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Bacterial PCB biodegradation

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Abstract

The environment has become polluted with a variety of xenobiotics, including PCBs, as a result of the industrial development of useful halogenated compounds. While the PCBs may not exhibit the acute toxicity originally ascribed to them, they and their attendant byproducts remain as significant factors for adverse effects in the ecological food-chain. The use of microorganisms for bioremediation of PCBs is reviewed. This paper further details three new isolates obtained by conventional enrichment technics which show significant degradation capabilities for Aroclor 1242. These were identified by morphology, staining, and fatty acid analysis as *Comamonas testosteroni*, *Rhodococcus rhodochrous*, and a strain of *Pseudomonas putida*. These isolates demonstrated somewhat selective degradations of the congeners within Aroclor 1242; comprising total losses of 13.8, 19.1, and 24.6%, respectively. Each organism can attack dichloro-through tetrachloro-biphenyls. Analysis of chromatographic patterns from anaerobically digested Aroclor 1242 samples treated by these bacteria demonstrated decreases in di- through penta-substituted biphenyls. Each of these isolates, with discrete specificities, showed preferences for 'open' 2,3-sites, indicative of the action of 2,3-dioxygenase enzymes. The identification of many intermediates in the foregoing transformations was established by GC-MS analyses. Several variations in metabolic pathways, centering on the *meta* cleavage product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) were suggested from these data. It is concluded that the described strains may be of future bioremediation use in processes which have an initial anaerobic dechlorination stage.

Introduction

Of a number of 'recalcitrant' organic halogen compounds in the environment that may pose significant threats to the global ecosystem, one such group is the polychlorinated biphenyls (PCBs). The parental backbone of the PCB molecule is biphenyl and PCBs are synthesized by its direct chlorination with anhydrous chlorine and iron filings or ferric chloride as a catalyst. This process creates a crude product which is then purified by treatment with alkali and distilled resulting in a mixture of chlorobiphenyls with different numbers of chlorine atoms per molecule. In theory, the biphenyl molecule can be substituted at any and all of its unlinked 10 carbons; this allows for 209 different congeners. However, mechanistic and statistical evidence indicate that all possible congeners are not formed (Huntzinger et al. 1974). Typically, a synthetic PCB mixture will contain between 60 and 80 different chlorinated biphenyls.

Such PCB mixtures have been synthesized since 1929 in a number of different countries (USA, Great Britain, France, Japan). In the United States and Great Britain, PCBs were produced by Monsanto and marketed under the trade name Aroclor. Different mixtures of Aroclor are referred to by use of a four-digit numbering system. The first two

digits, 12, represent the biphenyl parent structure, and the last two, -21,-42, for example, represent the weight percent of chlorine.

The physical and chemical properties of PCB mixtures made them industrially useful. Aroclor mixtures are extremely thermostabile (up to 350 °C), resistant to oxidation, acids, bases, and have excellent electrical insulating as well as dielectric characteristics. It has been estimated in the United States alone, that 57×10^7 kilograms of PCBs were produced between the early 1920s and 1978 (Brown et al. 1984), and it is assumed that an equivalent quantity was manufactured in Europe, Russia and Japan. Dependent on composition, Aroclor mixtures have been used as industrial fluids, i.e., in hydraulic systems, gas turbines, and vacuum pumps; as dielectric fluids (capacitors, transformers); as plasticizers (adhesives, textiles, surface coatings, sealants, printing, copy paper); and as heat exchangers. These properties contribute, in parallel, to their relative metabolic inertness in the environment. It has been estimated that some 10×10^7 kilograms reside in the biosphere as contaminants of soil, the air, in (and under) rivers and waste streams, and as components of the lipoidal compartments of plant and animal wildlife. Because of this lipophilicity, the PCBs became bioaccumulated in ecosystem food chains. These topics and related facets have been comprehensively reviewed in a three-volume work edited by Waid (1986).

Table 1. Effects of PCBs on cellular physiological responses.

→ Various (−) effects on aquatic organism reproduction. [photosynthetic bact; phytoplankton; protozoa, *Daphnia*, salmon eggs; sea lions; seals, beluga whales, etc.]
→ Various (−) effects on bird reproduction.
→ Various (−) effects on terrestrial mammal reproduction.
→ Chromosome damage, teratogenicity, substandard juvenile growth, embryonic mortality.
→ Various (+) or (−) effects on mammalian enzymes.
→ Various (+) or (−) effects on lipid metabolism.
→ Various (+) or (−) hormonal or pseudohormonal manifestations, *via* binding to specific receptors.
→ Various (−) effects on immunologic defense system.
→ Various dermatological effects ['chloroacne'].
→ Degenerative neurological symptoms.
→ Experimentally induced tumorigenic or carcinogenic effects.

The health effects of halogenated aromatic hydrocarbons of technical use, including PCBs were initially intensively delineated in a monograph (Nicholson & Moore 1979) published under the auspices of the New York Academy of Sciences. More recent reviews of human health effects (Kimbrough 1987) and of mutagenicity and carcinogenicity of the PCBs (Safe 1989) are also available. The documented effects of PCBs at the organismal, cellular, or molecular biological levels are summarized in Table 1. Additionally, the specific binding of PCBs to rat liver cytosol proteins has been documented (Buff & Brundl 1992). These materials have thus been a cause for concern and have indeed been implicated in a number of well-publicized catastrophic events (Kimbrough 1987). As a result, the U.S. Congress banned their manufacture in 1976; but these long-lived chemicals are still found in service around the world and continue to be suspect in various environmental intoxication situations. This is especially so for marine organism die-offs (Hileman 1992). Yet, PCBs may not be the distinct causal factors in such syndromes, and a number of experts in this area attribute acute toxicities seen in humans and other animals to contaminating manufacturing byproducts and/or to abiotic transformation products which have accrued during PCB usage (Stone 1992). Identification of such putative 'supertoxic' species among PCB congeners remains as a daunting analytical task (cf. Erickson 1985; Storr-Hansen 1991). Despite such uncertainties, it is clear that PCBs can covalently bind to DNA *in vivo* and *in vitro*, that PCB mixtures and single congeners exhibit discernible mutagenic activity, and that more highly chlorinated PCB mixtures (>50% Cl by weight) are hepatocarcinogens in rodents. While lower chlorinated mixtures are not strongly carcinogenic, equivocal results from occupational studies suggest that humans exposed to PCBs may have an excess rate of cancer at some sites.

Insofar as PCB activities in enzyme inductions and repressions, as competitors for hormone receptor sites, as teratogens, and as diverse metabolic agonists or antagonists are concerned, it would

appear (Table 1), besides total percent of chlorination, that substitution position for halogen is also critical. Thus, in terms of structure-function relationships, the possibility for the two phenyl rings to assume co-planarity is considered to be a parameter which influences the foregoing metabolic phenomena (Brunstrom 1989; Borlakaglu et al. 1990; Huang & Gibson 1992). It therefore cannot be gainsaid that the removal of PCBs from the environment represents a high priority challenge. Where these substances have accumulated in highly concentrated and accessible locales, direct incineration is the most likely practical approach. Additionally, solvent extraction processes have been patented and specific removal chemical interactions, for example, in transformer oil have been explored (Brunelle et al. 1985). However, for PCB depositions in soil, waste streams, and in or under lakes and rivers, alternative methodologies such as microbiol detoxification have also received considerable attention.

Microbial transformations of PCBs – descriptive overview

Studies dealing with the microbial breakdown of PCBs have been reported as early as 1973 (Ahmed & Focht 1973; Omori et al. 1973). Since that time, numerous experiments have been conducted to determine the nature of diverse microbial transformations of polychlorinated biphenyls. These transformations fall under two general categories; those which occur under anaerobic conditions and those which take place aerobically. In theory, the optimal biological process should mineralize PCBs to carbon dioxide, water and chlorine. In order for this to occur, chlorine must be removed from the biphenyl ring and resultant intermediates must be cleaved and oxidized. Under anaerobic conditions, one stage by which chlorine atoms can be removed from a chlorinated biphenyl is reductive dechlorination. Under aerobic conditions, oxidative dechlorination or hydrolytic dehalogenation, especially by members of the genus *Pseudomonas*, have been described (Scholten et al. 1991; Markus et al. 1986). Such biotransformations of halogenated organic compounds and their putative reaction mechanisms have been assessed by Hardman (1991) and by Chaudry & Chapalamadugu (1991). The aerobic and anaerobic biodegradations of PCBs have specifically been reviewed by Abramowicz (1990), Bedard (1990), and Mohn & Tiedje (1992).

Anaerobic biotransformations – field studies

Rhee and co-workers (1989) provided some initial evidence for the anaerobic degradation of polychlorinated biphenyls. Their studies (Chen et al. 1988) indicated the production of $^{14}CO_2$ when Hudson River sediments were incubated anaerobically with ^{14}C-labeled monochlorobiphenyls. Amending these sediments with biphenyl enhanced the degradation of the higher chlorinated congeners but not the lower ones (mono- and dichloro). Other important observations were that PCB degradation was seen only under a headspace of pure nitrogen, with no evidence for dechlorination or methanogenesis. However, at around the same time Brown et al. (1987) demonstrated in PCB contaminated sediments from the Hudson River that several dechlorination processes, presumably mediated by different populations of anaerobic microorganisms, appeared to be in operation. While none of the gas chromatographic distribution patterns showed the removal of *ortho*- substituted chlorines, they differed in the removal of *meta*- and *para*-substituted PCBs, and showed the accumulation of PCBs that were *ortho*-substituted. The authors hypothesized that the relevant microorganisms were selectively removing chlorines from PCB *meta* and *para* positions and that observed inconsistencies resulted from different microbial consortia with selective congener specificities.

Further studies of 'Silver Lake' sediments indicated the dechlorination of Aroclor 1260. This mixture consists primarily of hexa- and heptachlorobiphenyls, while tri- and tetrachlorobiphenyls account for less than 1%. Analysis of these sediments indicated 90 to 98% loss of the hexa- and heptachlorobiphenyls, and an increase in the amount of tri- and tetrachlorobiphenyls, which ac-

count for 57 to 82% of the PCBs in the dechlorinated sediment.

Laboratory studies of dechlorination

Evidence up to this point indicates that an undefined microbial metabolism was occurring in such sediments. In extension, researchers attempted to reproduce dechlorination in the laboratory to have a better understanding of this phenomenon. Three different groups have now shown the dechlorination of polychlorinated biphenyls using sediments under anaerobic, methanogenic conditions (Quensen et al. 1988; Abramowicz 1990; Vogel et al. 1989). The Quensen group has demonstrated dechlorination of Aroclor 1242, 1248, 1254 and 1260 by Hudson River sediment. Tiedje et al. (1989) showed the dechlorination of Aroclor 1242 and 1248 occurring as early as 8 weeks, with 80 to 90% loss of tetra- and pentachlorobiphenyls after 12 weeks. In following up these probes in order to identify responsible physiological groups of microorganisms Ye et al. (1992) found that heat- and ethanol-treated bacteria eluted from Hudson River sediments are indeed able to dechlorinate Aroclor 1242 under anaerobic conditions, as evidenced by a decrease in the highly chlorinated PCB congeners, with concurrent accumulations of the lesser chlorinated species. It was determined in this study that the cultures resistant to adverse experimental conditions, presumably anaerobic spore formers, gave PCB dechlorination patterns characterized by the removal of meta-chlorines and an increase in ortho- and para-substituted biphenyls. It has further been demonstrated by Van Dort & Bedard (1991) that reductive dechlorination of PCBs is effected by microbial anaerobes. Such findings are of significance because, by the application of numerous biological criteria (Table 1), the less substituted PCB congeners are usually less toxic; and furthermore, are more amenable (as seen below) to aerobic biotransformations. In exemplary studies, Pettigrew et al. (1990) demonstrated the mineralization of 4-chlorobiphenyl by a freshwater consortium of bacteria which included *Pseudomonas testosteroni, Ps. putida,* and an *Arthrobacter* isolate; and Kimbara et al. (1988) detailed a two-component *Pseudomonas* mixture which transformed highly chlorinated PCBs.

Aerobic catabolism

The aerobic biotransformations of PCBs essentially follow the steps which have been elucidated for biphenyl metabolism. The first step in this process is the conversion of biphenyl to cis 2,3-dihydro-2,3-dihydroxybiphenyl via a 2,3-dioxygenase. Then a NAD^+ dependent dehydrogenase converts this compound to 2,3-biphenyldiol. A dioxygenase enzyme causes meta cleavage to form 2-hydroxy-6-oxo-6-phenylhexa 2,4 dienoic acid (yellow compound) or ortho cleavage yielding 2-hydroxy-3-phenyl-6-oxohexa-2,4-dienoate and then phenylpyruvic acid. Further ring separation of the meta cleavage compound can occur by base hydrolysis to form acetophenones (Bedard et al. 1987a, 1987b) and a four carbon unit or benzoic acid and 2-oxo-penta-4-enoate. The first product, benzoate, then is converted by benzoate oxidase to catechol. Catechol is in turn acted on by a 1,2 oxygenase producing cis,cis muconate. Following the well known β-ketoadipate pathway, this compound is further converted to β-ketoadipate-enol-lactone by a muconolactonizing enzyme. This product is then hydrolyzed to β-ketoadipate. The final step is the cleavage of this structure by a thiolase yielding succinyl Co A and acetyl Co A, which are then shuttled off into the TCA cycle. The second product of the ring separation, 2-oxo-penta-4-enoate, forms the intermediates pyruvate and acetaldehyde. Alternatively, a 3,4-dioxygenase enzyme attack, to form a 3,4-dihydrodiol intermediate, has also been postulated (see below).

Early work by Catelani et al. (1973) identified this general pathway (Fig. 1) and since then, numerous attempts to isolate organisms and to identify the responsible enzymes for each step in this degradation scheme have been carried out. In addition, the genetic basis for possession and expression of the relevant enzymes have been extensively explored. The bulk of this research has been conducted over the past 25 years by a group led by

Furukawa in Japan. This work began with studies on the biodegradability of different PCBs by *Alcaligenes* sp. and then examined the effect of chlorine substitution on bacterial metabolism of PCBs (Furukawa et al. 1978, 1979). Furukawa & Chakrabarty (1982) then identified the genetic elements responsible for chlorobiphenyl degradation by the isolation of plasmids from *Acinetobacter* sp. P6, a strain previously reported to dissimilate 33 pure congeners of polychlorinated biphenyls and from *Arthrobacter* M5, a contaminant of the former isolate grown on 4-chlorobiphenyl. This early analysis of plasmids from chlorobiphenyl degrading organisms then led to the cloning of the chromosomal gene which encodes for chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes* and *Ps. aeruginosa*, along with purification of the translated, linked oxygenases (cf. Taira et al. 1992; and references therein). Biochemical and genetic characteristics of gram-positive bacteria (*Arthrobacter* sp., *Corynebacterium* sp., *Brevibacterium* sp., *Bacillus* sp.) and of gram-negative bacteria (*Pseudomonas, Alcaligenes, Achromobacter, Acinetobacter, Agrobacterium, Enterobacter* and *Flavobacterium* spp.) have also been delineated by Walia et al. (1988) and by Khan & Walia (1991). Experiments carried out by the Silvestre group in Canada (Sondossi et al. 1991) showed moreover, that a *Ps. testosteroni* isolate metabolized biphenyl, hydroxybiphenyl, chlorobiphenyl, and chloro-hydroxybiphenyl by a common metabolic pathway; and that this enzymatic route is specified by a conserved transmissible gene cluster. It was suggested that this metabolic pathway may be yet another manifestation of those bacterial genomes which are concerned with phenylpropanoid catabolism; and indeed, evidence for a super-family of proteins comes from the fact that there is a sequence homology between the *Pseudomonas* dihydrodiol dehydrogenase which metabolizes PCBs, and 'normal' dehydrogenases involved in polyol and sterol transformations (Baker 1990).

While there thus appears to be a conservation of the genes responsible for PCB degradation, different strains of bacteria show greatly different activity in regard to the congeners of PCBs which they degrade. Bedard (1990) isolated 25 different bacteria of which only three exhibited identical abilities to degrade mixtures of PCBs. From this study, Bedard and co-workers isolated two bacteria, *Pseudomonas putida* LB400 and *Alcaligenes eutrophus* H850, that exhibited the ability to degrade a large number or PCB congeners, including those substituted at the 2 and 3 carbons of both rings, a pattern which confers resistance to enzymatic degradation via 2,3-hydroxylation. Further analysis of these organisms indicated that the 2,3-dioxygenase in these organisms can attack *ortho*-chlorinated carbon (Bedard 1990). This capability obviously contributes to the significant degradative capabilities of these organisms. Another unique feature in these organisms and which may help to explain their ability to degrade such highly substituted PCB congeners is the additional presence of 3,4-dioxygenase enzyme activity.

Microcosms of microorganisms for the purpose of PCB mineralization thus continue to be sought. It has become apparent that there exists a gene pool in nature which may be exploited for such purpose *via* the current tools of genetic manipulation (Chakrabarty 1986; Furukawa & Suzuki 1988; Adams et al. 1992), by co-cultivation (Kimbara et al. 1988; Adriaens & Focht 1990), or by sequential applications to the substratum under appropriate physiological conditions (cf. Fathepure & Vogel 1991).

Current work in this laboratory

In keeping with the above considerations, we initiated a program to selectively obtain additional bacterial isolates which might contain elements both of anaerobic and aerobic PCB dissimilatory pathways, since it was shown that degradation of these compounds would probably greatly be improved by initial anaerobic dechlorination (see section *Laboratory studies of dechlorination*). We therefore proceeded to attempt to obtain facultative nitrate-respiring microorganisms (cf. Criddle et al. 1991) from biphenyl, biphenyl/PCB mixtures or from Aroclors. The technics (see below) essentially paralleled those detailed for ferulate catabolism as studied by Grbic-Galic (1985). While a number of

the isolates we obtained exhibited distinct nitrate-reducing capabilities, it was not possible to link this physiological attribute to PCB biotransformation. Along this line, it was also noted in a study on the dechlorination of substituted benzenes using anaerobic biotic columns that there was no acceleration of these reactions under denitrifying conditions (Bosma et al. 1988). However from our experiments, a number of new and potentially useful bacterial strains and consortia for aerobic catabolism of PCBs were obtained. Several of these have, to date, been explored in some detail.

Materials and methods

Enrichment technics and culture isolation

Enrichments were set up either with PAS medium (Bedard et al. 1987) or B + M medium and various sludges as innocula (10% v/v). B + M medium consisted of (mg/l): $NaNO_3$ (450.0), K_2HPO_4 (100.0), $MgSO_4 \cdot 7H_2O$ (20.0), $FeSO_4 \cdot 7H_2O$ (5.0), $CaCl_2$ (2.0), $MnCl_2 \cdot 4H_2O$ (0.2), $NaMoO_4$ (0.1). Two sewage sludges, obtained from the Metropolitan Syracuse Treatment Plant (Syracuse, New York) and the Tully Municipal Sewage Treatment Plant (Tully, New York) and a paper mill sludge, obtained from the Department of Paper Science and Engineering, SUNY College of ESF (Syracuse, New York) were used. Enrichments were amended with 100 to 2,000 ppm biphenyl (Sigma Chemical, St. Louis, MO) and incubated at 23 °C in the dark. After two weeks of incubation, the enrichments were screened using a method similar to that of Sylvestre (1980). The inoculum was first streaked onto a PAS agar plate and then a biphenyl/ether solution was applied to the surface using a sterile Pasteur pipet in a fume hood. After 10–15 seconds, the ether evaporated off, leaving a thin film of biphenyl on the plate. This method was also used with 2-chloro- and 4-chlorobiphenyl (Lancaster Synthesis, Windham, NH). These plates were incubated at 23 °C and observed daily. Colonies showing a zone of clearing, indicative of substrate degradation, were selected for further study.

Culture maintenance and identification

Isolates were maintained in PAS broth with 500 ppm biphenyl on PAS/biphenyl plates, or on nutrient agar (Difco, Detroit, MI) supplemented with 0.1% yeast extract (Difco). Mixed cultures were separated using nutrient agar/yeast extract plates. Once pure cultures were obtained, PAS/biphenyl plates were used for strain maintenance. Selected isolates were identified by cellular fatty acid analysis (Microbial ID, Inc. Newark, DE).

Analysis of growth on aroclor 1242

Comamonas testosteroni was cultured in PAS medium containing 200 ppm Aroclor 1242 (Monsanto, St. Louis, MO) at 23 °C. Samples were taken for dilution plating on tryptic soy agar (Difco) to determine colony forming units (CFU)/ml and to record absorbance at 396 nm (Perkin Elmer UV/VIS spectrophotometer, model 4B) to determine the accumulation of degradative metabolites, specifically 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA IV, Fig. 1) and its chlorinated analogs.

Degradative competence studies

Select strains were cultured in PAS with 500 ppm biphenyl and incubated at 30 °C on a rotary shaker (150 rpm). After 2–4 days of incubation, the cultures were filtered through sterile glass wool and centrifuged. The supernatants were decanted and the cells were washed with PAS buffer (PAS medium without yeast extract and trace metals) and recentrifuged. The cells were resuspended in PAS medium and distributed to sterile screw cap glass vials with teflon-lined septa (Supelco, Bellefonte, PA). Aroclor 1242 and biphenyl were added from concentrated acetone stocks to a final concentration of 10 ppm. After 2–4 days of incubation at 30 °C, perchloric acid was added (0.7% final concentration) to kill the culture. Controls had perchloric acid added at time zero. The cultures were extracted with 4 volumes of methylene chloride and subjected to gas chromatographic analysis.

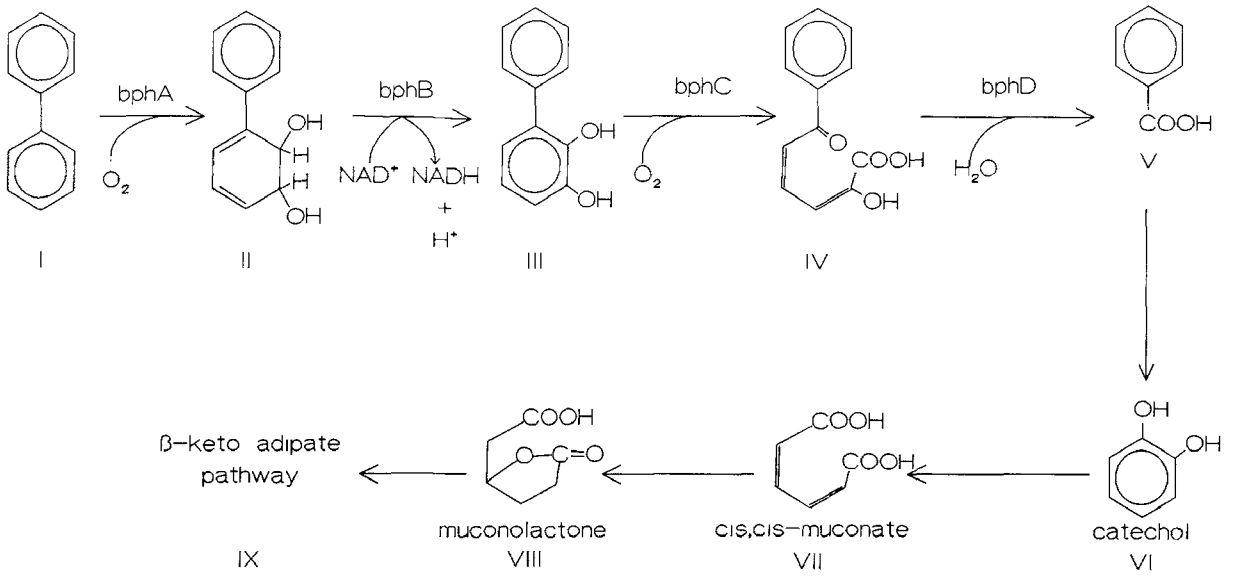

Fig. 1. Aerobic catabolic pathway for degradation of biphenyl and chlorinated biphenyls. The bph operon consists of at least seven genes and four expressed enzyme activities (Taira et al. 1992).

Metabolite production and recovery

Strains were inoculated into 2 l of PAS with 500 ppm biphenyl and 500 ppm Aroclor 1242 and incubated for 24–48 hours at 30 °C. The culture fluid was filtered through glass wool and centrifuged to pellet the cells. The supernatant was then acidified with concentrated H_2SO_4 to a pH of 1–2. The culture filtrate was then saturated with NaCl and extracted twice with ethyl acetate. The organic phase was collected, kept over anhydrous $NaSO_4$, and concentrated by flash evaporation. These preparations were then derivatized with methanolic HCl (Supelco, Inc.) to form the appropriate methyl esters for analysis by GC-MS.

Analytical methods

(i) Gas chromatography
Samples from degradation studies were analyzed on a Hewlett Packard 5890A GC equipped with SPB-5 fused silica capillary column (60 m, 0.2 mm I.D., Supelco, Inc.). The injector was set at 250 °C with a He carrier gas flow of 1.0 ml/min. Nitrogen was used as the make up gas at 30 ml/min. The electron capture detector was set at 300 °C. The temperature program was as follows: 150 °C held for 5 minutes then increased 2 °C/min to 250 °C and held for 10 minutes. Congener assignments for subsequent data given under 'Results and discussion' are tabulated in Table 2.

(ii) Gas chromatography-mass spectrometry
Samples for metabolite identification were analyzed using a Finnigan 9610 automated gas chromatography/EI-CI mass spectrometer system interfaced with a Varian 3400 GC equipped with a SPB-1 fused silica capillary column (30 m, 0.25 mm I.D.). The temperature program consisted of a 5 minute hold at 50 °C, then increased by 10 °C/min to 270 °C, with a 20 minute hold. Electron ionization was performed at 70 EV. Methane was the reagent gas for chemical ionization.

Results and discussion

Environmental bacterial isolates obtained by elective culturing of samples from contaminated locales are listed in Table 3. While practically all of these consortia (or single strains resolved therefrom) are nitrate reducers, it was not possible to propagate them anaerobically with nitrate on bi-

phenyl, Arochlor 1242, or their admixtures. However, as detailed (Table 3), a number of the isolates did possess the ability to degrade these substrates aerobically.

From a five-membered bacterial consortium designated TS-1 obtained from sewage sludge, *Comamonas testosteroni* was isolated in pure culture. This organism when grown in media supplemented with yeast extract and Aroclor 1242, biphenyl, or mixtures of these reached populations approaching 10^8 CFU (Fig. 2) within 47 hrs. Scans of supernatants from *C. testosteroni* grown on biphenyl, biphenyl/Aroclor 1242, and Aroclor 1242 exhibited UV wavelength maxima at 396 nm characteristic of the biphenyl-derived meta-cleavage product (HOPDA; IV, Fig. 1). These supernatants also exhibited characteristic keto-enol tautaumerism at pH values between 4 and 7. HPLC analyses of extracts from cultures grown on biphenyl, biphenyl/Aroclor 1242, and Aroclor 1242 revealed the presence of benzoic acid and chlorinated benzoic

Table 2. Congener assignments for chromatographic peak numbers.

Peak number	Congeners in peak		
1	2,6	2,2′	
2	2,4	2,5	
3	2,3′,		
4	2,4	2,3	
5	2,2′,6		
6	2,2′,5	2,2′,4	4,4′
7	2,3,6	2,3′,6	
8	2,2′,3	2,4′,6	
10	2,4,5		
11	2,3′,5		
12	2,3′,4		
13	2,4′,5	2,4,4′	
14	2,3,3′	2′,3,4	2,2′,5,6′
15	2,2′,4,6′	2,3,4′	
16	2,2′,3,6		
17	2,2′,3,6′		
19	2,2′,5,5′		
20	2,2′,4,5′		
21	2,2′,4,4′	2,2′,4,5	2,4,4′,6
23	2,2′,3,5′		
24	3,4,4′	2,3,3′,6	2,2′,3,4′
25	2,2′,3,4	2,3,4′,6	
27	2,2′,3,3′		
28	2,2′,4,4′,6	2,3′,4,5	
29	2,3,4′,5		
30	2,4,4′,5		
31	2,3′,4′,5		
32	2,3′,4,4′	2,2′,3,5′,6	
34	2,2′,3,4′,6		
35	2,3,4,4′	2,3,3′,4′	
38	2,2′,3,4′,5	2,2′,4,5,5′	
39	2,2′4,4′,5		
42	2,2′,3′,4,5		
43	2,2′,3,4,5′	2,3,4,4′,6	
44	2,2′,3,4,4′		
46	3,3′,4,4′	2,3,3′,4′,6	
47	2,2′,3,3′,4	2,2′,3,4′,5′,6	
50	2′,3,4,4′,5	2,2′,3,4′,5′,6	2,3′,4,4′,5

Table 3. Environmental isolates with the ability to degrade chlorinated biphenyls.

Isolates	Source[1]	Bacterial composition[2]	Activity[3]
H-1	Hudson River	2 Gram (−) rods	Bp, 2-Cl, 4-Cl
H-2	Hudson River	2 Gram (−) rods	Bp, 2-Cl
X*	Hudson River	1 Gram (−) rod	Bp, 2-Cl, 4-Cl
S-2	sewage sludge #1	*Erwinia herbicola*	Bp, 2-Cl, 4-Cl,
		Enterobacter aerogenes and *Corynebacterium sp.*	1242
S-4	sewage sludge #1	*Aureobacterium saperdae* and *Bacillus cereus*	Bp, 2-Cl
S-29	sewage sludge #1	*Xanthomonas campestris*, *Citrobacter freundii*, *Pseudomonas fluorescens*, *P. putida* and *Comamonas testosteroni*	Bp, 2-Cl, 4-Cl, 1242
TS-1			(see text)
NB-1	sewage sludge #2	*P. putida*	Bp, 1242
OB-1	paper sludge	*Rhodococcus rhodochrous*	Bp, 1242

[1] Samples from these sources were used as inocula for various enrichments (see 'Materials and methods')

[2] Determined as outlined in 'Materials and methods'.

[3] Based on colony formation on overlay plates with indicated single congeners (Bp = biphenyl, 2-Cl = 2-chlorobiphenyl, 4-Cl = 4-chlorobiphenyl) or GC analyses of cell assays for Aroclor 1242.

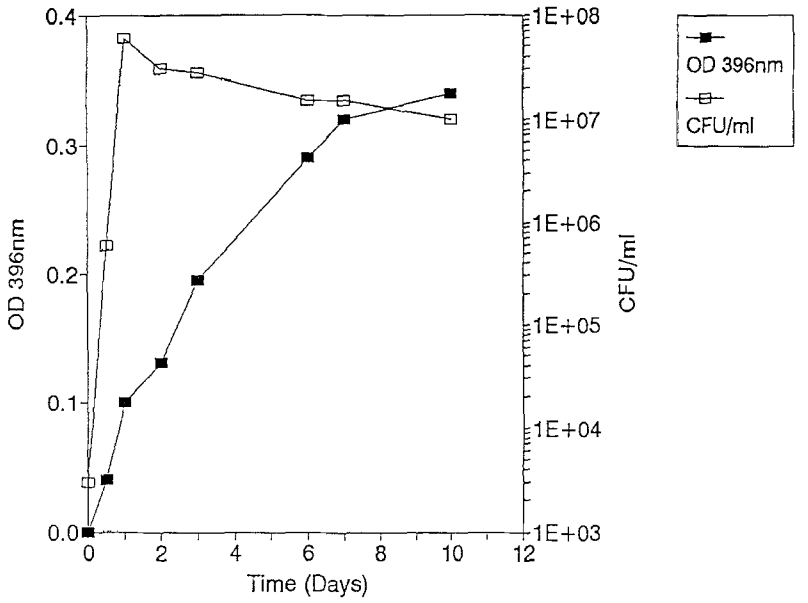

Fig. 2. Growth and meta-cleavage product formation by *C. testosteroni* on Aroclor 1242.

acid derivatives, respectively. Quantitation of products formed indicated that 190 mg/L and 138 mg/l of benzoic acid and chlorinated benzoic acid were formed within 5 days from an initial concentration of biphenyl and Aroclor 1242 at 1 g/l each. Analyses of individually resolved HPLC peaks by GC-MS indicated the presence of several expected metabolites including: 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA), 2,6-dioxo-6-phenylhexanoic acid, γ-benzoyl-butyric acid, γ-benzoyl-propionic acid, phenylacetic acid, benzoic acid, and 2-oxo-penta-4-enoate. Thus, metabolism of biphenyl by *C. testosteroni* resulted in the formation of several metabolites which have been previously described in the literature. Moreover (see below) growth of this organism on chlorinated biphenyls resulted in the production of chlorinated benzoic acids indicating metabolic attack on the less chlorinated ring.

Aroclor 1242 was tested at concentrations between 5 ppm and 50 ppm as a substrate. As summarized in Table 4, treatment of 5 ppm and 10 ppm samples with the pure cultures isolated in this study resulted in a 13–24% loss in total congener concentration. Minor changes (< 1%) were observed at 25 ppm and no changes were detected in the 50 ppm sample. In the lower concentration (5 ppm and 10 ppm) experiments, the congener selectivity of *C. testosteroni* was identical, with 100% loss of 2,5-dichlorobiphenyl, 2,4-dichlorobiphenyl, 2,3'-dichlorobiphenyl, 2,3',4-trichlorobiphenyl (possibly 2,3',5-trichlorobiphenyl and 2',3,5-trichlorobiphenyl); and an 87% loss in a peak representing 2,4'-dichlorobiphenyl and 2,3-dichlorobiphenyl. *C. testosteroni* was further grown in the presence of several congeners known to comprise Aroclor 1242. These included: 2-chlorobiphenyl, 4-chlorobiphenyl, 2,4-dichlorobiphenyl, and 4,4'-dichlorobiphenyl. In the presence of these Aroclor congeners, chlorinated benzoic acids were formed. From the dichlorobiphenyls, either 2- or 4-chlorobenzoic acid was identified as a product (indistinguishable by GC-MS).

Of some interest was the finding that this isolate could also grow on the following substrates as sole carbon sources: nitrobenzene, 4-nitrocatechol, 2-chloro-5-nitrobenzoic acid, p-nitrophenol, and 2,4,6-trinitrobenzene sulfonic acid. From these observations (Table 5) it can be concluded that our isolate of *C. testosteroni* contains dioxygenase(s) analogous to those demonstrated in *C. testosteroni* 'T-2' which catalyzes the conversion of p-sulfobenzoate into protocatechoic acid (Locher et al. 1991), or in a pseudomonad grown on 2,4-dinitrotoluene

which catalyzed the displacement of the 4-nitro group by molecular oxygen (Spanggord et al. 1991). The genetic interrelationships, functional group specificities, annd positional points of attack among these oxygenases are, as yet, unknown.

Workup of consortia S-29 or NB-1 provided isolates of *Pseudomonas putida* as the active component. Besides 'HOPDA' (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid), there was identified in the growth filtrate of this microorganism mono-, di-, and trichlorobenzoic acids (Table 6). In like fashion, from paper sludge OB-1, a gram-positive microorganism identified as *Rhodococcus rhodochrous* was obtained. The identifiable metabolites formed by this isolate are given in Table 7. These include, besides HOPDA, benzoylbutanoic acid, dichlorobenzoylpropionic acid, phenylacetic acid, hydroxybenzoic acid, benzoic acid, and mono- and dichlorobenzoic acids. The foregoing breakdown distributions are in keeping with those found earlier by Yagi & Sudo (1980) who studied PCB metabolism by an *Alkaligenes* strain which oxidized high concentrations of dichlorobiphenyls.

To examine the effects of sequential anaerobic/aerobic treatments, 'site H' Hudson River samples (courtesy of D. Abramowicz, General Electricc Co.) were spiked with 300 ppm Aroclor, amended with RAMM medium (Shelton & Tiedje 1984), and incubated for six weeks under an anaerobic headspace. Replicate samples were then subjected to *R. rhodochrous, P. putida* NB1, or a mixture of the two inocula and kept under aerobic conditions. This process led to the loss off 4.9, 18.1 and 18.8%, respectively, in PCB content. Analysis of sequential treatments with *R. rhodochrous* for example, showed decreases in the tri-, tetra-, and pentachlorobiphenyls below those found in the parental Aroclor 1242 mix. Despite this stepwise processing it is conceivable that biphenyl supplementation might be needed in order to fully induce degradative enzymes for further aerobic PCB breakdown by thhe microorganisms at hand.

However, isolation of the actinomycete *R. rhodococcus* amplifies the available spectrum of PCB oxidizing/dechlorinating microorganisms, which typically to date, have mainly included closely phylogenetically-related gram-negative rods. In this context, it should be noted that Smith & Ratledge (1988) showed that a *Nocardia* species dissimilated biphenyl, *via* HOPDA andd benzoate to catechol and then to cis, cis-muconate. Additionally, an isolate also designated as *R. rhodococcus* was obtained by Fuchs et al. (1991) which could co-metabolize with ethanol 2-methylaniline or 4-chloro-2-methylaniline into the TCA cycle. Both catechol-1,2 and catcheol 2,3-dioxygenases were inducible enzymes in this strain, but prolonged culture gave rise to mutants which had lost the gene for the latter. Degradation of 2-methylaniline proceeded by way of *meta*-cleavage, whereas 4-chloro-2-methylaniline was split by *ortho*-cleavage. A

Table 4. Degradation of Aroclor 1242 by *Comamonas testosteroni, Pseudomonas putida* NB 1 and *Rhodococcus rhodochrous.*

Strain	Peaks degraded[a]	Total % loss[b]
C. testosteroni	2,3,4,12,15	13.8
C. putida NB1	1,2,3,4,10,11,12,13,14,15,28	19.12
R. rhodochrous	1,2,3,4,10,11,12,13,14,15, 28,35	24.59

[a] See Table 2 for congener assignments.
[b] Total percent loss of Aroclor 1242 as calculated from controls.

Table 5. Growth of *Comamonas testosteroni* on various aromatic substrates.

Substrate	Qualitative growth
p-Nitroaniline	(−)
4-Nitroguaiacol	(+/−)
3,5-Dinitrosalicyclicacid	(+/−)
5-Nitrovanillin	(+)
Nitrobenzene	(++)
Picrysulfonic acid	(+/−)
4-Nitrocatechol	(++)
p-Toluenesulfonate	(−)
2,4,6-Trinitrobenzenesulfonate	(+++)
p-Nitrophenol	(++)
2-Chloro-5-nitrobenzoic acid	(+++)
2,4-Dinitrotoluene	(−)
Phenylpyruvic acid	(+++)
Phenylacetic acid	(+++)
Sulfanilic acid	(+++)

Microorganism tested with 450 ppm of substrate in PAS salts buffer. (−), no detectable growth; (+), (++), (+++), One, two, and three log growth patterns.

Table 6. Mass spectrometric data of metabolites produced by *Pseudomonas putida* NB1 grown on biphenyl and Aroclor 1242.

Metabolite	E.I.ions[a] (m/z)	C.I. ions[b] (m/z)	Proposed structure
1	218, 133, 120, 105, 77, 55	221, 249, 261	2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid
2	238, 168, 139, 105, 77	239, 267, 279	trichlorobenzoic acid[d]
4	204, 173, 145, 109, 74	N.D.[c]	dichlorobenzoic acid[d]
5	170, 139, 111, 75	N.D.	monochlorobenzoic acid[d]

[a] M^+ (first value) and diagnostic ions identified from electron impact mass spectra.
[b] M^+ +1, M^+ +29 and M^+ +41 ions from chemical ionization mass spectra.
[c] Not determined.
[d] Identified as the methyl ester of indicated compound.

composite of the identification of aerobic PCB breakdown intermediates formed by the several isolates obtained here is given in Fig. 3.

Significance

Since the turn of this century, the environment has become polluted with a variety of man-made xenobiotics resulting from the development of herbicides, pesticides, munitions, refrigerants, and other industrially useful compounds. Those materials which are chlorinated or nitrated hydrocarbons, their manufacturing byproducts, and their partial metabolic transformants are often most toxic to plant and animal life at very low concentrations; and moreover, are quite persistent within given ecosystems. These factors are especially true for polychlorinated biphenyls (PCBs). Thus, the work reported here, as well as efforts from other laboratories, has been directed toward selection of microorganisms, which in concert or under cometabolic conditions, may be capable to a greater or lesser degree of mineralizing these compounds. Our research to date has revealed in nature the existence of a number of new bacterial isolates which selectively remove specific polychlorinated congeners from among that mix known as PCBs. These isolates could form the basis as agents for future bioremediation process development. Furthermore, the next obvious goal for application of such biocatalysts in cost-effective and environmentally compatible bioremediations would be to enlarge and redirect their metabolic potential. Such goals could be realized by the complementation or addition of specific heterologous enzyme-forming genes, so as to produce derivative constructs which would more completely mineralize most of the PCB congeneric substances. Provided that such genetically-engineered strains could thrive effec-

Table 7. Mass spectrometric data of metabolites produced by *Rhodococcus rhodochrous* grown on biphenyl and Aroclor 1242.

Metabolite	E.I.ions[a] (m/z)	C.I. ions[b] (m/z)	Proposed structure
1	218, 133, 120, 105, 77, 55	N.D.[c]	2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid
2	206, 175, 147, 105, 77, 55	207, 235, 247	benzoylbutanoic acid
3	260, 229, 201, 173, 145	261, 289, 301	dichlorobenzoyl-propionic acid
4	204, 177, 145, 109, 74	N.D.	dichlorobenzoic acid[d]
5	170, 139, 111, 75	N.D.	monochlorobenzoic acid[d]
6	136, 105, 77, 51	N.D.	benzoic acid[d]
7	152, 120, 92	153, 181, 193	hydroxy benzoic acid[d]
8	150, 91, 65	151, 179, 191	phenylacetic acid[d]

[a] M^+ (first value) and diagnostic ions identified from electron impact mass spectra.
[b] M^+ +1, M^+ +29 and M^+ +41 ions from chemical ionization mass spectra.
[c] Not determined.
[d] Identified as the methyl ester of indicated compound.

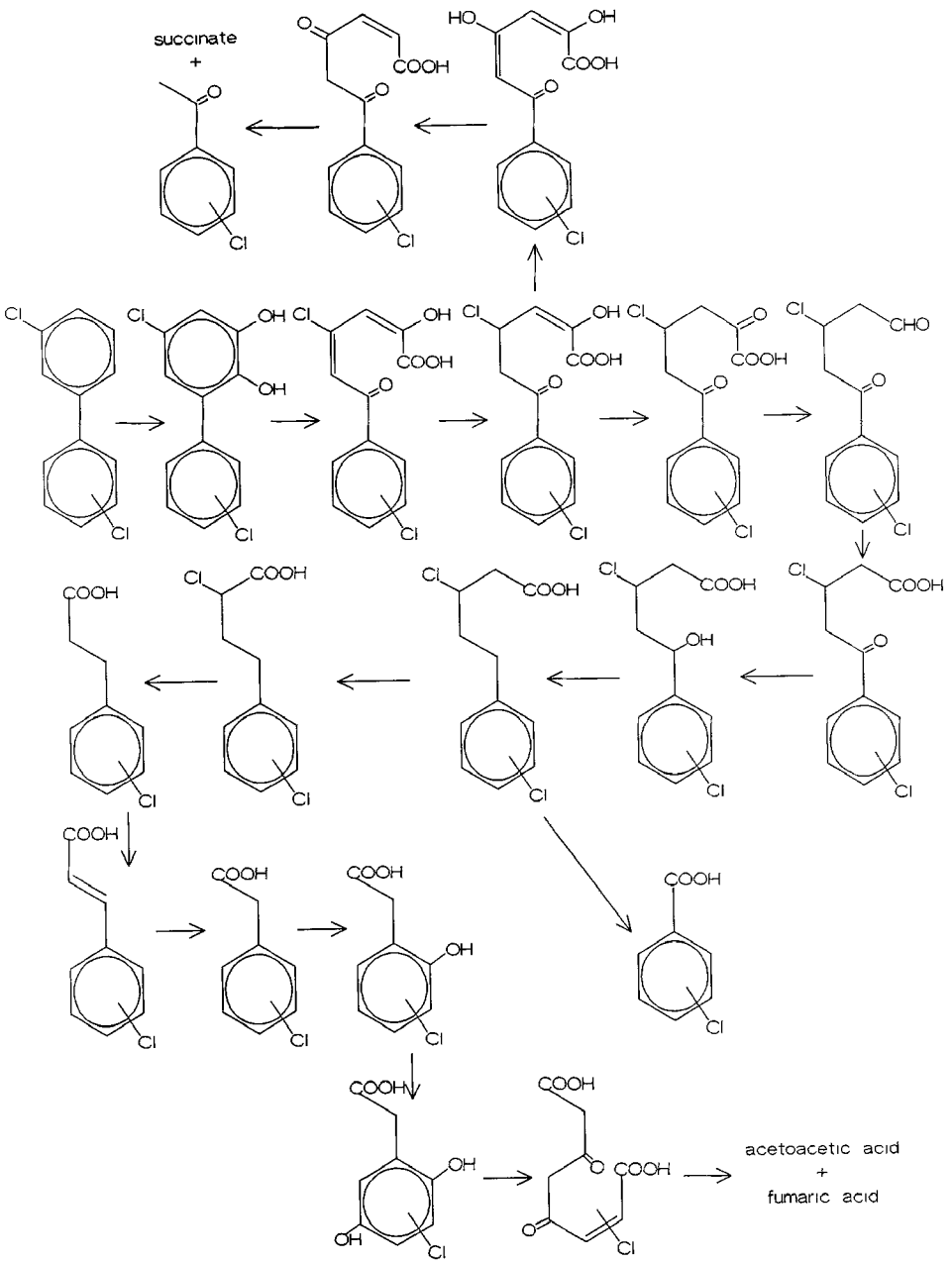

Fig. 3. Proposed composite reaction sequence to account for the metabolites identified in the oxidation of biphenyl or of chlorinated biphenyly by the isolates obtained in this study.

tively under field conditions and also overcome the current political barriers towards their use, it should, in theory, be possible to foresee their use in meeting legislative regulations regarding the clean-up of such hazardous wastes.

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