THE GENETIC CONSTRUCTION OF TWO PCB-DEGRADING BACTERIA

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ABSTRACT

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Polychlorinated biphenyls (PCBs) are a class of widespread environmental pollutants, which are extremely resistant to degradation by any means, including biodegradation. Their accumulation and persistance has stimulated research into novel means for their eradication. This study describes the attempt to genetically engineer PCB-degrading bacteria, through the introduction of recombinant plasmids containing genes for dehalogenation of chlorobenzoates (CBAs), the major products of PCB breakdown. The genes introduced were the *ohb* genes of *Pseudomonas aeruginosa* 142, and *fcb* genes of *Arthrobacter globiformis* KZT1, which encode for the dechlorination of 2-CBA and 4-CBA respectively. These plasmids were successfully introduced and expressed in a biphenyl-degrading host strain, *Comamonas testosteroni* strain VP44. The resulting recombinant strains were capable of growth on and dechlorination of both chlorobenzoates, and their corresponding monochlorobiphenyls, 2-chlorobiphenyl and 4-chlorobiphenyl. However, no significant growth or dechlorination were observed on any biphenyls containing more than one chlorine, even those yielding only chlorobenzoates amenable to degradation.

To my beloved family, who has always supported me unconditionally

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LIST OF ABBREVIATIONS

CB ..... chlorobiphenyl

CBA ..... chlorobenzoic acid *or* chlorobenzoate

DCB ..... dichlorobiphenyl

DCP ..... dichlorophenol

DNA ..... deoxyribonucleic acid

DDT ..... dichlorodiphenyltrichloroethane

EPA ..... Environmental Protection Agency

FAME ..... fatty acid methy ester

GC ..... gas chromatography

GEM ..... genetically-engineered microorganism

IUPAC ..... International Union of Pure and Applied Chemistry

MS ..... mass spectroscopy

MSU ..... Michigan State University

PCB ..... polychlorinated biphenyl

PCR ..... polymerase chain reaction

RNA ..... ribonucleic acid

TSCA ..... Toxic Substance Control Act

CHAPTER 1

DEGRADATION OF PCBs: A REVIEW

THE PCB PROBLEM

Polychlorinated biphenyls (PCBs) are a class of widespread environmental pollutants which have traditionally been considered extremely recalcitrant both to biodegradation and chemical degradation methods such as incineration. They are a group of 209 isomers synthesized by chlorination of biphenyl . Each PCB isomer, called a congener, may contain from 1 to 10 chlorine atoms covalently bonded to the carbon skeleton of a biphenyl molecule. By IUPAC convention, congeners are named by assigning unprimed numbers to the positions of chlorines on the more-chlorinated ring, and primed numbers on the other, less-chlorinated ring, in addition to a numerical prefix indicating total chlorines (20). More simply, each congener has been assigned a single number ranging from 1 (2-chlorobiphenyl) to 209 (decachlorobiphenyl) (Fig. 1).

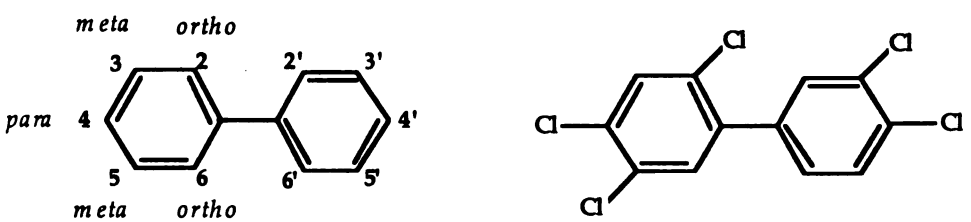

Figure 1. Nomenclature of PCBs
At left, the numbering scheme and positional references of PCBs, relative to the opposing phenyl ring. At right, an example PCB molecule known as 2,4,5,3'4'-pentachlorobiphenyl by the IUPAC nomenclature, or simply congener number 118.

Although first synthesized in 1881, PCBs did not become common until the 1930's, when they began replacing the flammable petroleum products found in a variety of industrial applications, including transformers, capacitors, hydraulic fluids, lubricants, and plasticizers. They were marketed under a variety of trade names in different countries: Clophen (Bayer, West Germany), Phenoclor (Caffaro, Italy), Kanechlor (Kanegafuchi, Japan), Pyralene (Prodelec, France), and Solvol (USSR) (29). Each product is a mixture of PCB congeners. The most common mixture available in the United States was the Aroclor series, manufactured by Monsanto. While there are 209 congeners theoretically possible, less than half are actually found in commercial PCB mixtures, though more than 60 different congeners have been detected in some mixtures. These products are usually assigned a number that reflects their composition in some way. For example, the Aroclor series utilizes a four-digit numbering system where the first two numbers (12- for PCBs) designate the 12-atom carbon skeleton, and the last two numbers (-21, -42, -48, -54, -60) refer to the chlorine content as a percentage by weight (20).

During the years of their manufacture, some 1.4 billion pounds of PCBs were manufactured in the United States alone, with similar quantities estimated to have been produced in the USSR, Japan, and Europe (60). There was little concern over the fate of PCBs in the environment until 1966, when unexplained chromatographic peaks, seen worldwide in soil and water during testing of environmental samples for DDT accumulation, were finally identified as PCBs (47, 48). All PCB manufacture in the United States was finally banned in 1977 by the Toxic Substances Control Act (TSCA), Public Law 94-469 (22, 60), although some PCBs can still be found in a variety of closed applications.

In spite of their discontinued manufacture and industrial application, PCBs persist. Because of their hydrophobicity and resistance to degradation, these compounds have accumulated in the environment, especially in soils and aquatic sediments. As might be

expected, PCB concentrations are highest around industrial and urban areas, but contamination is not limited to these places. Transported by air and water currents, PCBs have found their way to even the most isolated parts of the planet, often accumulating in remote polar regions. Their ubiquitous presence and biomagnification through the food chain has contaminated virtually everyone on earth, and can be detected in the body fat of people worldwide (see refs. in 23).

The health effects of this contamination are not completely understood. Because of their low chemical reactivity, PCBs are considered to be of a low order of toxicity when administered as a single dose (27). Two major outbreaks of PCB poisoning have been reported, following the ingestion of rice oil contaminated with Kanechlor 400 (equivalent to Aroclor 1248, consisting primarily of tetra- and pentachlorobiphenyls) in Japan and Taiwan. This syndrome has been named Yusho ("rice-oil disease"). Acute symptoms included chloracne (a persistent skin disease), discoloring or pigmentation of skin, nails, and hair, eye discharge, numbness of the limbs, and fever (50). Such incidents are rare, and no human deaths resulting from PCB exposure have ever been reported. PCBs, especially the more-highly chlorinated congeners, have long been suspected human carcinogens. From a regulatory standpoint, all PCBs are considered equivalent, and some have argued that this concern is overstated (1).

The effects of polychlorinated biphenyls in the human body over the long-term are more open to question, and potentially far more dangerous. In the United States today, most exposure to PCBs occurs by consuming fish from contaminated waters, particularly those of the Great Lakes (27, 50). The consequences of such continuous exposure are unclear, although PCBs have been implicated in diverse health problems including neurological, digestive, immunosuppressive, developmental, and reproductive disorders. In fact, children born to mothers who were pregnant, or became pregnant shortly after the Yusho incidents, have shown marked problems in psychological, neurological, and

cognitive development. (27, 50, see refs. in 23). Particularly troubling is the suspected ability of these compounds to act as mimics of the hormone estrogen, and they have been considered a possible cause for declining sperm counts in men worldwide over the last 50 years (21). Recent studies have shown individual PCB congeners can act synergistically, producing much greater effects in combination than they may independently (74).

NATURAL PCB DEGRADATION

PCBs are among the most stable organic compounds known. Their excellent thermal stability, resistance to extremes of pH, low dielectric constant, chemical unreactivity, and especially their non-flammability made them popular in a wide variety of industrial applications. It is this same general inertness which also makes them extremely recalcitrant to degradation. Although the rates and quantities are not known, microbial metabolism is believed to be one of the major routes of PCB degradation in the environment, and both aerobic and anaerobic processes are involved (15, 29).

In anaerobic environments, the major contribution of microbes is reductive dechlorination of PCBs. Since the first report of such a process (18), much work has been concentrated on understanding anaerobic dechlorination, through the congener-specific analysis of PCBs in a number of laboratory and *in situ* studies. Several distinct patterns of anaerobic dechlorination have been identified, ranging from very selective dechlorination of only certain congeners, to the removal of nearly all *meta-* and *para-* chlorines of complex PCB mixtures (Table 1).

Table 1. Reductive dechlorination processes

Dechlorination Process	Specificity
C	[combination of processes M and Q]
H	flanked *para* Cl, doubly flanked *para* Cl *meta* Cl of 2,3,4-CB
H'	flanked and doubly flanked *para* Cl meta Cl on 2,3-CB, 2,3,4-CB, and possibly 2,3,6-CB
M	flanked and unflanked *meta* Cl
N	flanked *meta* Cl, doubly flanked *meta* Cl
P	flanked *para* Cl, doubly flanked *para* Cl
Q	flanked and unflanked para Cl meta Cl of 2,3-CB and maybe 2,3,4-CB

Table adapted from Bedard & Quensen, 1995 (15)

Several factors influence whether a given chlorine atom will be removed from any particular congener, including **a)** the microbial populations present, **b)** the position (*ortho*, *meta*, or *para*) of the chlorine relative to the opposite phenyl ring, **c)** the surrounding chlorine configuration, **d)** the chlorine configuration on the opposite ring, and **e)** the incubation conditions (the Aroclor added, temperature, carbon substrate and availability, electron acceptors present, salinity, oil, other contaminants, etc.) (15). Incubation temperature has been proposed to be major influence during anaerobic dechlorination (82). The anaerobic removal of *ortho*- chlorines appears to be an especially refractile process, having been recently reported for estuarine sediments, and described as a "rare and unique" activity (17). In fact, the most abundant products of anaerobic dechlorination processes are all chlorinated at the *ortho* position (15, 82) (Table 2).

Table 2. Major products of anaerobic PCB dechlorination

Monochlorobiphenyls		*2 CB
Dichlorobiphenyls	*2,6 CB	2-3' CB
	*2-2' CB	*2-4' CB
Trichlorobiphenyls	2,4-2' CB	2,4-4' CB
	2,5-2' CB	2,5-3' CB
		2,5-4' CB

*Note: these congeners together make up over 80% of total PCBs by mass in samples of Aroclor 1242 following anaerobic dechlorination (by process C)

The discovery and confirmation of anaerobic dechlorination provides a means of modifying the highly chlorinated congeners which are recalcitrant to aerobic PCB metabolism. Neither the less-chlorinated congeners produced from reductive dechlorination, nor the carbon skeleton of PCBs are transformed under anaerobic conditions, and they accumulate as end-products. In tandem with an efficient aerobic degradative process, reductive dechlorination may provide a means for complete mineralization of these compounds. This idea of creating such a two-phase aerobic/anaerobic system for total degradation of PCBs has been proposed by a number of investigators (2, 11, 38, 43).

Many reports describing the aerobic biodegradation of PCBs have been published, primarily on the basis of disappearance of these compounds, or of certain specific congeners (14, 19, 32, 51). While disappearance of PCBs has been described in many cases, it is important to differentiate between cometabolic transformation and mineralization, since both processes can result in disappearance of PCBs. Until recently, little distinction has been made in the literature.

During cometabolism, biphenyl-degrading microbes fortuitously break down susceptible congeners and the degradative dead-end products accumulate. These

compounds are mainly chlorobenzoates, which PCB-cometabolizers are not capable of degrading further, and the pentadiene products of ring cleavage which may also be chlorinated (5, 51, 56), Other chloroaromatic end products have also been observed, including various hydroxylated intermediates of the biphenyl degradation pathway (10, 24, 56). PCB transformation by cometabolism (via the biphenyl degradative pathway, see below) is typically slow because the initial metabolism of the biphenyl ring structure provides no carbon or energy to the degradative microbes, which are rarely able to metabolize the chlorinated products they produce.

A few microbes have been reported to grow, albeit poorly, on certain chlorobiphenyls. However it is important to note that these substrates all contained one non-chlorinated ring. Since there was an accumulation of chlorobenzoates and no release of chloride, growth most likely occurred through metabolism of the pentadiene product which resulted from cleavage of the nonchlorinated ring (3, 5, 14, 32, 33, 38). This type of transformation process, while serving to break down biphenyls into simpler compounds, is ineffectual for the elimination of chlorinated aromatics, or even removal of the chlorines which make them so recalcitrant. Little attention has been focused on the fate of the pentadiene product of PCB cometabolism, although a set of genes from *Pseudomonas* sp. LB400 encoding genes for metabolism of the pentadiene into TCA cycle intermediates has been tentatively identified (45). A region of similar size and unknown function within the *bph* operon of *Pseudomonas putida* KF707 is believed to encode the same pathway (39, 45).

In contrast to cometabolism, bacteria able to grow on chlorobiphenyls have shown significantly higher rates of PCB degradation, without the concomitant accumulation of chlorinated aromatic compounds as end products. In this case, the chlorobiphenyls are a source of carbon and energy for the microbes that are able to break them down. To date, only monosubstituted chlorobiphenyls have been completely mineralized by bacteria,

primarily 4-chlorobiphenyl (4-CB), by both mixed (30, 38, 51, 66, 72) and pure cultures (10, 66, 75). More significantly, there has been a dearth of strains capable of growth on 3-CB, and especially 2-CB. Havel and Reineke (38) reported several isolates which grew readily on 4-CB, but only poorly on 2-CB and 3-CB with no elimination of chloride from any of these substrates. Bedard *et al.* (14) reported an *Alcaligenes eutrophus* strain H850 capable of slow growth on 2-CB, and poor growth on 4-CB and 3-CB, although there was no mention of chloride release or chlorobenzoate production. Likewise, an isolate of Hickey and Focht (42) was also capable of growth on 2-CB without release of chloride. None of these strains were capable of growth on chlorobenzoates. The abundance of 4-CB degradation compared to that of 2-CB and 3-CB may stem from the relative toxicity of the products of the corresponding chlorobenzoates (38) (see below for more detail).

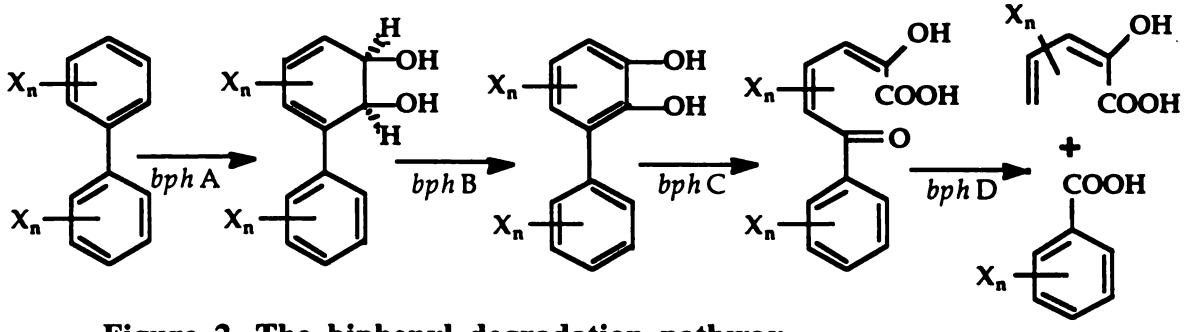

Figure 2. The biphenyl degradation pathway
The pathway for degradation of biphenyl and chlorobiphenyls is shown above. The genes coding for each step are printed below the arrow for each reaction. In the case of biphenyl, X represents only hydrogen atoms, but for chlorobiphenyls, X represents at least one chlorine atom per molecule. As shown, chlorobiphenyls may produce both chlorobenzoates and pentadienoates which are chlorinated. The degree of substitution (*i.e.* number and positions of chlorine atoms) tolerated by the *bph* operon varies among different species and strains.

Microbial attack on PCB molecules typically proceeds via the same oxidative route used for the degradation of biphenyl. The genes encoding this pathway appear to be highly conserved in a number of diverse organisms, even between Gram-positive and Gram-negative strains, suggesting widespread horizontal transfer of these genes (16, 32, 33, 45,

54, 65). The products of the *bph* operon typically catalyze a 2,3- dioxygenase attack and subsequent *meta* cleavage of one ring of the biphenyl molecule (Fig. 2).

In the case of chlorobiphenyls this attack usually occurs on the less-chlorinated or non-chlorinated ring, although the ring specificity of this initial attack varies between strains (13). In addition, an unusual 3,4- dioxygenase activity has been observed in certain bacterial strains (14, 62), and is suspected to allow improved degradation of certain congeners, particularly those substituted at the *meta*- and *ortho*-positions which are resistant to traditional 2,3-dioxygenase attack. Recently the *bphA* genes of various biphenyl-degrading organisms were compared, for insight into their congener specificities (62). The *bphA* gene encodes the large subunit of the terminal iron-sulfur protein of biphenyl dioxygense, which performs the initial oxidation of biphenyl, and largely determines the congener specificity of the enzymes. Sequence analysis and site-directed mutagenesis revealed that specific amino acid changes within the carboxy terminus of the enzyme could dramatically increase substrate range.

The number and position of chlorine atoms on the biphenyl skeleton affects its metabolism by microbes, especially if chlorination occurs on the 2- or 3- positions which encounter the initial dioxygenase attack (11). Many differences in substrate specificity occur among strains. In one case, fortuitous dehalogenation during the initial dioxygenase attack was reported (4). The positions of any chlorines present on the molecule may affect the enzymatic reaction catalyzed by the *bph* enzymes (11, 32, 33).

CONSTRUCTION OF PCB-DEGRADING STRAINS

Given the apparent inability of any single bacterial isolate to metabolize PCBs, the idea to combine the complementary pathways of PCB cometabolism and chlorobenzoate

degradation in a single transgenic microbe was proposed (30). Efforts to expand the substrate range of a bacterium through genetic engineering had begun in the late 1970's. In the first use of a transgenic strain for degradation of xenobiotics, Reineke and Knackmüss demonstrated that the introduction of certain genes of the TOL plasmid from *Pseudomonas putida* mt-2 could extend the substrate range of another strain of *Pseudomonas* to degrade 4-chloro- and 3,5-dichlorobenzoate, compounds which the parent strain could not metabolize (69, 70). It was only a matter of time before attention focused on employing these techniques for PCB degradation.

To date, there have been several attempts at engineering PCB-degrading strains (a more detailed review of these efforts can be found in reference 80). These efforts have followed two similar strategies, either introduction of biphenyl- or chlorobiphenyl-degradation genes (*i.e. bph* or related genes) into bacteria capable of degrading chlorobiphenyl metabolites, or introduction of genes for metabolite degradation (typically chlorobenzoate or chlorocatechol) into biphenyl-degrading strains. In most cases, some sort of mating procedure was used to transfer genetic material between strains. However, in one report the *bph* genes from three different bacteria were cloned into a plasmid vector and transferred via a transposable element (75). In addition to recombinant strains able to degrade PCBs, there have been a number of reports describing expression of *bph* genes in bacterial host strains such as *Escherichia coli* (4, 55, 62, 65) and *Pseudomonas* sp.(4, 30, 39), capable of various degrees of PCB transformation.

Successful efforts at engineering novel PCB-degrading microbes have been limited to a very narrow range of substrates. The first such transgenic microbe was a *Pseudomonas* sp. capable of mineralizing 3-CB, resulting from transfer of chlorocatechol-degrading genes into a biphenyl-degrading strain by a simple mating (61). Shortly thereafter, another recombinant strain was reported with the ability to grow well on 4-CB, but poorly on 2-CB, 3-CB, 2,4-dichlorobiphenyl (DCB) and 3,5-DCB (38). A mating

experiment reported the following year between a chlorobiphenyl-degrading *Arthrobacter* strain and a chlorobenzoate-degrading *Pseudomonas* host yielded a transconjugant capable of growth and chloride release on 2-CB and 2,5-DCB, although both growth and chloride release were slow, requiring over two weeks to mineralize 2 mM 2-CB (43). Until this time, transgenic strains were only capable of mineralizing monochlorobiphenyls, or dichlorobiphenyls with both chlorines on the same ring. In addition, the transgenic strains which resulted from this research provided little, if any, progress towards the development of any sort of viable remediation process. The 3- and 4-CBs upon which these strains grew bore little resemblance to the *ortho*-substituted products of anaerobic dechlorination, processes which transform PCBs into mixtures more amenable to aerobic degradation (see Table 2).

The only reported recombinant bacterium with the ability to mineralize a PCB congener chlorinated on both rings is *Pseudomonas acidovorans* M3GY, which is able to grow on 3,4'-DCB (58). Several intermediates were identified in the degradative pathway of this substrate, including 4-chlorobenzoate (4-CBA), 4-chlorocatechol (4-CC), and chloroacetate (Fig. 3). Although this strain utilized 3,4'-DCB as a growth substrate, it could not grow on 4-CBA, a key intermediate in dichlorobiphenyl metabolism. Neither could it grow on 3,3'-DCB, presumably because of the production of 3-chlorocatechol, which would be toxic to the cell if metabolized by the *meta* cleavage pathway (see below). Like the recombinant strains before it, M3GY would have little or no effect on the congeners remaining after anaerobic dechlorination. In fact, the *meta*- and *para*- substituted congeners which it could metabolize are among the first PCBs dechlorinated in anaerobic sediments (15), making them less important for aerobic degradation in any potential two-phase process.

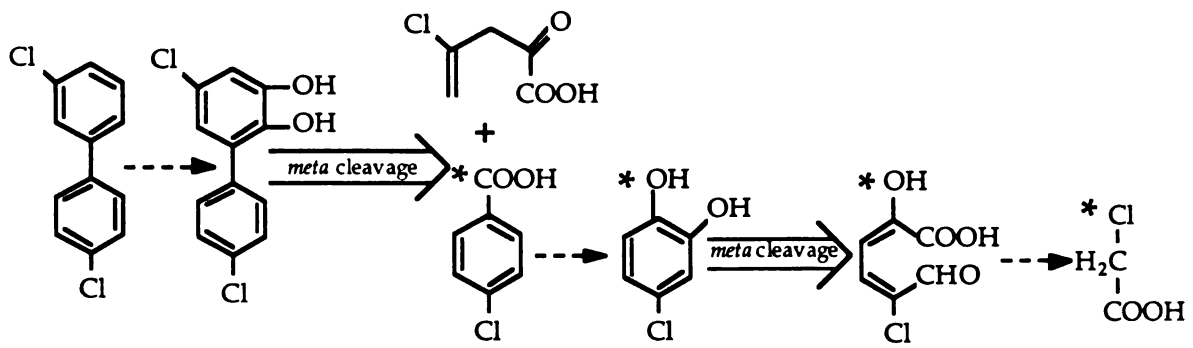

Figure 3. The M3GY degradative pathway
The proposed pathway for degradation of 3,4'-dichlorobiphenyl in *Pseudomonas acidovorans* M3GY. Those compounds marked with an asterisk (*) were identified by GC-MS or GC-IR-MS. The remainder of the pathway is proposed according to the *meta* fission pathway (from ref. 58).

PROBLEMS IN TRANSGENIC DEVELOPMENT

While the concept of combining the metabolic pathways for chlorobiphenyl transformation with chlorobenzoate degradation in a single organism has been endorsed by a number of researchers, this approach has met with limited success. A major early impediment to the development of transgenic, broad-substrate PCB degrading strains was the fundamental incompatibility of the *ortho* and *meta* cleavage pathways, especially in dealing with the chlorocatechol intermediates of chlorobenzoate degradation. More recently, the concern has shifted to the lack of dechlorination genes and pathways, and their integration into PCB-cometabolizing strains.

One problem recognized early in the discussion of transgenic PCB degradation was the problem of conflicting ring-cleavage pathways. Just as unsubstituted aromatics like benzoate, the product of the upper pathway for degradation of biphenyl (the *bph* operon), are funneled into the central metabolic intermediate catechol, so their chlorinated analogs are converted into the corresponding chlorocatechols. While catechol itself may be metabolized by either the *ortho* or the *meta* cleavage pathways, chlorocatechols generally cannot. The

construction of transgenic bacteria containing both the catechol *ortho* and *meta* cleavage pathways demonstrated that simultaneous functioning of these two pathways could not occur in the presence of certain chlorinated compounds like chlorocatechols. While the simultaneous induction and functioning of ring-cleavage enzymes of both pathways has been noted before (36), this is simply not possible in the presence of these compounds.

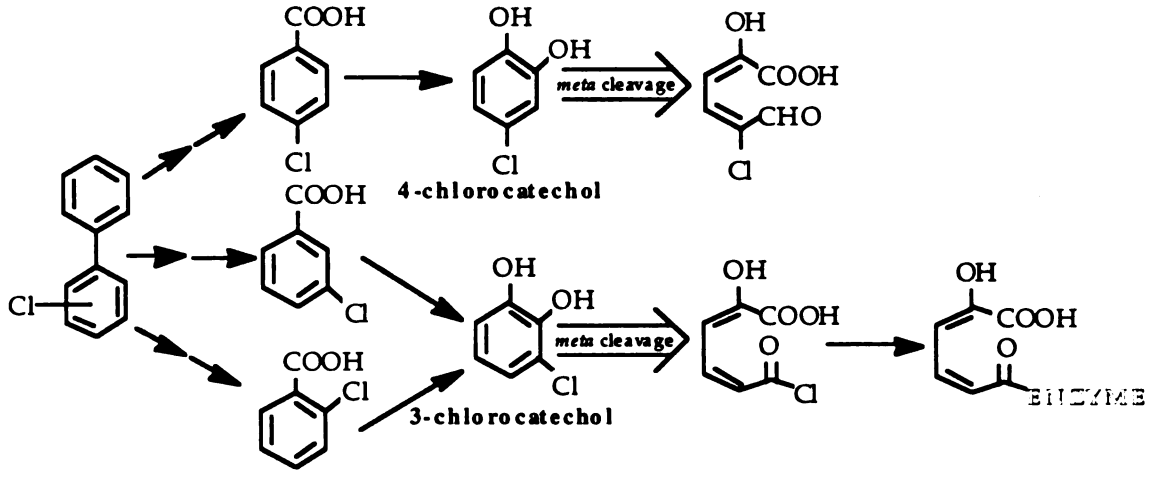

Figure 4. The *meta* cleavage of biphenyls
Proposed pathways of metabolism of various monochlorobiphenyls via *meta* cleavage. The *para-* chlorinated compounds yield 4-chlorocatechol as an intermediate, which is benign to *meta* cleavage enzymes. The *ortho* and *meta* substituted CBs and CBAs produce a 3-chlorocatechol intermediate, an acyl chloride, which can react with cellular macromolecules following *meta* cleavage.

This phenomenon was first noted following the construction of transgenic strain WR211 (69), when researchers observed an inexplicable loss of enzyme activity. This strain was the result of mating TOL plasmid pWW0, encoding genes for the catechol *meta* cleavage pathway, into *Pseudomonas* sp. B13 (WR1) which harbored its own chromosomal genes for 3-chlorobenzoate (3-CBA) degradation via the chlorocatechol modified *ortho* cleavage pathway. The resulting transconjugant displayed an initially puzzling loss of 2,3-catechol dioxygenase activity, the key enzyme in the *meta* cleavage pathway of catechol. Subsequent investigation revealed the toxic effects of 3-chlorocatechol to the *meta*-cleavage pathway. 3-Chlorocatechol is formed as an

intermediate during 3-CBA degradation in these cells, Upon ring cleavage of this intermediate by the 2,3-catechol dioxygenase, an extremely reactive acyl halide is formed which could condense to cellular macromolecules such as the cleavage enzyme itself, or other molecules in close proximity (see Fig. 4). Reaction of the acyl halide with this enzyme would effectively kill it. Later work by other investigators revealed similar processes, with the same toxic effect in other organisms (6, 9). In contrast to the toxicity of these 3-chlorocatechols, the 4-chlorocatechols produced from the metabolism of other congeners are benign to the *meta* cleavage pathway (7) (Fig. 4). One notable exception to this rule is the a recently described catechol 2,3-dioxygenase from *Pseudomonas putida* GJ31, which apparently has a *meta*-cleavage enzyme which is resistant to inactivation by the acylchloride (54).

IN SITU REMEDIATION

In considering the use of genetically engineered microorganisms (GEMs) for release into a natural ecosystem, there are a number of questions which must be answered. Aside from the regulatory concerns of such an introduction, there are scientific issues which must be addressed first. Most of these questions are applicable even for the release of GEMs into closed systems. Dwyer *et al* (26) outlined five basic factors to be considered prior to release of a GEM:

- survival of the GEM in its target environment
- stability of the cloned genetic material
- potential for transfer of genetic material to indigenous microorganisms
- efficient functioning of the GEM for its designated purpose
- effects upon the target ecosystem

Perhaps the greatest uncertainty regarding GEMs has been that of their ability to survive *in situ*. Cultivation of microbes in a laboratory environment, addition of foreign genes, and potential disruption of normal physiological process have been suggested as causes for reducing competitiveness of an organism in a natural environment (79). One early study describing the fate of various microbial strains in model ecosystems yielded mixed results. Seven different species of potential interest in genetic engineering, were inoculated into lake water, sewage, or soil and showed differing patterns of survival in each these environments, attributed to their varying abilities to survive abiotic stresses, maintain viability when starved, and coexist with antagonists (53). However, a subsequent study on the persistence of plasmid-bearing microbes in aquifer material yielded more consistent, and encouraging, results. Several species of bacteria, including two strains of *Pseudomonas putida* bearing plasmids TOL and RK2, which were introduced into experimentally contaminated aquifer material showed stable populations and concomitant removal of pollutants throughout an 8-week incubation period (46). Moreover, the inoculated strains stably maintained their plasmids even in the absence of selective pressure for specific genotypes.

The stability of introduced genetic material in GEMs has been under constant scrutiny. Although parameters such as genetic stability and plasmid maintenance are variable with individual strains and plasmids, previous efforts at genetically engineering microbes has shown these concerns to be exaggerated. At least one report has noted significant loss of a catabolic plasmid under non selective conditions *in vitro* (49). However, a number of studies with a variety of organisms and vectors have demonstrated that microorganisms bearing foreign DNA on recombinant plasmids can be stably maintained over extended periods of time (55, 64, 68). Genetic stability may be quite variable and should necessarily be examined on an individual case-by-case basis.

Horizontal transfer of genetic material from GEMs has been considered the greatest potential hazard of deliberate GEM release, especially for recombinant plasmids. Although direct intermicrobial genetic transfer is a valuable technique *in vitro*, even having been used to construct bacterial strains for xenobiotic catabolism (3, 38, 43, 58, 61, 69, 74), it is still considered a risk *in situ*. Transfer of recombinant plasmids in environmental microcosms has been reported previously, even for some which are not self-transmissible, through mobilization by indigenous plasmids (57, 64). One possibility rarely considered is the potential for beneficial results of such genetic transfer. In at least one case, native bacteria which had acquired a recombinant catabolic plasmid exhibited higher rates of xenobiotic breakdown than the introduced strain (57).

Efficient functioning of GEMs *in situ* is critical for effective experimental release. One of the most thoroughly studied recombinant microorganisms is *Pseudomonas* sp. strain B13, engineered for the simultaneous biodegradation of chloro- and methylbenzoates (71). This organism significantly enhanced the rate of substituted aromatic degradation in microcosms of two different aquatic sediments (41, 67), activated sludge (64), and marine sediment (52). However, there have also been several reports of a GEM failing to function adequately *in situ*, even after expressing significant activity *in vitro* (35, 57). These failures underscore the necessity of extensively testing GEMs in a simulated natural environment prior to actual release. Appropriate microcosms can simulate natural environments, and may retain enough of the complexity of the target ecosystems to provide accurate predictions of GEM behavior prior to actual release (64).

A final consideration pending introduction of GEMs into a natural environment, is the effect of the introduction on that environment itself. While for biodegradative organisms the removal of target pollutants would represent a profound impact on the target ecosystem, this would be the intended function of the organism. Assessment of potential negative impacts may not be readily available, given the difficulty of reproducing

sufficiently reliable environmental models in a laboratory setting. There has been at least one previously reported instance of a negative impact, where the introduction of a plasmid-bearing *Pseudomonas* strain converted 2,4-dichlorophenoxyacetate (2,4-D) into the toxic metabolite 2,4-dichlorophenol (2,4-DCP), resulting in adverse effects on the native microbial populations (73). Such an example illustrates the potential impact of GEM introduction, and underscores the need for understanding the *in situ* activity of an organism prior to environmental release.

In the field of PCB degradation, research on introduction of microorganisms has been quite limited. Early work on bioaugmentation for enhancing PCB degradation included inoculation with PCB-metabolizing strains, as well as analog enrichment. An early study performed in contaminated soils revealed inoculation with the PCB-degrading bacterium *Acinetobacter* (now *Rhodococcus*) P6 gave no significant improvement over native microflora in the mineralization rate of Aroclor 1242 (20). Degradation rates were significantly improved following amendment with biphenyl, and even greater when coupled with microbial inoculation. While amendment of PCB-contaminated samples with biphenyl may enhance degradation rates, this practice is undesirable, since biphenyl itself is an EPA priority pollutant, in addition to being an expensive cosubstrate for use in large scale field applications.

The first *in situ* study of PCB degradation evaluated several variables on PCB degradation in soil (59). After 18 weeks, control samples showed no evidence of PCB removal, while plots inoculated with *Pseudomonas* (now *Burkholderia*) strain LB400 showed a 10% reduction of PCBs with stirring, and a 25% reduction in the top 3 cm of soil without stirring. These rates are about 50% of what had been observed in laboratory experiments, and much of this difference was attributed to poor control of soil temperature, soil desiccation, and subsequent decrease in viability of the inoculum. A similar *in situ* study published the same year concluded that treatment of contaminated soil with water,

minerals, and yeast extract stimulated resident microorganisms to degrade 4-chlorobiphenyl as efficiently as an inoculum of the known PCB-degrading strain, *Alcaligenes* A5 (44). In this study, the addition of biphenyl as a substrate analog enriched the soil for biphenyl-degrading microorganisms, and increased microbial activity, as measured by incorporation of acetate into cellular lipids, but did not increase the rate of 4-CB mineralization.

Bioavailability of PCBs has been considered one of the limiting factors in degradation of these compounds. A strong association with the organic component of sediments and soils may leave PCBs inaccessible for microbial metabolism. The utility of surfactants to increase the aqueous solubility of nonionic organic compounds (including PCBs) has been demonstrated in soil-water systems (76, 77). One study reported that while use of surfactants increased desorption of PCBs from sand by 30 to 85%, as compared to water only (81). However, the authors concluded that while the use of surfactants dramatically improved solubility of PCBs, their inhibitory effects towards PCB-degrading microorganisms, even at low concentrations, precluded their use in a combined degradation strategy. A subsequent study in which soil samples were inoculated with both a PCB-degrader and a surfactant-producing strain, revealed no increased PCB degradation. The effect of biphenyl additions was also examined, and resulted in the highest rate of degradation when a low level of the compound was maintained, most likely by inducing the *bph* operon for PCB cometabolism (8).

Perhaps the most complete *in situ* study of PCB biodegradation was undertaken in the sediments of the upper Hudson River, long known to be contaminated with PCBs following years of dumping from industrial sources upstream. These experiments revealed that indigenous biphenyl-metabolizing populations were active against mono- and dichlorobiphenyls, but most isolates could not degrade the more highly-chlorinated congeners. Introduction of the well-characterized *Alcaligenes eutrophus* H850, which is

better able to attack the more highly-chlorinated congeners, gave no significant improvement in PCB degradation, likely due to its poor survival *in situ* (36).

SIGNIFICANCE

Several lines of evidence suggest that PCB degradation should occur in the environment, yet these compounds accumulate and persist in soils and sediments. The aerobic metabolism of PCBs is an energetically favorable process, and a potential source of carbon and energy for microbes. In addition, many bacterial strains capable of cometabolizing a wide range of PCBs have been isolated. Some of these isolates possess broad substrate specificities, and many have potent activities against these compounds, although attack on more highly-chlorinated congeners is still quite limited. Chlorobenzoates, the major breakdown products of PCB metabolism, have been described as readily biodegradable under aerobic conditions (37, 40, 42), and in PCB-contaminated sediments, the low concentrations of CBAs found are consistent with these studies (28). In spite of these observations suggesting that PCBs should degrade naturally, they continue to be troublesome compounds.

One major impediment to successful breakdown of PCBs exists in the first steps for their aerobic catabolism. Many bacteria have been isolated which possess the ability to cleave the carbon skeleton of PCBs into simpler compounds via the biphenyl degradative pathway, but this is typically a fortuitous reaction accomplished by cells already grown on some other substrate, usually biphenyl. The organisms which perform this reaction obtain no benefit from it. Addition of biphenyl to PCB-contaminated soils, while improving native PCB degradation, is undesirable. Aside from its status as an EPA priority pollutant, biphenyl is prohibitively expensive for broad application as a co-substrate in practical remediation efforts. Genuine growth on PCBs would not require such additions, and may

potentially provide energy necessary for cometabolism of congeners which do not permit growth. In fact, oxidation of PCBs may be an unexploited ecological niche in contaminated environments.

The primary obstacle to PCB metabolism however, has been thought to be the incompatibility of degradative pathways. The *meta* cleavage which occurs during the breakdown of biphenyls to benzoates, and which may occur during catechol metabolism, generates toxic acyl chlorides from 3-chlorocatechols, which irreversibly inactivate the same 2,3-dioxygenases (9). While at least one *meta* cleavage enzyme resistant to this inactivation has been isolated and described (54), this activity remains rare and is not well characterized. Avoiding initial production of toxic intermediates remains the most widely applicable, and perhaps simplest, solution to this problem.

This study describes the *in vitro* construction of a bacterium capable of growth on PCBs accomplished through the introduction of specific dehalogenases to avoid toxic intermediates. These novel pathways are be aimed at funneling chlorinated benzoates to non-chlorinated analogs for which degradative pathways are already present in a host organism, capable of degrading biphenyl, as well as cometabolizing a wide range of PCB congeners.

Such a strategy and methodology for constructing a transgenic PCB-degrading microbe differs from prior efforts in several ways. While a microbe which can degrade all existing PCB congeners is a bit unrealistic, this recombinant microorganism is intended to be a part of a two-phase degradative plan, in which an anaerobic consortium would first be employed to dechlorinate the most recalcitrant congeners of PCB mixtures to lesser-chlorinated congeners, followed by aerobic degradation of these lesser-chlorinated congeners by the recombinant organism. Given such a scheme, the target congeners for the recombinant microbe are clear: the *ortho*-chlorinated PCBs which constitute the majority of products following anaerobic dechlorination, in contrast to the seemingly random

substrates of earlier transgenic microorganisms. Unlike some prior efforts, this work stresses the use of recombinant DNA technology to introduce specific genes and functions into host bacteria. This allows a clear understanding of what functions are being transferred into the recombinant organisms, and what their physiological fate is within a host cell. The genes introduced serve to complement existing pathways. Since the host organism in these studies already possesses pathways for biphenyl, benzoate, and catechol degradation, as well as central pathways for the post-cleavage processing of these compounds, the introduced genes are not intended to replace any of these. Rather, they are intended to complement them by modifying chlorinated substrates to biochemically benign analogs amenable to breakdown by existing pathways.

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CHAPTER 2

CONSTRUCTION AND CHARACTERIZATION OF TWO RECOMBINANT PCB-DEGRADING BACTERIAL STRAINS

INTRODUCTION

The accumulation of polychlorinated biphenyls (PCBs) in soils and sediments worldwide first attracted attention in 1966 (16, 17), and has led to intense efforts aimed at understanding their breakdown in nature. The chemical stability which made PCBs popular for a wide variety of industrial applications has also made them very resistant to degradation, and a persistent environmental concern.

Although rates and quantities are uncertain, microbial activity is believed to be a major route of PCB degradation (5, 11). Under anaerobic conditions, such as those existing in freshwater and marine sediments where PCBs often accumulate, the primary contribution of bacteria is reductive dechlorination, which may remove nearly all chlorines at the *meta-* and *para-* positions. The resulting *ortho*-chlorinated congeners often accumulate in PCB-contaminated sediments (5, 9). Although anaerobic removal of *ortho* chlorines has been reported recently, it has been described as a "rare and unique" activity (7, 33).

Aerobic PCB degradation occurs mainly through the fortuitous action of biphenyl-degraders, however both the degradative ability of individual strains and their effectiveness against specific PCB congeners, vary greatly (3, 4, 24). Bacterial degradation typically proceeds via the oxidative biphenyl pathway encoded by the *bph* genes, resulting in the accumulation of chlorobenzoates as dead-end products. PCB transformation in this manner

is usually slow since the initial metabolism of the biphenyl molecule provides no carbon or energy to the degradative microbes, which rarely metabolize the chlorinated products they produce.

Given the apparent inability of any single bacterial isolate to metabolize PCBs, complementation of degradative pathways for PCB cometabolism and chlorobenzoate degradation in a single transgenic microbe has been proposed as a means to achieve complete degradation (12). To date, there have been several attempts at engineering PCB-degrading strains, primarily by mating strains with complementary metabolic activites.

This is the first study using introduction of specific dechlorination genes to effect growth on PCBs in a recombinant bacterium, in contrast to previous efforts at strain construction using matings and coculture. This approach offers the advantage that only peripheral enzymatic activities, *i.e.* dehalogenases, need to be added, rather than entirely new pathways, hence radical changes in the host organism may be avoided. The use of well-defined genetic fragments permits targeting of specific compounds for degradation, and prediction of expected intermediates and products.

While there have been some transgenic bacteria able to metabolize a few PCB congeners (13, 14, 20, 23), the *meta-* and *para-* substituted congeners upon which these strains grow bear little resemblance to the *ortho*-substituted products of anaerobic dechlorination, processes which make PCB mixtures more amenable to aerobic degradation. These strains are also limited by specificity for a single congener (20, 23), substrate concentration (13), or incomplete mineralization (13, 14, 20, 23). The results presented here suggest that manipulation of PCB-cometabolizing bacteria may be directed at the degradation of specific congeners of interest, such as those remaining after reductive dechlorination, and that a better understanding the regulation of biphenyl breakdown is needed.

MATERIALS AND METHODS

Chemicals: Chlorobiphenyls (CBs) used herein (99% pure), were purchased from AccuStandard (New Haven, CT). 2-Chlorobenzoate (2-CBA), 4-chlorobenzoate (4-CBA), and all other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Media. Bacteria were routinely grown at 30°C in LB medium (22). Transformants were grown on mineral medium K1 (35) containing chlorobenzoates and chlorobiphenyls as indicated in text, and with 1.5% Bacto-agar added when grown on solid media. Tetracycline (10 $\mu g \cdot ml^{-1}$, w/v) was added where indicated. Chlorobenzoates were added to media from 0.5 M sterile-filtered stock solutions. Chlorobiphenyls were added to flasks from acetone stock solutions (188 $g \cdot liter^{-1}$), and the acetone was allowed to evaporate prior to addition of mineral medium.

Plasmid construction Three recombinant broad host-range plasmids were constructed from the RK2-derived low-copy plasmid pTJS75 (28). Cloning plasmid pSP329 was constructed by cloning the *Hae* II fragment from pUC18 (34), containing the pUC18 polylinker and *lacZ* α complementation fragment , into the *Hae* II site of pTJS75. Plasmid pE43 was constructed by cloning a 3.4 kb minimized functional fragment, bearing the *ohb* genes for oxygenolytic *ortho*-dehalogenation of chlorobenzoates, from *Pseudomonas aeruginosa* 142 (25, 26) into the *Sma* I site of plasmid pSP329. Plasmid pPC3 was constructed by cloning of the *fcb* genes of *Arthrobacter globiformis* KZT1 (25, 32, 36), which code for ATP and CoA-dependent dechlorination of 4-CBA, into the *Sma* I site of plasmid pSP329. Both plasmids contained the P_{lac} promoter upstream of the introduced genes (T.V. Tsoi, personal communication).

Strain construction. Host cells of *Comamonas testosteroni* strain VP44 (24) were made competent for electrotransformation by growth in LB medium to mid-log phase. The culture was centrifuged at 3000 x*g* for 10 min, and the resulting cell pellet was

resuspended in an equal volume of sterile, deionized water, chilled to 4°C. This procedure was repeated three times, followed by resuspension in a volume of 20% glycerol approximately equal to that of the cell pellet. Competent cells were divided into 100 µl aliquots, frozen in liquid nitrogen, and stored at -70°C until use, when thawed on ice.

Transformation was performed by addition of up to 1 µg of plasmid in sterile, deioionized water to cells and transferred to a chilled cuvette. Cells were exposed to current (180 kV in a BioRad E.coli Pulser, Model 1652102) and 1 ml of SOC medium (22) was immediately added. Following transformation, cells were grown overnight in SOC medium, then spread onto LB plates containing tetracycline (5 $\mu g \cdot ml^{-1}$). Colonies growing within two days were picked and transferred onto K1 plates amended with 5 mM chlorobenzoates, then incubated at 30°C. After eight weeks, colonies of VP44 presumptive for containing plasmids pE43 and pPC3 were visible on 2-CBA and 4-CBA plates, respectively, and were picked for further investigation. Strains were maintained on LB medium or K1 medium supplemented with chlorobenzoates, containing tetracycline (10 $\mu g \cdot ml^{-1}$), and routinely checked for ability to metabolize biphenyl, naphthalene, and benzoate.

Growth assays. Recombinant strains were tested for the ability to utilize chlorobenzoates and chlorobiphenyls by inoculation into 125-ml Erlenmeyer flasks containing the respective substrates (1 or 2 $g \cdot liter^{-1}$) and 25 ml of K1 medium. Flasks were placed on a rotary platform shaker and shaken at 200 rpm. Growth was measured by increased optical density (600 nm) measured on a Hewlett Packard Model 5284A diode-array spectrophotometer. Protein concentration, inorganic chloride, and chlorobenzoates were detected by removal of 1 ml samples from growing cultures.

Determination of inorganic chloride. Samples (1 ml) taken from liquid cultures for chloride measurement were centrifuged for 10 min at 13,000 x*g* in 1.5 ml Eppendorf tubes, prior to analysis. Inorganic chloride was measured colorimetrically,

using the mercuric thiocyanate procedure (6).

Determination of protein. Protein concentration was evaluated by the method of Lowry after alkaline hydrolysis (31). Samples were prepared by addition of 10 µl of 10 M NaOH, to 100 µl of culture fluid and incubating at 90°C for 10 min. Protein concentration was determined by comparison with bovine serum albumin (BSA) standards assayed under the same conditions.

Analytical methods. Samples (1 ml) taken from liquid cultures for chlorobenzoate analyses were filtered through 0.4 µm pore diameter filters, prior to analysis. Chlorobenzoates were analyzed by reverse phase HPLC, on a 30 cm HiBar LiChrosorb RP-18 column (10 µm) (E. Merck Darmstadt, Germany), using an acidified (0.15% H_3PO_4) 33% acetonitrile/water mixture, with a Gilson Holochromic detector set at 220 nm. CBA concentrations were based on authentic standards, calculated from peak areas as determined by a Model 3390A Integrator (Hewlett Packard, Corvallis, OR).

RESULTS

Following transformation, the recombinant strains were observed to be morphologically and phenotypically very similar to the parental strain *Comamonas testosteroni* VP44, appearing as motile, gram-negative rods. All recombinants retained the ability to grow on biphenyl, naphthalene, benzoate, 4-hydroxybenzoate, and catechol as previously described (24).

Recombinant colonies which arose on chlorobenzoates were picked and grown in liquid K1 medium containing chlorobenzoates. All recombinants maintained resistance to tetracycline, and the ability to grow on chlorobenzoates, even following repeated growth on nonselective media.

Growth of recombinant strains on chlorobenzoates and chlorobiphenyls was measured by increased optical density, chloride release, substrate disappearance, and protein concentration. Strain 44(pE43) grown on 2-CBA showed dramatic increase in A_{600} absorbance over a 4-day period (Fig. 1). During growth, the concentration of 2-CBA in the medium dropped below detectable levels, and stoichiometric amounts of inorganic chloride accumulated in the culture supernatant. Analogous results were observed for two lower concentrations of 2-CBA (2.5 mM and 5 mM), which exhibited complete substrate disappearance, proportional increase in A_{600}, and stoichiometric chloride accumulation. Neither the parental strain or recombinant 44(pPC3) could utilize or dehalogenate 2-CBA.

Recombinant strain 44(pPC3) grown on 4-CBA showed results comparable to 44(pE43) on 2-CBA, at concentrations of 4-CBA up to 10 mM (Fig. 2). As A_{600} rose over a 3-day period, the concentration of 4-CBA in the culture supernatant dropped below detection limits. Chloride accumulated to concentrations corresponding to initial levels of 4-CBA. Neither the parental strain VP44, nor recombinant 44(pPE3) was able to utilize or dehalogenate 4-CBA.

The parental strain VP44 exhibited growth on all three monochlorobiphenyls, but there was no release of chloride, and the corresponding chlorobenzoates accumulated in the culture supernatant. Increased substrate concentrations showed a corresponding increase in growth yield, as well as an increase in accumulated benzoates (data not shown). The host strain appears to have no ability to metabolize the chlorobenzoates, and the growth that did occur must come from the five-carbon product from the other (cleaved) ring.

Recombinant strains 44(pE43) and 44(pPC3) showed similar patterns of growth on 2-CB and 4-CB, respectively. Strain 44(pPC3) exhibited rapid growth on 4-CB, with transient production of yellow color and 4-CBA in the culture supernatant during log phase, with concomitant release of inorganic chloride (Fig. 3a). Maximal accumulation of

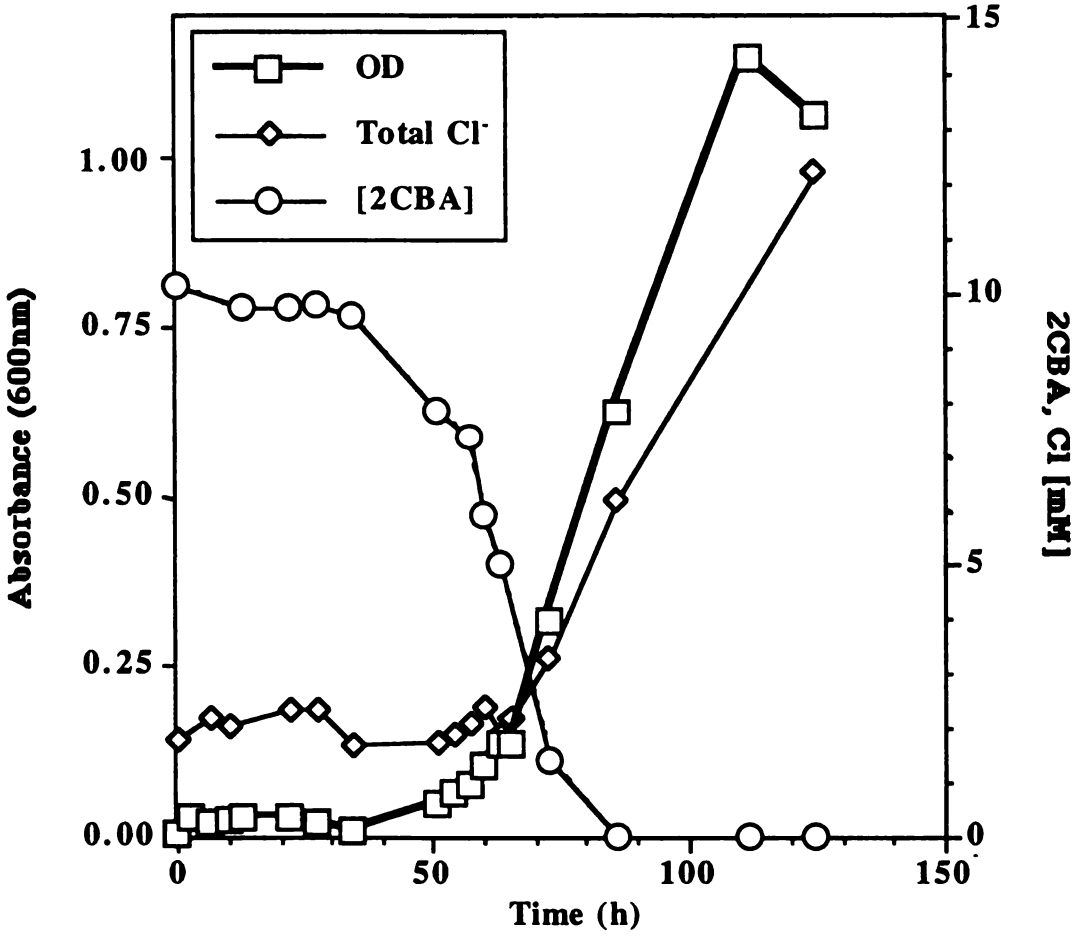

Figure 1. Growth of VP44(pE43) on 2-CBA
Growth of a recombinant strain VP44(pE43) on 10 mM 2-CBA, as measured by increase in optical density at 600 nm (left axis). The disappearance of 2-CBA corresponds to accumulation of chloride in the supernatant (right axis).

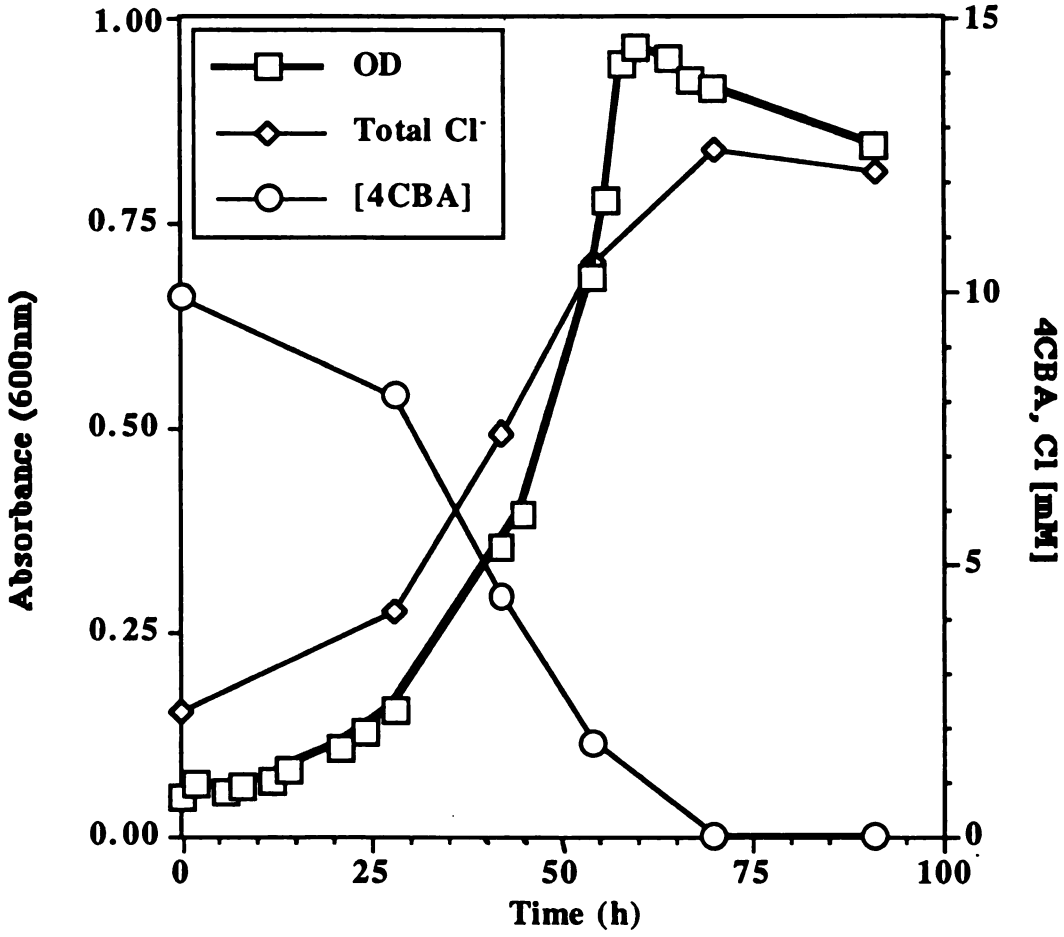

Figure 2. Growth of Growth of VP44(pPC3) on 4-CBA
Recombinant strain VP44(pPC3) growing on 10 mM 4-CBA, as measured by increase in optical density at 600nm (left axis). During growth, the disappearance of 4-CBA can be observed, corresponding to accumulation of chloride in the supernatant (right axis).

4-chlorobenzoate was about 50 μM, or 5% of the original concentration of 4-CB in the medium. In contrast to strain 44(pPC3) growing on 4-CB, growth of strain 44(pE43) on 2-CB was slightly slower with a greater accumulation of 2-CBA (125 μM, or 12.5% of original 2-CB concentration) which persisted slightly longer (Fig. 3b). All strains exhibited growth on 3-CB, with no release of chloride, a lower growth yield, and 3-CBA accumulated stoichiometrically in the culture supernatant (data not shown).

Accumulation of protein and chloride by the recombinant strains on these substrates is shown in Table 1. Protein levels in chlorobenzoate-grown cultures of the recombinant strains were similar to those found in benzoate-grown cultures, with stoichiometric accumulation of chloride relative to starting substrate concentrations. VP44(pE43) produced approximately the same concentration of protein on 2-CB as compared to growth on biphenyl and released stoichiometric amounts of chloride. Protein yield and chloride release were linear up to at least 10 mM 2-CB (Fig. 4), corresponding to approximately 68 μg protein•ml^{-1}•$mmol^{-1}$ 2-CB•$liter^{-1}$. When this recombinant was grown on 4-CB, the inappropriate substrate for this recombinant, it produced no chloride, roughly half as much protein, and stoichiometric concentrations of 4-CBA accumulated.

Recombinant strain VP44(pPC3) yielded comparable protein concentrations when grown on either 4-CBA or benzoate, with chloride accumulation observed in 4-CBA cultures. Growth on 4-CB was marked by stoichiometric chloride release, and protein yield comparable to that observed on biphenyl. Protein yield on 4-CB was linear only at low concentrations of substrate, with maximal production reached at up to 5 mM 4-CB (Fig. 4). Likewise, when this recombinant was grown on the inappropriate substrate, 2-CB, no chloride release was observed, and only half as much protein was detected, with accumulation of 2-CBA in the supernatant.

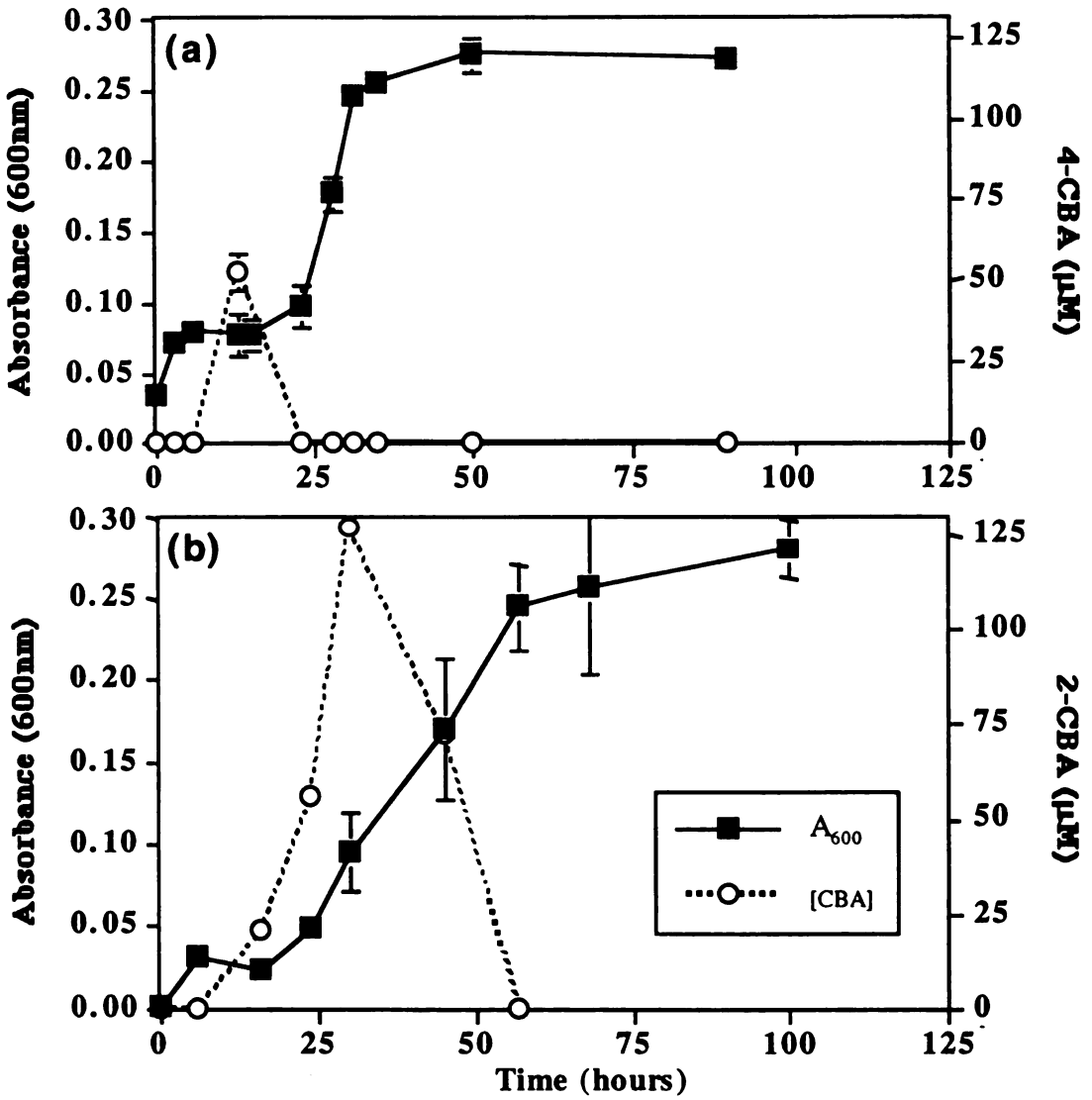

Figure 3. Growth of Recombinants on Monochlorobiphenyls
(**a**) Growth of VP44(pPC3) on 1 mM 4-CB. Lag time appears short, with rapid metabolism of substrate, transient accumulation of 4-CBA. (**b**) Growth of VP44(pE43) on 1 mM 2-CB. Lag time appears to be longer than for the other recombinant strain, and appearance of the chlorobenzoate intermediate persists longer, and to a higher concentration. All data represent mean values of three replicate cultures.

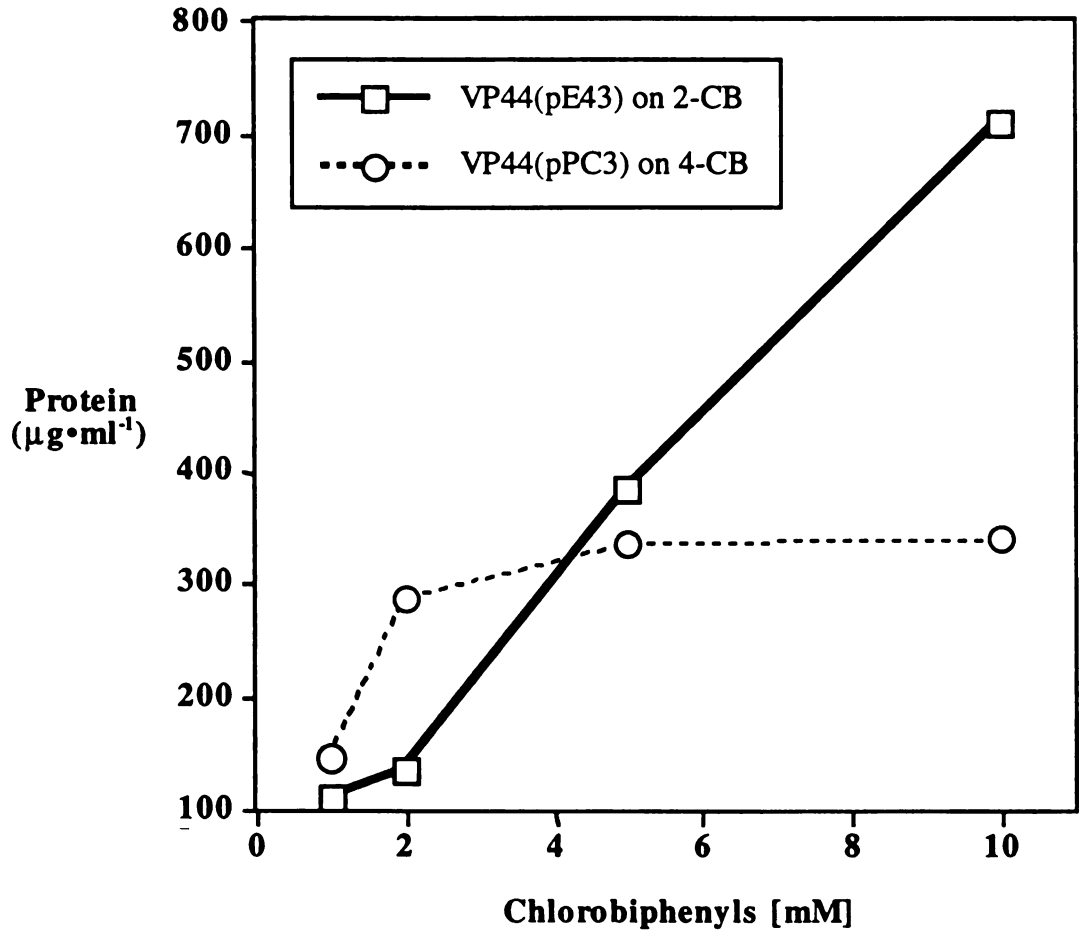

Figure 4. Protein Yield with Increasing Substrate Concentration
Recombinant strains VP44(pE43) and VP44(pPC3) were grown in liquid medium K1 containing from 1 to 10 mM concentrations of 2-CB and 4-CB, respectively, and observed for protein production. Accumulation of chloride in the medium corresponded with protein yield (data not shown). A black precipitate formed in cultures containing 4-CB at concentrations of 5 mM and 10 mM.

TABLE 1. Protein and chloride accumulation by recombinant strains VP44(pE43) and VP44(pPC3) grown on benzoates and biphenyls

Substrate (mM)	44(pE43)		44(pPC3)	
	Protein [$\mu g \cdot ml^{-1}$] (±SD)	Chloride [mM] (±SD)	Protein [$\mu g \cdot ml^{-1}$] (±SD)	Chloride [mM] (±SD)
Benzoate (5)	181 (39)	0.4 (0.1)	228 (24)	0.7 (0.1)
2-CBA (5)	169 (15)	5.3 (0.4)	<10	<0.1
2-CBA (10)	373 (16)	10.1 (1.2)	<10	<0.1
4-CBA (5)	<10	<0.1	158 (9)	7.1 (0.2)
4-CBA (10)	<10	<0.1	257 (16)	10.4 (0.1)
Biphenyl (2)	154 (10)	0.5 (0.1)	188 (9)	0.1 (0.1)
2-CB (2)	177 (4)	1.9 (0.2)	69 (7)[b]	0.1 (0.1)
4-CB (2)	84 (6)[a]	0.7 (0.3)	160 (39)	1.8 (0.2)

[a]4-chlorobenzoate (4-CBA) accumulated to 2.02 (±0.21) mM in the supernatant
[b]2-chlorobenzoate (2-CBA) accumulated to 1.88 (±0.13) mM in the supernatant

Each recombinant strain was introduced as a 1% inoculum into several flasks containing individual PCB congeners. No growth or dechlorination was observed on any of the following compounds: 2,4-CB, 2,6-CB, 2-2'-CB, 2-4'-CB, 4-4'-CB, 2,4-2'-CB, and 2,4-4'-CB. No significant chloride accumulation, or chlorobenzoates were detected in the supernatants of any of these cultures. Several of these congeners had previously been demonstrated to be co-oxidized by biphenyl-grown VP44 (24).

DISCUSSION

Two independent dechlorination operons (*ohb* and *fcb*) were cloned into broad-host-range plasmids (pE43 and pPC3, respectively), and successfully expressed in the biphenyl- degrading bacterium *Comamonas testosteroni*, strain VP44. The resulting transgenic strains were resistant to tetracycline and capable of dechlorination of the respective chlorobenzoates, in addition to retaining the ability of the parental strain to degrade biphenyl and benzoate. Previously, transgenic strains for degradation of particular PCB congeners were generated by either mating strains with complementary pathways (13, 14, 20, 23), or through introduction of the *bph* operon into other strains of interest (1, 8, 10, 18, 21, 29).

This study demonstrates an alternative approach for the construction of PCB degrading bacteria, *i.e.* using genes encoding peripheral enzymatic activities for modification of xenobiotics into substrates for the central metabolic pathway for degradation of aromatic compounds. The *ortho*-halobenzoate dioxygenase encoded by the *ohb* genes acts by incorporation of molecular oxygen into an aromatic ring, to produce catechol (26). During degradation of 2-CBA by VP44(pE43), catechol was never observed as an intermediate, suggesting that catechol was rapidly metabolized following

dechlorination. Similarly, the dechlorination of 4-CBA by the *fcb* genes proceeds via 4-hydroxybenzoate, which was not detected during 4-CBA degradation by VP44(pPC3).

The growth of VP44(pE43) and VP44(pPC3) on 2-CBA and 4-CBA respectively, demonstrates the complete dechlorination and mineralization of these substrates, even at high substrate concentration (10 mM). While host strain VP44 was incapable of either growth or dechlorination of chlorobenzoates, the introduced genes encoding dehalogenation of these compounds permitted both. Degradation of *ortho*-chlorinated PCB congeners, and the resulting 2-CBA, is especially significant given their predominance among the products of anaerobic PCB dechlorination. Up to 80% of the PCBs present following anaerobic dechlorination of Aroclor 1242 consist strictly of *ortho*-chlorinated congeners, and greater than half that total is 2-chlorobiphenyl alone (5).

The host strain VP44 was capable of growth on all of the monochlorobiphenyls, with accumulation of the corresponding chlorobenzoates in the culture supernatant. Both growth and chlorobenzoate accumulation was stoichiometric, showing clear dependence on the concentration of starting substrate. Growth on biphenyls via the *bph* pathway for biphenyl degradation yields a benzoate and pentadienoate as key intermediates, either or both of which may be chlorinated, depending on the PCB congener (15). The stoichiometric accumulation of chlorobenzoates in the culture supernatant of strain VP44 during growth on monochlorobiphenyls indicates the ability of this strain to metabolize and grow on the unsubstituted pentadiene moiety. The accumulation of the chlorobenzoate makes this strain ideally suited for introduction of the dehalogenase genes, since the chlorinated ring should then be fully utilized for growth.

Growth on 4-CB has been previously reported in both mixed and pure cultures, including recombinant microorganisms (see Chapter 1). The degradation of 4-CB by VP44(pPC3) in this report demonstrates the proof of concept, namely the introduction of chlorobenzoate utilizing genes allows the organism to grow on the second (chlorobenzoate)

ring. Mineralization of 4-CB by VP44(pPC3) was rapid and complete, with only transient accumulation of a chlorobenzoate intermediate. This strain maintained the ability to grow on 2-CB with accumulation of 2-CBA, indicating that the pentadienoate produced by the biphenyl pathway is metabolized by this strain. The inability of this strain to metabolize high concentrations of 4-CB (as seen in Figure 4) remains unexplained, and requires further evaluation. The accumulation of a black precipitate is consistent with polymers of aromatic compounds, especially those derived from catechols.

The recombinant strain 44(pE43) growing on 2-CB exhibited rapid growth with transient appearance of 2-CBA, and stoichiometric accumulation of inorganic chloride in the culture supernatant. The analogous strain 44(pPC3) grew on 4-CB at a slightly faster rate, with a lesser amount of the chlorobenzoate intermediate, 4-CBA. Chloride also accumulated in the growth medium, indicating complete mineralization and chlorine removal. The longer lag time, slower growth, and greater transient chlorobenzoate concentration may represent a difference in the ability of the *bph* enzymes of strain VP44 to attack each of these substrates, or of the respective chlorobiphenyls to induce this pathway.

Both recombinant VP44 strains compared favorably to strains previously constructed to grow on chlorobiphenyls. Strain VP44(pPC3) grown on 2 mM 4-CB exhibited 90% chloride release, a doubling time of about 4, and protein accumulation of 80 $\mu g \cdot ml^{-1} \cdot mM^{-1}$ 4-CB (up to about 4 mM). Strain VP44(pE43) grown on 2 mM 2-CB exhibited 95% chloride release, a doubling time of about 7, and protein accumulation of 68 $\mu g \cdot ml^{-1} \cdot mM^{-1}$ 2-CB. By comparison, strain M3GY (20) grown on 0.89 mM 3-4'-CB, released 67% of the total chlorine, had a doubling time of approximately 20 days, and accumulated protein to a final concentration of 58 $\mu g \cdot ml^{-1}$. Strain JHR22 (13) was reported to grow on several chlorobiphenyls including 2-CB and 4-CB. It exhibited doubling times of approximately 16 and 10, as well as 80% (up to 4 mM) and 50% chlorine release, respectively. Another strain, UCR2 (14), was reported to mineralize both 2-CB and 2,5-

CB, with doubling times of 20 and 48, and dechlorination of 90% and 48.9%, respectively. All three strains had been generated through intergenic mating.

Introduction of specific dechlorination operons can permit growth on otherwise recalcitrant substrates, and may be more easily predicted than methods previously used to generate recombinant PCB degraders. Thus, the inability of these recombinant organisms to grow on any congeners with more than one chlorine seems somewhat surprising, since several of the di- and tri-chlorinated congeners tested are expected to yield either 2-CBA or 4-CBA as intermediates. Clearly the introduction of genes which permit intermediary metabolism is insufficient for degradation of polychlorinated congeners. The complete absence of any intermediates suggests that none of these congeners was capable of inducing the *bph* operon. An alternative explanation is that there may be some toxic effect, either of the individual PCB congeners themselves, or of some breakdown products.

While it has been observed that more highly chlorinated PCBs may not be capable of inducing the biphenyl-degradative pathway (18), it seems surprising that even lesser-chlorinated di- and tri-chlorobiphenyls would be insufficient to effect its expression. If this is the case, it may suggest why there appears to be a lack of naturally-occurring bacteria capable of growth on PCBs. Two separate incidents appear to be necessary: the acquisition of a chlorobenzoate degradation pathway, and changes in the regulation of the *bph* operon to allow induction by polychlorinated congeners. Individually, each of these events would be unlikely to confer any selective advantage to an organism, making independent acquisition of both traits in a single organism, and therefore growth on PCBs, unlikely.

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APPENDICES

APPENDIX A

TAXONOMIC CHARACTERIZATION OF STRAIN VP44

Strain VP44, which figured prominently in this work, was obtained from our laboratory collection. This strain was originally isolated by Vivian Pellizari from river sediment in Sâo Paulo, Brazil, and investigated because of its ability to degrade biphenyl and naphthalene (7). A preliminary characterization of this strain by FAME analysis, Gram stain, and morphology suggested this strain was member of the genus *Comamonas* (7), but a species-level designation was never made, and some questions remained about the accuracy of this assignment. To verify this identification, the 16S rRNA gene from this organism was isolated and partially sequenced.

VP44 was grown overnight in LB broth, and chromosomal DNA was isolated from 5 ml of culture by the CTAB Miniprep procedure (p. 2.4.1 in ref. 1). The 16S ribosomal RNA gene was amplified from this sample by PCR, using primers fD1 and rD1*, as previously described. These primers are capable of amplifying nearly full-length 16S ribosomal DNA from many eubacterial genera (10). Each PCR reaction contained 1 µl genomic DNA template solution (at various dilutions), 25 µM primer rD1, 25 µM primer fD1, 200 µM of each nucleotide, 3 mM $MgCl_2$, 0.5 µl (5 Units μl^{-1}) of *Taq* polymerase (Gibco BRL, Gaithersburg, MD), and 3 µl of 10X PCR reaction buffer (Gibco BRL, Gaithersburg, MD) in a total aqueous volume of 30 µl. Electrophoresis of this sample revealed a single PCR product consistent with the approximate size of a 16S gene (about 1.6 kilobases).

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*primer sequences are as shown below:
fD1 5'-CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G-3'
rD1 5'-CCC GGG ATC CCA AGC TTA AGG AGG TGA TCC AGC C-3'

A dye termination sequencing reaction of this 16S PCR product was performed by the MSU DNA Sequencing Facility, using primers LFP1 (5'-AGAGTTTGATCCTGGCTCAG-3'), which is complementary to positions 529 through 515 in the *E. coli* 16S sequence, and 529R (5'-CGCGGCTGCTGGCAC-3'), which corresponds to positions 8 through 27 (10). Primers LFP1 and 529R are designed to hybridize with highly conserved regions within 16S genes, and provide sequences of approximately the first one-third of this gene. This region of the 16S gene contains a mix of regions with different degrees of sequence conservation between organisms (2). Each end consists of highly conserved "U" (universally conserved) regions, which permit accurate alignment with other known 16S sequences, and provide a reference to a standard *Escherichia coli* sequence. In between these is a third "U" region, as well as several "S", semi-conserved regions, and "V", variable, or non-conserved regions. These latter segments allow for distinction between strains, and provide a means for making taxonomic assignments.

Two independent sequences, one in each direction, were obtained from this region. These sequences revealed perfect complementation over 475 base pairs. The Ribosomal Database of the University of Illinois at Urbana-Champaign (4) was accessed via the worldwide web (http://rdp.life.uiuc.edu) to provide sequence alignment and phylogenetic analysis. Based on the assignment of this database, this DNA sequence corresponds to bases 31 through 506, with the closest related organism being *Comamonas testosteroni* RH 1104 (S_{ab}=0.974). The only discrepancy in the 16S sequence between these two organisms occurred at position 497, where RH 1104 contains a cytosine residue, while VP44 contains an adenosine residue. *Comamonas testosteroni* RH 1104 is the type strain of this species, first isolated in 1952 (5, 8), and deposited in the American Type Culture Collection (ATCC) under accession number 11996 in January, 1955 (3).

In conclusion, strain VP44 is determined to be a member of the species *Comamonas testosteroni*, based on this partial 16S rDNA sequence, earlier analysis by

FAME, and conservation of morphology and metabolic activity consistent with the type strain of the species described previously (6, 9, 11).

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APPENDIX B

EXPLORING INDUCTION OF THE *bph* OPERON BY PCBs

The concept of using complementary metabolic pathways to produce novel routes for degradation of aromatic compounds in bacteria has existed for several years (10), and has been proposed as a potential solution to the problem of PCB degradation (5). In spite of many efforts to produce PCB-degrading strains through genetic recombination, no strain with any significant ability to grow on PCBs has been found. The most common tack has been introduction of genes (via mating or transformation) encoding for the modification of PCB metabolites such as chlorobenzoates, into biphenyl-degrading strains of bacteria. While this approach is theoretically plausible, the actual results of such efforts have been disappointing, yielding only strains capable of growth on a very limited range of PCB congeners.

A number of bacteria with broad substrate ranges and high activities against chlorinated biphenyls have been isolated (e.g. *Burkholderia* sp. LB400, *Alcaligenes eutrophus* H850, *Pseudomonas putida* KF707, *Comamonas testosteroni* VP44), and introduction of genes which would presumably allow growth on downstream metabolites into some of these strains has proven to be only a partial contribution towards viable degradation of these compounds. One key factor in the degradation of PCBs has been omitted: the genetic regulation of the degradative pathways involved has not been considered. More precisely, the regulation of the *bph* genes, which encode the upper pathway for degradation of biphenyls into simpler monoaromatic compounds, has largely been ignored.

There are several indications that chlorine substitution of the biphenyl molecule may result in failure to induce the *bph* operon. No naturally-occurring organisms have been found which are capable of growth on biphenyls containing chlorine at more than one position, and transgenic strains have been limited to only one or two congeners. In the case of recombinant strains of *Comamonas testosteroni* VP44, growth was observed on all three monochlorobiphenyls, but no growth was observed when more than one chlorine was present (see Chapter 2).

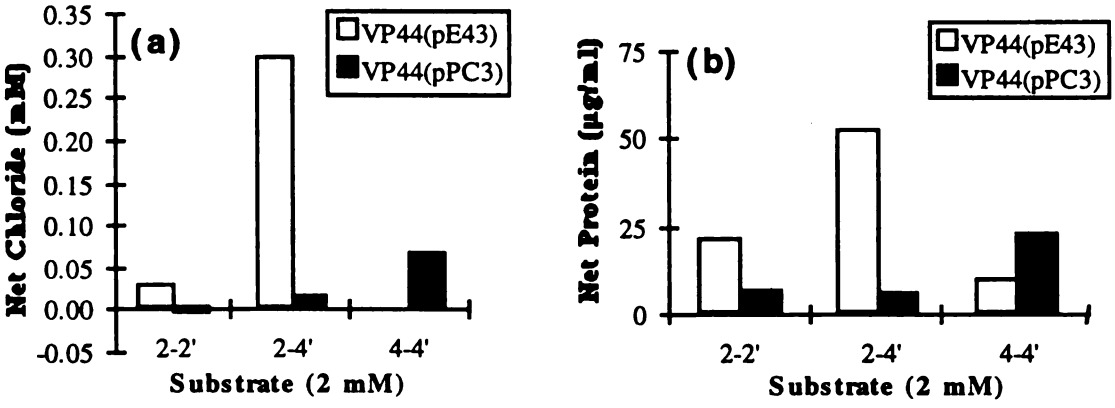

Figure 1. Chloride and protein production during growth on PCBs **(a)** Net chloride produced from single-congener cultures of VP44(pE43) and VP44(pPC3). Initial chloride concentrations in these cultures were 0.22 mM and 0.42 mM, respectively. **(b)** Net protein produced from single cultures of VP44(pE43) and VP44(pPC3). Initial protein concentrations in these cultures were 110 µg ml^{-1} and 115 µg ml^{-1}, respectively. All values shown are averages from duplicate cultures.

Recombinant strains VP44(pE43) and VP44(pPC3) from cultures actively growing (i.e. in log-phase) on 2-CB and 4-CB respectively, were inoculated at high levels (10% by volume) into K1 medium containing individual PCB congeners (at a concentration of 2 mM). Some growth and dechlorination of PCBs was observed (Fig. 1). Gross chloride and protein were very high in these cultures, carried over from the large inoculum, so I show net increases, with initial levels reported in the figure legend. HPLC analyses were performed as previously described (see Chapter 2). 2-CBA was detected at a concentration

of approximately 200 μM in one of two replicate cultures of VP44(pE43), growing on 2-4'-CB. No chlorobenzoates were detected in any other culture, but ring-cleavage products could be observed (based on their characteristic yellow color) but were not quantified, since authentic standards are not available. Low density inoculation (1% of total volume) of PCB cultures yielded no growth or dechlorination (see Chapter 2).

The congeners used in this experiment were chosen based on their predicted susceptibility to these transgenic strains. The individual congenters 2-2'-CB and 4-4'-CB should yield exclusively 2-CBA and 4-CBA respectively, when metabolized by the biphenyl pathway. 2-4'-CB should yield either 2-CBA or 4-CBA depending on the preference of the VP44 oxidative enzymes, although oxidation on the 4-chlorinated ring is more likely, given the typical preferences of the biphenyl dioxygenases (1). This pathway is supported by the accumulation of 2-CBA in one of the VP44(pE43) cultures. Biphenyl-grown *Comamonas testosteroni* VP44 has previously been shown to degrade all three of these congeners, effecting the disappearance of greater than 90% of each congener within 24 hours (9). The inability of recombinant strains of VP44 to significantly grow on any of these substrates seems surprising. These data suggest that the *bph* operon of VP44 can be induced by monochlorobiphenyls, but the presence of more than one chlorine on a biphenyl molecule **prevents** induction. The slight growth and partial dechlorination observed may be the result of enzymatic activity remaining in growing cells prior to repression in the absence of a suitable inducer.

The apparent inability of naturally-occurring microorganisms to use PCBs as growth substrates appears to be the result of several factors acting in concert. One obvious trait missing in many biphenyl degraders is a means for attacking the chlorinated downstream metabolites of biphenyl degradation. This may be the result of selective pressure against strains which experience pathway incompatibilities (see Chapter 1). While one PCB congener may be a benign substrate, another may prove to be a metabolic poison. Another necessary constituent of a successful PCB-degrader would be the ability to induce

the degradative pathway in the presence of PCBs, which does not seem to occur in natural isolates. Without the ability to degrade downstream metabolites, there is no selective advantage for pathway induction by PCBs. It may be this contradiction that has prevented the natural evolution of a PCB-degrading bacterium.

Regulation of the *bph* operon in bacteria remains a mystery. While transcriptional analysis has revealed likely promoter regions (4), the mechanism of regulation is still unknown. However, there are several clues about how expression of these genes may be controlled. The relationship of the *bph* operon of *Pseudomonas* (now *Burkholderia*) sp. LB400 to the *tod* operon for toluene degradation in *Pseudomonas putida* strain F1 has been noted before. While the similarity of genetic organization, size, and homology in both operons is obvious (see Figure 2) (4, 6), this likeness extends even into enzymatic activity. Furukawa et al. (6) demonstrated that most of the genes in both operons were interchangeable, with substrate specificity localized in the large subunit of the respective terminal oxidases (*bphA* and *todC1*).

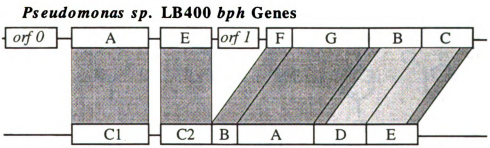

Figure 2. Genetic organization of the *bph* and *tod* operons
Comparison of the regions containing genes for biphenyl and toluene degradation in strains LB400 and F1, respectively. Open boxes indicates genes, labeled by letter designation. Darker-shading illustrates comparisons of *tod* sequences with LB400 *bph* sequences, and lighter shading represents comparisons with *P. pseudoalcaligenes* KF707 *bphBC*. (Figure adapted from references 4 and 6).

The region upstream of the *tod* operon was found to contain a potential regulatory protein (*todR*), with significant sequence similarity to the LysR family of transcriptional regulators (11, 14). Unknown open reading frames (ORFs) located in the LB400 *bph* operon are conserved in the *bph* operon of at least three other organisms (*Alcaligenes eutrophus* H850 (4), *P. pseudoalcaligenes* KF707 (12), and *P. putida* KT2442 (3)) and may represent similar regulatory proteins, although there is no apparent similarity of their deduced amino acid sequences to the putative regulatory protein of the *tod* operon (14).

Despite the apparent inability of PCBs to induce biphenyl degradation in bacteria, several plant products have been suspected to do so. A class of compounds known as terpenes (Fig. 3) were determined to stimulate PCB degradation by *Arthrobacter* sp. strain B1B, presumably through activation of the *bph* operon (7). These compounds are suspected to be the natural substrates for biphenyl-degrading bacteria, and have been found to dramatically enhance bacterial transformation of Aroclor 1242 in soils (8). Especially surprising was the ability of isoprene to induce PCB degradation, since it is nonaromatic and lacks a ring structure entirely.

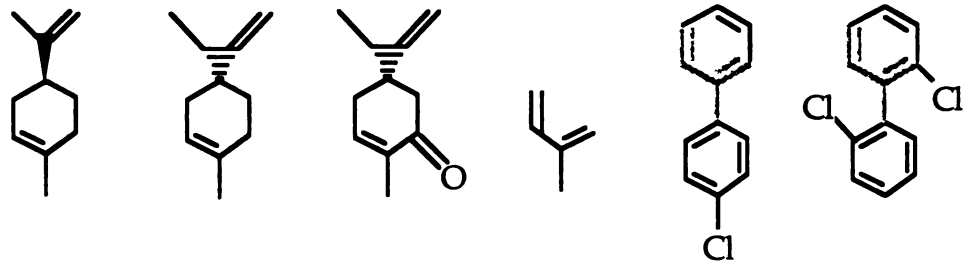

Figure 3. Terpene and Chlorobiphenyl Structures
(a) (*S*)-(-)-limonene, found in pine needle oil; (b) (*R*)-(+)-limonene, found in citrus, juniper, and dill seed; (c) (*S*)-(+)-carvone, found in spearmint; (d) isoprene; (e) 4-chlorobiphenyl; (f) 2-2' chlorobiphenyl. The isoprene motif is emphasized as shaded lines in chlorobiphenyl structures (e) and (f).

Given the ability of the isoprene molecule to activate biphenyl degradation, it is suspected that recognition of a similiar structural motif may be responsible for for inducing

biphenyl degradation. While this pattern is clearly evident in biphenyls, it is not difficult to imagine how chlorine substitutions might inhibit effective recognition of this structure by some regulatory molecule or protein, either through changes in the electronic structure of the molecule, or through steric hindrance. While all three monochlorobiphenyls have been observed to induce the PCB degradation, no congener with a chlorine atom at more than one position appears capable of such regulation (see Chapter 2).

The future directions of this work lie primarily in elucidation, and possible optimization, of the regulatory mechanism(s) controlling initial PCB metabolism. The most direct and versatile means of studying *bph* operon regulation is through the use of a reporter gene. Work has already begun in our laboratory using the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (2) as a reporter gene to elucidate induction of the biphenyl pathway. GFP is an attractive reporter gene, because it is independent of the metabolic state of the bacterium, can be detected *in situ* and without cell lysis, and has been used successfully in other related organisms (13). With the GFP gene inserted within the *bph* operon of *Burkholderia* sp. strain LB400, noninvasive detection of gene expression in the presence of PCBs, biphenyl, and terpenes will be possible. It may also be possible to elucidate the mechanism of regulation through random mutagenesis, or knockouts of the unidentified ORFs present in the *bph* operon, and their effects on reporter gene expression.

It may be useful to study the regulation of the *bph* operon using the recombinant strains or methods, employed in this study. Since they contain a viable pathway for the metabolism of PCB breakdown products, these organisms possess a selective advantage over those strains which do not. Mutagenesis of these strains, and selection for growth on polychlorinated congeners may yield changes in regulation of the *bph* operon, allowing for induction by various PCB congeners.

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