mineralization of 4-chlorobiphenyl. J. Bacteriol. 163: 882–889

Slater, JH (1985) Gene transfer in microbial communities. In: HO Halvorson, D Pramer, M Rogul (Eds) pp 89–98. Engineered Organisms in the Environment: Scientific Issues. American Society for Microbiology, Washington

Sondossi M, Sylvestre M & Ahmad D (1992) Effects of chlorobenzoate transformation on the *Pseudomonas testosteroni* biphenyl and chlorobiphenyl degradation pathway. Appl. Environ. Microbiol. 58: 485–495

Springael D, Diels L, Hooyberghs L, Kreps S & Mergeay M (1993) Construction and characterization of heavy metal-resistant haloaromatic-degrading *Alcaligenes eutrophus* strains. Appl. Environ. Microbiol. 59: 334–339

Springael D, Kreps S & Mergeay M (1993) Identification of a catabolic transposon, Tn*4371*, carrying biphenyl and 4-chlorobiphenyl degradation genes in *Alcaligenes eutrophus* A5. J. Bacteriol. 175: 1674–1681

Springael D, Diels L & Mergeay M (1994) Transfer and expression of PCB-degradative genes into heavy metal resistant *Alcaligenes eutrophus* strains. Biodegradation 5: 343–357 (this issue)

Stewart GJ, Sinigalliano CD & Garko KA (1991) Binding of exogenous DNA to marine sediments and the effect of DNA/sediment binding on natural transformation of *Pseudomonas stutzeri* strain ZoBell in sediment columns. FEMS Microbiol. Ecol. 85: 1–8

Sylvestre M, Masse R, Ayotte C, Messier F & Fauteux J (1985) Total biodegradation of 4-chlorobiphenyl (4CBP) by a two-membered bacterial culture. Appl. Microbiol. Biotechnol. 21: 191–195

Sylvestre M, Mailhiot K & Ahmad D (1989) Isolation and preliminary characterization of a 2-chlorobenzoate degrading *Pseudomonas* . Can. J. Microbiol. 35: 439–443

Thomas CM & Smith CA (1987) Incompatibility group P plasmids: Genetics, evolution, and use in genetic manipulation. Ann. Rev. Microbiol. 41: 77–101

Timmis KN, Lehrbach PR, Harayama S, Don RH, Mermod N, Bas S, Leppik R, Weightman AJ, Reineke W, Knackmuss HJ (1985) Analysis and manipulation of plasmid encoded pathways for the catabolism of aromatic compounds by soil bacteria, p.719–739. In: Helinski DR, Cohen SN, Clewell DB, Jackson DA & Hollaender A (Eds), Plasmids in Bacteria. Plenum Publishing Corporation, New York

Timmis KN, Rojo F & Ramos JL (1988) Prospects for laboratory engineering of bacteria to degrade pollutants. In: Omenn GS (Ed), Environmental Biotechnology: Reducing Risks from Environmental Chemicals through Biotechnology (pp 61–79) Plenum Publishing, New York

van der Meer JR, de Vos WM, Harayama S & Zehnder AJB (1992) Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiol. Rev. 56: 677–694

van der Meer JR, Eggen RIL, Zehnder AJB & de Vos (1991a) Sequence analysis of the *Pseudomonas* sp. strain P51 *tcb* gene cluster, which encodes metabolism of chlorinated catechols: evidence for specialization of catechol 1,2-dioxygenases for chlorinated substrates. J.Bacteriol. 173: 2425–2434

van der Meer JR, Frijters ACJ, Leveau JHJ, Eggen RIL, Zehnder AJB & de Vos WM (1991b) Characterization of the *Pseudomonas* sp. strain P51 gene *tcb*R, a LysR-type transcriptional activator of the *tcbCDEF* chlorocatechol oxidative operon, and analysis of the regulatory region. J.Bacteriol. 173: 3700–3708

van der Meer JR, van Neerven ARW, de Vries EJ, de Vos WM & Zehnder AJB (1991c) Cloning and characterization of plasmid-encoded genes for the degradation of 1,2-dichloro-, 1,4-dichloro-, and 1,2,4-trichlorobenzoate of *Pseudomonas* sp. strain P51. J.Bacteriol.173: 6–15

van der Meer JR, de Vos WM, Harayama S. & Zehnder AJB (1992) Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiol. Rev. 56: 677–694

van Dort HM & Bedard DL (1991) Reductive *ortho* dechlorination of a polychlorinated biphenyl congener by anaerobic microorganisms. Appl. Environ. Microbiol. 57: 1576–1578

Wagner-Döbler I, Pipke R, Timmis KN & Dwyer WF (1992) Evaluation of aquatic sediment microcosms and their use in assessing of introduced microorganisms on ecosystem parameters. Appl. Environ. Microbiol. 58: 1249–1258

Walia S, Khan A & Rosenthal N (1990) Construction and applications of DNA probes for detection of polychlorinated biphenyl-degrading genotypes in toxic organic-contaminated soil environments. Appl. Environ. Microbiol. 56:254–259

Weightman AJ, Don RH, Lehrbach PR & Timmis KN (1984) The identification and cloning of genes coding haloaromatic catabolic enzymes and the construction of hybrid pathways for substrate mineralization (pp 47–80). In: Omenn GS & Hollaender A (Eds), Genetic Control of Environmental Pollutants. Plenum Publishing, New York

Weisshaar MP, Franklin FCH & Reineke W (1987) Molecular cloning and expression of the 3-chlorobenzoate-degrading genes from *Pseudomonas* sp. strain B13. J. Bacteriol. 169: 394–402

Wyndham RC, Singh RK & Straus NA (1988) Catabolic instability, plasmid gene deletion and recombination in *Alcaligenes sp.* BR 60. Arch. Microbiol. 50: 237–243

Yates JR & Mondello FM (1989) Sequence Similarities in the Genes Encoding Polychlorinated Biphenyl degradation by *Pseudomonas* strain LB400 and *Alcaligenes eutrophus* H950. J. Bacteriol. 171: 1733–1735

Zaitsev GM & Karasevich YN (1984) Utilization of 2-chlorobenzoic acid by *Pseudomonas cepacia*. Mikrobiologiya 54: 356–359