 doi:10.1111/j.1462-2920.2007.01533.x

Evidence of aerobic utilization of *di-ortho*-substituted trichlorobiphenyls as growth substrates by *Pseudomonas* sp. SA-6 and *Ralstonia* sp. SA-4

Sunday A. Adebusoye,[1,2]* Flynn W. Picardal,[2] Matthew O. Ilori[1] and Olukayode O. Amund[1]
[1]*Department of Botany and Microbiology, University of Lagos, Lagos, Nigeria.*
[2]*Environmental Science Research Center, School of Public and Environmental Affairs Indiana University, Bloomington, IN 47405, USA.*

Summary

Robust and effective bioremediation strategies have not yet been developed for polychlorinated biphenyl (PCB)-contaminated soils. This is in part a result of the fact that *ortho*- or *ortho*- and *para*-substituted congeners, frequent dead-end products of reductive dechlorination of PCB mixtures, have greatly reduced aerobic biodegradability. In this study, we report substantial evidence of utilization of *diortho*-substituted trichlorobiphenyls (triCBs) as growth substrates by *Ralstonia* sp. SA-4 and *Pseudomonas* sp. SA-6 in which *ortho*-substitution resulted in no obvious patterns of recalcitrance. These stains exhibited unusual preferences for growth on congeners chlorinated on both rings. Substrate uptake studies with benzoate-grown cells revealed that the isolates attacked the 2-chlorophenyl rings of 2,2′,4- and 2,2′,5-triCB. Between 71% and 93% of the initial 0.23–0.34 mM dose of congeners were transformed in less than 261 h concomitant with non-stoichiometric production of respective dichlorobenzoates and chloride ion. In enzyme assays, activity of 2,3-dihydroxybiphenyl-1,2-dioxygenase was constitutive. Additionally, these strains harboured no detectable plasmids which, coupled with exponential growth on the two triCB congeners, suggested chromosomal location of PCB degradative genes. In addition to the fact that there is a paucity of information on degradation of PCBs by tropical isolates, growth on triCBs as a sole carbon and energy source has never been demonstrated for any natural or engineered microorganisms. Such isolates may help prevent accumulation of *ortho*-substituted congeners in natural systems and offer the hope for development of effective bioaugmentation or sequential anaerobic–aerobic bioremediation strategies.

Received 19 June, 2007; accepted 15 November, 2007. *For correspondence. E-mail sadebusoye@yahoo.com; Tel. (+234) 80 34388871; Fax (+234) 1 5820432.

Introduction

Despite active research in developed nations spanning three decades, extensive regulatory action and an effective ban on their production since the late 1970s, polychlorinated biphenyls (PCBs) remain a focus of research and environmental attention. They are generally recalcitrant, disperse throughout the biosphere, and have bioaccumulated in many species (Clark *et al*., 1979; Safe, 1992; Bruhlmann and Chen, 1999). Natural attenuation of PCBs is attributed to microbial activity and, as a result, bioremediation has been considered a potential strategy for long-term, *in situ* attenuation of PCB contamination. Support for this strategy comes from studies suggesting that PCBs degradation occurs in a variety of soil types including aerobic and anaerobic soils, and that the organisms responsible for PCB degradation are enriched with repeated application of Aroclors 1221, 1242 and even 1254 (Bedard *et al*., 1986; 1987; Quensen *et al*., 1988; Abramowicz, 1990; Kim and Picardal, 2000).

Consequently, the use of a sequential anaerobic–aerobic treatment has often been proposed as a potential bioremediation strategy for treatment of soils and sludges contaminated with PCBs (Abramowicz, 1990; Anid *et al*., 1993; Tiedje *et al*., 1993). Using this scenario, an anaerobic treatment would be used to reductively dechlorinate highly chlorinated congeners. The lightly chlorinated congener products would then be degraded aerobically. One difficulty associated with such a sequential treatment is that aerobic biodegradation is thought to require a primary growth substrate, e.g. biphenyl, as it has been generally believed that congeners containing more than one chlorine cannot serve as an aerobic growth substrate and must therefore be degraded co-metabolically (Focht, 1997). This presents additional problems as biphenyl is expensive, difficult to disperse throughout a contaminated matrix, and can be subject to regulatory restrictions as well. The use of biphenyl or other inducer of biphenyl dioxygenase as a

primary substrate might be circumvented therefore, if aerobic bacteria could be isolated that could grow on a range of dichlorobiphenyls (diCBs) or trichlorobiphenyls (triCBs) while co-metabolically degrading more highly chlorinated congeners.

In addition to physicochemical properties of PCBs that affect bioavailability, e.g. hydrophobicity, development of an effective sequential anaerobic–aerobic treatment is also impeded by various microbiological factors. Reductive dechlorination of PCBs rarely occurs at the *ortho* positions and generally results in the accumulation of *ortho*- or *ortho*- and *para*-substituted congeners (Quensen *et al.*, 1990; Morris *et al.*, 1992). Unfortunately, *ortho*-chlorinated PCB congeners which have been found to elicit a wide range of toxic responses (Arcaro *et al.*, 1999; Chauhan *et al.*, 2000; van der Plas *et al.*, 2000) are also often resistant to chemical and aerobic biodegradation (Furukawa, 1982; Abramowicz, 1990; Dai *et al.*, 2002; Korte *et al.*, 2002; Weber *et al.*, 2002). This may be a result of inhibition by *ortho*-chlorinated PCB metabolites of the enzyme responsible for aromatic ring cleavage, 2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3DHBD) (Dai *et al.*, 2002). Isolation of bacterial strains that can utilize *ortho*-substituted congeners as a sole carbon and energy source may be useful, whether used alone or in conjunction with other treatments, to remediate environments contaminated with highly chlorinated congeners.

The isolation of aerobes capable of growth on *ortho*-substituted dichlorobiphenyls has, until relatively recently, been elusive. Only few organisms, including the organisms dealt with in this study, can grow with 2,2′- and 2,4′-diCB as a sole source of carbon and energy (Potrawfke *et al.*, 1998; Kim and Picardal, 2001; Adebusoye *et al.*, 2007a). It is uncertain if these isolates were anomalies or if the occurrence of bacteria able to grow on diCBs was more widespread than believed for the last several decades. At the moment, utilization of triCBs, whether *ortho*-substituted or not, as the single source of carbon and energy by a single strain bacterium has not yet been reported. This work describes the ability of axenic cultures of tropical strains of *Ralstonia* sp. SA-4 and *Pseudomonas* sp. SA-6 to utilize 2,2′,4- and 2,2′,5-triCB as sole sources of carbon for their growth. To the best of our knowledge, this is first substantive evidence for the abilities of natural microorganisms to grow on triCBs. These bacteria preferentially attacked the *ortho*-substituted ring and the *ortho*-substitution resulted in no obvious patterns of recalcitrance.

Results

Degradation of 2,2′,4- and 2,2′,5-triCB as a sole carbon and energy source during growth by SA-4 and SA-6 is shown in Figs 1 and 2 respectively. During growth on

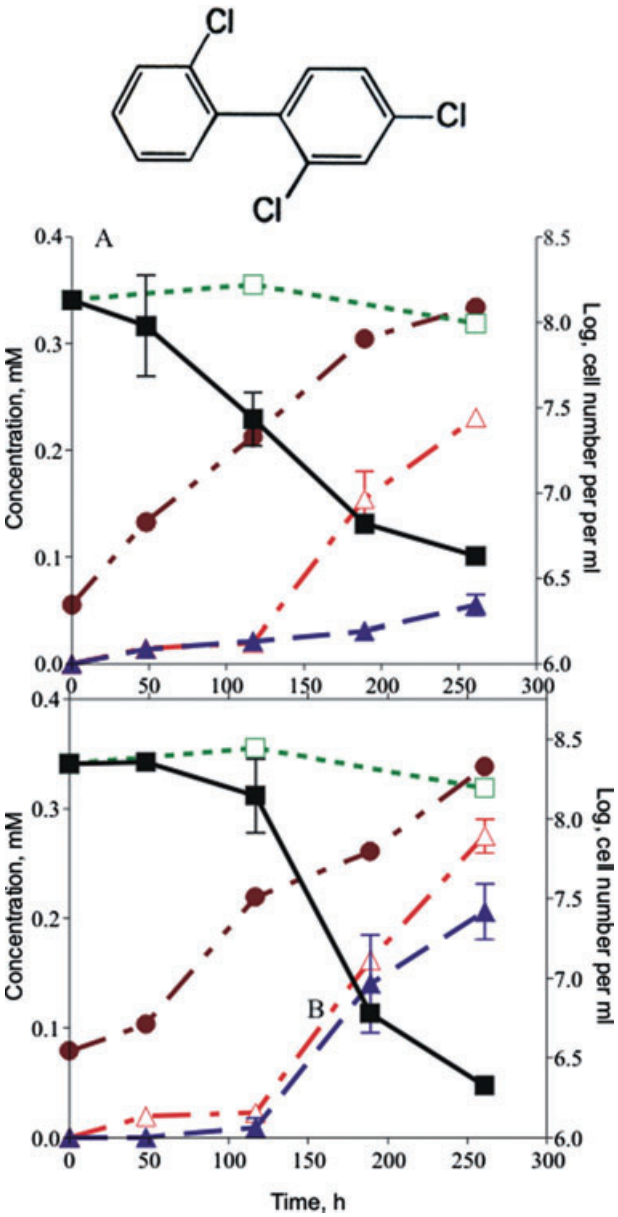

Fig. 1. Time-course for 2,2′,4-triCB metabolism and production of benzoate and chloride by SA-4 (A) and SA-6 (B). (■) 2,2′,4-triCB concentration in experimental tubes; (□) triCB concentration in non-inoculated controls; (▲) 2,4-CBA; (Δ) chloride; (●) log cell number. In the controls without cells, 2,2′,4-triCB was not metabolized and minimal abiotic loss occurred. Data represent the averages and standard deviations of triplicate determinations. Large error bars were due to differential response of cells to substrate in replicate tubes.

2,2′,4-triCB, the isolates occasionally produced a light yellow to yellow–green-coloured compound, signalling production of the *meta*-cleavage product, 2-hydroxy 6-oxo-6-(2′,4′-dichlorophenyl)hexa 2,4-dienoic acid (HOPDA). As growth progressed, the colour faded and disappeared from the growth medium. No yellow colour was produced during the metabolism of 2,2′,5-triCB by

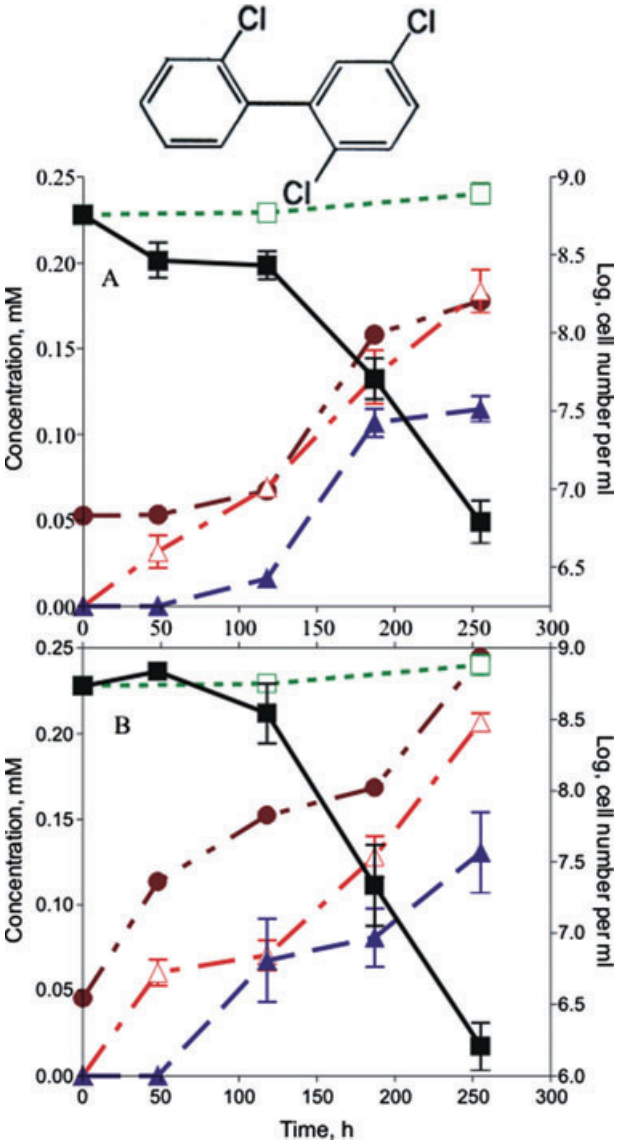

Fig. 2. Time-course for 2,2′,5-triCB metabolism and production of benzoate and chloride by SA-4 (A) and SA-6 (B). (■) 2,2′,5-triCB concentration in experimental tubes; (□) triCB concentration in non-inoculated controls; (▲) 2,5-CBA; (Δ) chloride; (●) log cell number. In the controls without cells, 2,2′,5-triCB was not metabolized and minimal abiotic loss occurred. Data represent the averages and standard deviations of triplicate determinations. Large error bars were due to differential response of cells to substrate in replicate tubes.

either isolate. Figures 1 and 2 also display the evolution of the degradation metabolites, dichlorobenzoates (diCBAs) and chloride, as a function of time during the degradation of 2,2′,4- and 2,2′,5-triCB. Over the 255–261 h of incubation, between 70.5% and 92.5% of the initial triCB was transformed (Figs 1 and 2, Table 1). The lack of significant disappearance of the congeners in replicate, uninoculated control tubes demonstrated that the removal was not due to physical-chemical losses. As our prolonged hexane

Table 1. Aerobic metabolism of trichlorobiphenyl isomers and production of metabolites by tropical strains of SA-4 and SA-6.

	SA-4[b]						SA-6[b]				
PCB congener tested[a]	Incubation period (h)	g (h)[c]	% Depletion[d]	CBA found (mM)[d]	% CBA found	Chloride found (mM)[d]	Incubation period (h)	g (h)[c]	% Depletion[d]	CBA found (mM)[d]	% CBA found	Chloride found (mM)[d]
2,2′,4-triCB	261	44	70.5 ± 2.4	2,4-CBA 0.06 ± 0.02	22.9 ± 7.1	0.23 ± 0.02	261	44	86.2 ± 3.2	2,4-CBA 0.21 ± 0.04	70.1 ± 15.0	0.28 ± 0.03
2,2′,5-triCB	255	55	78.5 ± 9.4	2,5-CBA 0.12 ± 0.01	64.2 ± 7.3	0.18 ± 0.01	255	32	92.5 ± 10.6	2,5-CBA 0.13 ± 0.03	62.1 ± 19.4	0.21 ± 0.01

a. 2,2′,4-triCB and 2,2′,5-triCB were supplied at respective concentrations of 0.34 ± 0.01 mM and 0.23 ± 0.00 mM.
b. Cells were pre-grown with benzoate and washed before inoculated into MS medium supplemented with individual congeners.
c. Mean generation time.
d. Data values are averages of triplicate samples sacrificed at each time point ± standard deviation. Per cent degradation values have been evaluated with reference to the amount recovered from non-inoculated tubes.

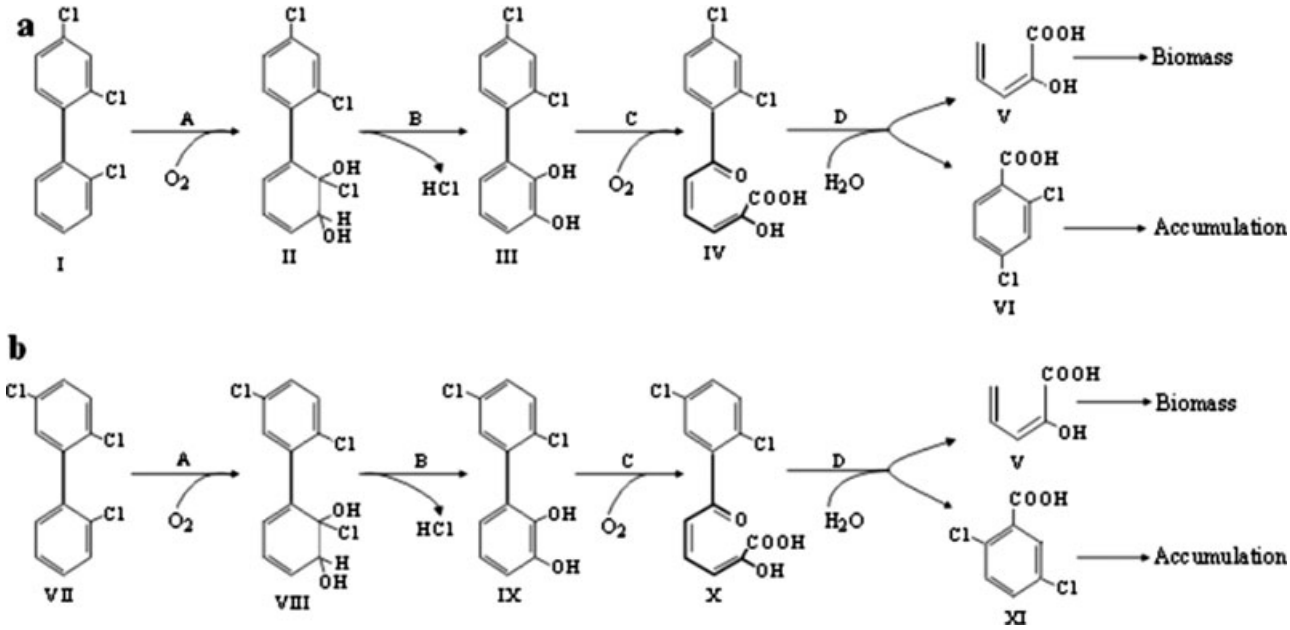

Fig. 3. Proposed catabolic pathway for aerobic degradation of 2,2′,4-triCB (a) and 2,2′,5-triCB (b) by strains SA-4 and SA-6. A, biphenyl 2,3-dioxygenase; B, 2,3-dihydroxy 2,3-dihydroxybiphenyl 2,3-dehydrogenase; C, 2,3-dihydroxybiphenyl 1,2-dioxygenase; D, HOPDA hydrolase; I, 2,2′,4-triCB; II, 2,3-dihydro 2,3-dihydroxy 2,2′,4′-trichlorobiphenyl; III, 2,3-dihydroxy 2′,4′-dichlorobiphenyl; IV, 2-hydroxy-6-oxo-6-(2′,4′-dichlorophenyl)hexa 2,4-dienoate; V, 2-hydroxypenta 2,4-dienoate; VI, 2,4-diCBA; VII, 2,2′,5-triCB; VIII, 2,3-dihydro 2,3-dihydroxy 2,2′,5′-trichlorobiphenyl; IX, 2,3-dihydroxy 2′,5′-dichlorobiphenyl; X, 2-hydroxy-6-oxo-6-(2′,5′-dichlorophenyl)hexa 2,4-dienoate; XI, 2,5-diCBA. Spectrophotometric scanning of resting cell assay showed that both *meta*-cleavage products IV and X did not accumulate in the culture fluids. Product V was converted to biomass while VI and XI were produced as primary metabolites which accumulated although further biotransformation to chlorinated (non-)aromatic intermediates cannot be discounted.

extraction lyses cells and also extracts congeners sorbed to cells, disappearance of triCBs was not a result of cell sorption. Production of metabolites in the presence of cells unambiguously demonstrated that triCB disappearance was a result of biodegradation.

Growth of the organisms as measured by direct cell count was near-exponential, except in the case of SA-4 during growth on 2,2′,5-triCB. In that case, cell numbers did not increase dramatically until after 100 h of incubation. Utilization of triCBs was accompanied by a two- to threefold increase in cell number over an incubation period of 255–261 h. The doubling time (Table 1) was calculated from linear portions of the growth curve on the respective triCBs. SA-4 had doubling times of 44 h and 35 h, respectively, while growing on 2,2′,4- and 2,2′,5-triCB. In the case of SA-6, corresponding generation times obtained were 44 h and 32 h. There was no significant growth in control SA-4 and SA-6 cultures containing 2,2,4,4,6,8,8-heptamethylnonane (HMN) but lacking triCBs as described previously (Adebusoye *et al.*, 2007a).

We observed identical, mean doubling times for SA-4 and SA-6 during growth on 2,2′,4-triCB, although SA-6 degraded a greater percentage of the added triCB (Table 1). In spite of an initially slow rate of 2,2′,4-triCB metabolism by this strain, with less than 10% conversion in 117 h (Fig. 1B), extensive transformation was subsequently observed, culminating in 80% degradation (Table 1). Production of 2,4-diCBA and chloride was less than stoichiometric for both isolates and accumulation of these metabolites was minimal until after 117 h (Fig. 1). By the conclusion of the experiment, significantly more chloride and 2,4-diCBA were produced by SA-6 than by SA-4 (Fig. 1, Table 1). The release of 2,4-diCBA shows that initial oxygenation of the substrate occurred on the monochlorinated ring that bore chlorine only at the *ortho* position, eventually leading to 2,4-diCBA via the *meta*-fission pathway as depicted in Fig. 3.

Data obtained from time-course utilization of 2,2′,5-triCB are summarized in Fig. 2 and Table 1. By the end of the incubation, the cultures had transformed between 79% and 93% of this congener. As observed during growth on 2,2′,4-triCB, products obtained from the culture fluids, i.e. 2,5-diCBA and chloride, indicated that the organisms preferentially attacked the *ortho*-substituted 2-chlorophenyl ring rather than the 2,5-chlorophenyl ring (see Fig. 3). We did not detect accumulation of 2-CBA in culture fluids during growth by either isolate on either congener.

Assays of extracts from benzoate-grown cells revealed the presence of the biphenyl *meta*-cleavage enzyme, 2,3DHBD in both isolates. Specific activities obtained for SA-4 and SA-6 were 0.1 and 0.25 μmol min^{-1} (mg of

protein)$^{-1}$ respectively. In addition, the activities of the *ortho*- and *meta*-cleavage enzymes for the metabolism of catechol were also quantified. The specific activities of catechol 1,2-dioxygenase were 0.06 and 0.12 μmol min^{-1} (mg of protein)$^{-1}$ for SA-4 and SA-6 respectively. Specific activities of catechol 2,3-dioxygenase were slightly higher and were, respectively, 1.31 and 0.15 μmol min^{-1} (mg of protein)$^{-1}$ for SA-2 and SA-6. We did not attempt a full characterization of all upper- and lower-pathway enzymes or determine the response of enzyme activities to different growth conditions or inducers.

There was no detection of the presence of extra chromosomal elements in the two bacterial strains utilized in this study. Electrophoretic runs of the two protocols for plasmid isolation showed no detectable plasmid DNA. Organisms were incubated at varying periods, e.g. 12, 16 and 24 h. Furthermore, antibiotics such as ampicillin and kanamycin were routinely included in the culture media for the purpose of stimulating plasmid replicable if indeed the strains carried plasmids. These notwithstanding, negative results were obtained for plasmids.

Discussion

Although bioremediation of PCB-contaminated soils, sediments and sludges presents a number of biological, physicochemical and engineering challenges, one significant roadblock has been the lack of known isolates capable of degrading lightly chlorinated (one to three chlorines) congeners as a primary growth substrate under aerobic conditions. This has resulted in the use of strategies involving co-metabolic degradation that require a primary substrate, e.g. biphenyl, capable of inducing bph-pathway enzymes. In this study, we report the ability of axenic cultures of *Ralstonia* sp. SA-4 and *Pseudomonas* sp. SA-6 to mediate transformation of two triCBs having at least one chlorine on each ring when supplied as sole substrate. This ability was unequivocally demonstrated on the basis of increased cell number, loss of triCB in cultures but not in controls, and recovery of diCBAs and chloride as metabolites. As previously reported, these isolates share a similar substrate range and can also grow on all three monochlorobiphenyls (CBs) and five diCBs (Adebusoye *et al*., 2007a,b). Such isolates may be able to grow in a reductively dechlorinated, PCB-contaminated matrix and cause substantial degradation of lightly chlorinated congeners without the need for biphenyl addition. Although we have yet to test the co-metabolic potential of these isolates, co-metabolic degradation of non-growth congeners while growing on mono-, di- and triCBs would result in even more extensive degradation.

Earlier studies reported that microbial degradation of PCBs generally occurred via oxidation of the non-chlorinated ring, suggesting that the chlorinated ring was recalcitrant to enzymatic attack (Ahmed and Focht, 1973; Abramowicz, 1990). More recent work, however, has demonstrated that growth on certain diCBs, e.g. 2,4′-diCB and 2,2′-diCB, with chlorine substitution on both rings is possible, usually yielding a chlorobenzoate (CBA) (Potrawfke *et al*., 1998; Kim and Picardal, 2001; Adebusoye *et al*., 2007a). In this work, we similarly show that chlorine substitution on both triCB rings did not prevent degradation. In addition, contrary to results of other researchers that *ortho*-substitution can limit aerobic PCB degradation (Furukawa *et al*., 1978; Furukawa, 1982; Bedard *et al*., 1987; Brenner *et al*., 1994; Dai *et al*., 2002), *ortho*-substitution showed no obvious negative effect on utilization of these triCBs by our bacterial strains.

The common aerobic degradation pathway for biphenyl and PCBs (bph-pathway) involves 2,3-dioxygenation of the less substituted aromatic ring, followed by ring cleavage to produce a HOPDA, and subsequent hydrolysis to a CBA and a 5-carbon fragment (Ahmed and Focht, 1973; Omori *et al*., 1986; Abramowicz, 1990; Bevinakatti and Ninnekar, 1993; Kobayashi *et al*., 1996). Metabolism of triCB substrates by our isolates likely follows a similar pathway (see Fig. 3). Although a mass balance of products was not obtained, production of 2,4-diCBA and 2,5-diCBA together with the lack of 2-CBA recovery suggest metabolism of the 2-chlorophenyl ring. In addition, the concentration of chloride recovered from the culture media (0.18–0.28 mM) was approximately equal to the triCB transformed which also suggested dechlorination, and perhaps mineralization, of the least substituted ring as illustrated in Fig. 3. In spite of the observed differences in transformation of the two triCB congeners by each strain, analysis of variance indicated no significant difference in their catabolic competence at 5% level of probability.

Partial recovery of 2,5-diCBA or 2,4-diCBA may be a result of incomplete transformation at various steps in the bph-pathway or limited degradation of the diCBA products. Although some incubations produced a yellow colour indicative of 2-hydroxy 6-oxo-6-(2′,4′-dichlorophenyl)hexa 2,4-dienoic acid formation that faded with time, it was considered possible that remaining 2-hydroxy 6-oxo-6-(2′,4′-dichlorophenyl)hexa 2,4-dienoic acid or 2-hydroxy 6-oxo-6-(2′,5′-dichlorophenyl)hexa 2,4-dienoic acid at low concentrations might account for the non-stoichiometric diCBA production. This was evaluated by spectral scanning of culture fluids according to the method of Maltseva and colleagues (1999). Results (data not shown) revealed no accumulation of these products. Similarly, previous reports have indicated that attack of certain *para*- and *ortho*-substituted congeners did not form persistent HOPDAs and ultimately transformed the congeners into equimolar amounts of CBAs (Bedard and Haberl, 1990; Maltseva *et al*., 1999).

As these isolates are unable to grow on CBAs or diCBAs as a primary substrate (Adebusoye *et al.*, 2007a) and production of chloride was insufficient for complete diCBA transformation by a co-metabolic mechanism, mineralization of either 2,5-diCBA or 2,4-diCBA can be discounted. We did not, however, attempt to measure the formation of chlorocatechols or other partial transformation products. Although this is not a feature commonly encountered in biphenyl degraders, such phenomena have been reported for *Burkholderia* sp. LB400 (Bedard and Haberl, 1990) and *Pseudomonas* testosteroni B-356 (Sondossi *et al.*, 1992). Incomplete degradation of PCBs to CBA products is not unusual and the formation of other intermediates has also been reported in the experiments of other researchers (Fava and Marchetti, 1991; Arensdorf and Focht, 1994; Bruhlmann and Chen, 1999).

As the first analysis of triCB transformation was performed about 50 h following inoculation (Figs 1 and 2), little can be said about initial rates of degradation. In most cases, however, the data show that triCB degradation was more rapid after 100 h of incubation. This could be a result of both the increase in cell numbers over that period and greater expression of necessary enzymes. Although the current studies did not attempt to evaluate enzyme activity following exposure to triCBs, we did determine that important upper- and lower-pathway enzymes were expressed by the benzoate-grown cells used in these studies. Previous growth on biphenyl was therefore not needed for either enzyme expression or triCB degradation.

Enzymes of both the catechol *ortho*- and *meta*-cleavage pathway were detected with the latter exhibiting a higher activity. It is plausible that aromatic metabolism takes place readily through the *meta*-catabolic route in these strains. This inference is best supported by occasional, transient production of a greenish-yellow metabolite when the isolates are incubated with benzoate. Activities of 2,3DHBD may be independent of the substrate on which the cells were grown, i.e. constitutive, as results were obtained on benzoate, a substrate not known to induce bph-pathway enzymes. Similar findings were reported by Commandeur and colleagues (1996).

The capacity to transform PCBs and other xenobiotics was not lost when the isolates were grown in the absence of these pollutants, i.e. on benzoate or nutrient agar to check for purity. The high rate of phenotypic conservation suggests that degradation may not be plasmid DNA-mediated. No extra chromosomal elements were detected when the isolates were screened for plasmids even though one of the methods used was one developed for the recovery of relatively large plasmids which are typically difficult to isolate (Kado and Liu, 1981). In contrast to our observations, Hickey and colleagues (1992) reported spontaneous loss of the ability to mineralize 2-CB when a pseudomonad that harboured no plasmid was cultured in the absence of 2-CB. Involvement of plasmids in degradation of PCBs has been demonstrated previously (Furukawa and Chakrabarty, 1982; Masse *et al.*, 1984; Shields *et al.*, 1985 Pettigrew *et al.*, 1990). Pettigrew and colleagues (1990) and Shields and colleagues (1985) confirmed that degradation pathways in 4-CB-mineralizing bacterial strains was coded by plasmids, whereas, Masse and colleagues (1984) were unable to relate the plasmids carried by *Achromobacter* sp. B-218 to 4-CB biodegradation. Nevertheless, the absence of detectable plasmids in the isolates utilized in the present study strongly suggests that required catabolic genes are bone on the chromosome, a feature that would confer stability of the PCB-degrading phenotype.

The reason for our success at isolating these triCB-degrading organisms and several others capable of growth on diCBs (Adebusoye *et al.*, 2007a) from the same site in Nigeria is subject to conjecture. Our enrichment method utilized HMN as a carrier to increase mass transfer of hydrophobic compounds into the aqueous phase. Such a carrier may assist in maintaining aqueous concentrations at levels that permit enrichment and growth that would otherwise not be possible. Indeed, the much higher solubility and resultant bioavailability of CBs relative to di- and triCBs may partially contribute to the greater ease and success of isolating CBs degraders. In addition, we cannot rule out the possibility that HMN influenced our isolations or results in some unknown manner. As the tropical soil used as the inoculum has been heavily contaminated with PCBs and polychlorinated benzenes for more than six decades, development of these bacteria could have also resulted from selective pressure exerted by prolong exposure to PCBs and other chlorinated aromatic pollutants.

It is noteworthy that the triCB congeners utilized by SA-4 and SA-6 are poorly degraded co-metabolically by many of the bacterial strains previously described by others (Furukawa, 1982; Bedard *et al.*, 1986; Maltseva *et al.*, 1999). The initial characterization of the isolates described in the present report demonstrates that aerobic biodegradation of triCBs in the absence of other carbon substrates is possible. Further investigation into the biochemical capabilities and genetic composition of these isolates is needed to better evaluate their potential role in the natural attenuation or engineered bioremediation of PCB contamination. The existence of natural, non-genetically modified, isolates with the capability to assimilate *ortho*-substituted triCB congeners suggests the possibility of bioaugmentation of contaminated soils or sludges, and the potential for a low-cost bioremediation strategy.

Experimental procedures

Bacterial strains and culture conditions

The strains, i.e. SA-4 and SA-6 utilized in this study, were isolation by batch enrichment culture on Askarel fluid (a blend of PCBs and polychlorinated benzenes) as the sole carbon source as described elsewhere (Adebusoye *et al.*, 2007a). The origin of the inoculum was a soil contaminated with electrical transformer fluid at National Electric Power Authority (NEPA) Transformer Workshop, Ijora, Lagos, Nigeria. The strains were preserved in cryogenic vials containing nutrient broth with 20% glycerin fortified with yeast extract and frozen at −80°C. The isolates are Gram-negative, non-motile rods. According to their phenotypic characteristics using API 20 *E*-test systems (bio Merieux Vitek, Hazelwood, MO, USA) and sequencing of the 16S rRNA gene, strains SA-4 and SA-6 were assigned to the genera *Ralstonia* and *Pseudomonas* respectively (Adebusoye *et al.*, 2007b). these sequence data have been submitted to the GenBank database under Accession No. DQ854841 and DQ854841, respectively, for SA-4 and SA-6. For the purpose of this study both organisms were grown and maintained at 25°C on phosphate-buffered chloride-free mineral salts (MS) medium (Kim and Picardal, 2000) to which biphenyl dissolved in a heptamethylnonane carrier was added to a final concentration of 100 p.p.m. as the sole carbon and energy source. The purity of these strains was controlled by frequent plating on nutrient agar (Difco Laboratories, Detroit, MI, USA) and MS agar supplemented with 2.5 mM benzoate.

Chemicals

The purity of PCB growth substrates used in this study such as 2,2′,4-triCB and 2,2′,5-triCB was greater than 99% and were all procured from AccuStandard (New Haven, CT, USA). As reported by the manufacturer, biphenyl was not present as a contaminant. All standard solutions of PCBs (more than 95% purity) for construction of standard curves were acquired from Ultra Scientific (North Kingstown, RI, USA). Biphenyl (> 99% purity), all mono- and dichlorobenzoates, catechol, sodium dodecyl sulfate (SDS) and Tris 2-amino-2-hydroxymethyl-1,3-propanediol were obtained from Sigma-Aldrich (St Louis, MO, USA), while high-purity 2,3-dihydroxybiphenyl was purchased from Wako Pure Chemical Industries (Osaka, Japan). 2,2,4,4,6,8,8-Heptamethylnonane (HMN) and analytical grade solvents for HPLC analysis were acquired from Fisher Scientific (Springfield, NJ, USA). Noble agar was obtained from Difco Laboratories (Detroit, MI, USA). All other chemicals were of analytical grade.

Trichlorobiphenyl utilization

Inocula used for substrate utilization experiments were prepared from log-phase cultures grown in MS medium amended with 2.5 mM benzoate, washed twice in MS medium lacking benzoate, and finally re-suspended in the same medium at an optical density of 0.5–0.6 at 600 nm. Each strain was grown on the selected triCB isomer as the sole growth substrate in MS medium as previously described by Kim and Picardal (2000). Briefly, strains were grown in three replicate sets of Balch tubes containing 6 ml of triCB-supplemented MS medium and crimp-sealed with Teflon-lined, butyl rubber stoppers. The final concentrations of the triCBs in the medium were 0.34 and 0.23 mM, respectively, for 2,2′,4- and 2,2′,5-triCB. Trichlorobiphenyls were added from stock solutions prepared by dissolving the chlorobiphenyl in HMN, a multibranched alkane carrier to improve mass transfer of triCBs into the aqueous phase. HMN is not known to be biodegradable or to induce enzymes needed for degradation of PCBs. Sealed tubes were incubated horizontally in the dark at room temperature (25°C) on a shaker table (Labline Instruments) at 100 r.p.m. The air contained in the headspace volume (21 ml) was sufficient to maintain aerobic conditions over the course of the study. To assess the dynamics of degradation and production of metabolites, the entire contents of three sets of culture tubes were sacrificed at selected time points and analysed as described below. Growth of the cultures was determined using an acridine orange direct counting method as previously described (Kepner *et al.*, 1994).

Chlorobiphenyl extraction and analysis

Residual chlorobiphenyls in the reaction tubes were extracted with 5 ml of hexane as previously described (Adebusoye *et al.*, 2007a). Following addition of hexane, the tubes were resealed, vortexed for 1–2 min and mixed continuously on a tube rotator for 12 h. As no solid phase was present other than biomass, our extraction method allowed almost complete triCB recovery without the need for Soxhlet extraction. The extracts were analysed on an HP 5890 Series II gas chromatograph fitted with an ECD and a DB-5 megabore capillary column (J & W Scientific, Folsom, CA, USA; 0.53 mm internal diameter, 2.5 μm film thickness). Trichlorobiphenyls were quantified using four-point calibration curves constructed using standards bracketing the concentrations expected in sterile control samples showing no degradation. Typical correlation coefficients, r^2, for standard curves was 0.95–0.99. Gas chromatograph efficiency and accuracy was constantly ascertained by routine injections made from standards prior to sample analysis.

For quantification and identification of diCBA metabolites, the aqueous fractions following hexane extraction were centrifuged (Hermle z 180 M Labnet) at 13 000 *g* and filtered prior to analysis. The filtered sample was analysed by HPLC (Waters, Milford, MA, USA) using a YMC-pack ODS-AQ column (YMC, Kyoto, Japan). Dichlorobenzoates were monitored by UV detection λ_{238} and identified with reference standard congeners by retention time. Chloride released during growth was similarly measured on the same liquid chromatograph but used a conductivity detector and IonPac AS17 analytical column (4 × 250 mm; Dionex). Chloride was also occasionally determined spectrophotometrically at 460 nm with mercury thiocyanate and ferric sulfate after the method of Bergman and Sanik (1957) and as modified by Coleman and colleagues (2002).

Determination of enzyme activities

Cell extracts were prepared from cells grown in MS medium supplemented with 2.5 mM benzoate at 25°C on a rotary

shaker. Cells were harvested by centrifugation, washed twice with phosphate buffer (50 mM, pH 7.5), re-suspended in the same buffer and subsequently disrupted with a French press (20 000 lb in^{-2}; Aminco, SLM Instruments, Urbana, IL, USA). The crude cell extracts were clarified by centrifugation (12 000 *g*, 30 min, 4°C). Cell extracts were used immediately without further purification.

Catechol 1,2-dioxygenase was assayed spectrophotometrically by the absorbance of the *ortho* fission product (*cis,cis*-muconate at $\lambda = 260$ nm, as described by Nakasawa and Nakasawa (1957) and modified by Ka-Leung and colleagues (1990). Catechol 2,3-dioxygenase was determined by via the absorbance of the *meta*-fission product (2-hydroxymucomic semialdehyde) at 375 nm as described by Kataeva and Golovleva (1990) and slightly modified by Kaschabek and colleagues (1998). 2,3-Dihydroxybiphenyl 1,2-dioxygenase was measured by the absorbency of 2-hydroxy-6-oxo-6-phenylhexa-2,4,-dienoic acid (HOPDA) at $\lambda = 434$ nm according to McKay and colleagues (2003). These activities are expressed as μmoles of fission-product formed per minute per milligram of protein at 25°C. Protein concentrations were determined by the modified Lowry method (Galli and McCarty, 1989). Bovine serum albumin was used as a protein standard to develop standard curves.

Plasmid screening procedure

The plasmid screening method of Kado and Liu (1981) was used for the detection of plasmid DNA with minor modifications. In addition, a commercial plasmid preparation kit (MoBio Laboratory, Solana Beach, CA, USA) was used. Axenic cultures of isolates were grown separately on Luria–Bertani (LB) medium and MS medium amended with 2.5 mM benzoate for various periods. Electrophoresis was performed in a horizontal 0.7% agarose (Seakem ME agarose, Cambrex Bio Science, Rockland, USA) gel using a Bio-Rad subcell unit, with tris-acetate (40 mM)-EDTA (21 mM) (pH 7.8) used as the buffer system.

Acknowledgements

S.A. Adebusoye would like to acknowledge the financial support of the ICSC-World Laboratory, Lausanne, Switzerland and the School of Public and Environmental Affairs, Indiana University, Bloomington, IN, USA.

References

Abramowicz, D.A. (1990) Aerobic and anaerobic biodegradation of PCBs: a review. *Crit Rev Biotechnol* **10:** 241–251.

Adebusoye, S.A., Picardal, F.W., Ilori, M.O., Amund, O.O., Fuqua, C., and Grindle, N. (2007a) Growth on dichlorobiphenyls with chlorine substitution on each ring by bacteria isolated from contaminated African soils. *Appl Microbiol Biotechnol* **74:** 484–492.

Adebusoye, S.A., Picardal, F.W., Ilori, M.O., Amund, O.O., and Fuqua, C. (2007b) Characterization of multiple novel aerobic polychlorinated biphenyl (PCB)-utilizing bacteria strains indigenous to contaminated African soils. *Biodegradation*. DOI: 10.1007/s10532-007-9122-x

Ahmed, M., and Focht, D.D. (1973) Degradation of polychlorinated biphenyls by two species of *Archromobacter*. *Can J Microbiol* **19:** 47–52.

Anid, P.J., Ravest-Webster, B.P., and Vogel, T.M. (1993) Effect of hydrogen peroxide on the biodegradation of PCBs in anaerobically dechlorinated river sediments. *Biodegradation* **4:** 241–248.

Arcaro, K.F., Yi, L., Seegal, R.F., Vakharia, D.D., Yang, Y., Spink, D.C., *et al.* (1999) 2,2′,6,6′-Tetrachlorobiphenyl is estrogenic *in vitro* and *in vivo*. *J Cell Biochem* **72:** 94–102.

Arensdorf, J.J., and Focht, D.D. (1994) Formation of chlorocatechol *meta* cleavage products by a pseudomonad during metabolism of monochlorobiphenyls. *Appl Environ Microbiol* **60:** 2884–2889.

Bedard, D.L., and Haberl, M.L. (1990) Influence of chlorine substitution pattern on the degradation of polychlorinated biphenyls by eight bacterial strains. *Microbiol Ecol* **20:** 87–102.

Bedard, D.L., Unterman, R., Bopp, L.H., Brennan, M.J., Haberl, M.L., and Johnson, C. (1986) Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. *Appl Environ Microbiol* **51:** 761–768.

Bedard, D.L., Wagner, R.E., Brennan, M.J., Haberl, M.L., and Brown, J.F., Jr (1987) Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutrophus* H850. *Appl Environ Microbiol* **53:** 1094–1102.

Bergmann, J.C., and Sanik, J. (1957) Determination of trace amounts of chlorine in naphtha. *Anal Chem* **29:** 241–243.

Bevinakatti, B.G., and Ninnekar, H.Z. (1993) Biodegradation of 4-chlorobiphenyl by *Micrococcus* species. *World J Microbiol Biotechnol* **9:** 607–608.

Brenner, V., Arensdorf, J.J., and Focht, D.D. (1994) Genetic construction of PCB degraders. *Biodegradation* **5:** 359–377.

Bruhlmann, F., and Chen, W. (1999) Transformation of polychlorinated biphenyls by a novel BphA variant through the *meta*-cleavage pathway. *FEMS Microbiol Lett* **179:** 203–208.

Chauhan, K.R., Kodayanti, P.-R.S., and McKinney, J.D. (2000) Assessing the role of *ortho*-substitution on polychlorinated biphenyl binding to transthyretin, a thyroxine transport protein. *Toxicol Appl Pharmacol* **162:** 10–21.

Clark, R.R., Chain, E.S.K., and Griffin, R.A. (1979) Degradation of polychlorinated biphenyls by mixed microbial cultures. *Appl Environ Microbiol* **37:** 680–685.

Coleman, N.V., Mattes, T.E., Gossett, J.M., and Spain, J.C. (2002) Biodegradation of cis-dichloroethane as the sole carbon source by a β-proteobacterium. *Appl Environ Microbiol* **68:** 2726–2730.

Commandeur, L.C.M., May, R.J., Mokross, H., Bedard, D.L., Reineke, W., Govers, H.A.J., and Parsons, J.R. (1996) Aerobic degradation of polychlorinated biphenyls by *Alcaligenes* sp. JB1: metabolites and enzymes. *Biodegradation* **7:** 435–443.

Dai, S., Vaillancourt, F.H., Maroufi, H., Druoin, N.M., Neau, D.B., Snieckus, V., *et al.* (2002) Identification and analysis

of a bottleneck in PCB degradation. *Nat Struct Biol* **9:** 934–939.

Fava, F., and Marchetti, L. (1991) Degradation and mineralization of 3-chlorobiphenyl by a mixed aerobic bacterial culture. *Appl Microbiol Biotechnol* **36:** 240–245.

Focht, D.D. (1997) Aerobic biotransformation of polychlorinated biphenyls. In *Manual of Environmental Microbiology*. Hurst, C.J., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D., and Walter, M.V. (eds). Washington, DC, USA: American Society for Microbiology Press, pp. 33–57.

Furukawa, K. (1982) Microbial degradation of polychlorinated biphenyls. In *Biodegradation and Detoxification of Environmental Pollutants.* Chakrabarty, A.M. (ed.). Florida: CRC Press, Inc., pp. 33–57.

Furukawa, K., and Chakrabarty, A.M. (1982) Involvement of plasmids in total degradation chlorinated biphenyls. *Appl Environ Microbiol* **44:** 446–626.

Furukawa, K., Matsumura, F., and Tonomura, A. (1978) *Alcaligenes* and *Acinetobacter* strains capable of degrading polychlorinated biphenyls. *Agric Biol Chem* **42:** 543–548.

Galli, R., and McCarty, P.L. (1989) Biotransformation of 1,1,1-trichloroethane, trichloromethane, and tetrachloromethane by a *Clostridium* sp. *Appl Environ Microbiol* **55:** 837–844.

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

Kado, C.I., and Liu, S.-T. (1981) Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol* **145:** 1365–1373.

Kaschabek, S.R., Kasberg, T., Muller, D., Mars, A.E., Janssen, D.B., and Reineke, W. (1998) Degradation of chloroaromatics: purification and characterization of a novel type of chlorocatechol 2,3-dioxygenase of *Pseudomonas putida* GJ31. *J Bacteriol* **180:** 296–302.

Kataeva, I.A., and Golovleva, L.A. (1990) Catechol 2,3-dioxygenase from *Pseudomonas aeruginosa* 2x. *Methods Enzymol* **188:** 115–121.

Kepner, J., Raymond, L., and Pratt, J.R. (1994) Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. *Microbiol Rev* **58:** 603–615.

Kim, S., and Picardal, F. (2000) A novel bacterium that utilizes monochlorobiphenyls and 4-chlorobenzoate as growth substrates. *FEMS Microbiol Lett* **185:** 225–229.

Kim, S., and Picardal, F. (2001) Microbial growth on dichlorobiphenyls chlorinated on both rings as a sole carbon and energy source. *Appl Environ Microbiol* **76:** 1953–1955.

Kobayashi, K., Katayama-Hirayama, K., and Tobita, S. (1996) Isolation and characterization of microorganisms that degrade 4-chlorobiphenyl to 4-chlorobenzoic acid. *J Gen Appl Microbiol* **42:** 401–410.

Korte, N.E., West, O.R., Liang, L., Gu, B., Zutman, J.L., and Fernando, Q. (2002) The effect of solvent concentration on the use of palladized-iron for the step-wise dechlorination of polychlorinated biphenyls in soil extracts. *Waste Manag* **22:** 343–349.

Ka-Leung, N., Neidle, E.L., and Ornston, N.L. (1990) Catechol and chlorocatechol 1,2-dioxygenases. *Methods Enzymol* **188:** 122–130.

McKay, D.B., Prucha, M., Reineke, W., Timmis, K.N., and Pieper, D.H. (2003) Substrate specificity and expression of three 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Rhodococcus globerulus* strain P6. *Appl Environ Microbiol* **185:** 2944–2951.

Maltseva, O.V., Tsoi, T.V., and Quensen, J.F., III (1999) Degradation of anaerobic reductive dechlorination products of Aroclor 1242 by four aerobic bacteria. *Biodegradation* **10:** 363–371.

Masse, R., Messier, F., Peloquin, L., Ayotte, C., and Sylvestre, M. (1984) Microbial biodegradation of 4-chlorobiphenyl, a model compound of chlorinated biphenyls. *Appl Environ Microbiol* **47:** 947–951.

Morris, P.J., Mohn, W.W., Quensen, J.F., III, Tiedje, J.M., and Boyd, S.A. (1992) Establishment of a polychlorinated biphenyl-degrading enrichment culture with predominantly *meta* dechlorination. *Appl Environ Microbiol* **58:** 3088–3094.

Nakasawa, T., and Nakasawa, A. (1957) Pyrocatechase (*Pseudomonas*). *Methods Enzymol* **17A:** 518–522.

Omori, T., Sugimura, K., Ishigooka, H., and Minoda, Y. (1986) Purification and some properties of a 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA) reducing enzyme from *Pseudomonas crucivae* S93, B1 involved in the degradation of biphenyl. *Agric Biol Chem* **50:** 931–937.

Pettigrew, C.A., Breen, A., Corcoran, C., and Sayler, G.S. (1990) Chlorinated biphenyl mineralization by individual populations and consortia of freshwater bacteria. *Appl Environ Microbiol* **56:** 2036–2045.

van der Plas, S.A., Sundberg, H., van der Berg, H., Scheu, G., Wester, P., Jensen, S., *et al.* (2000) Contribution of planar (0-1 *ortho*) and nonplanar (2-4 *ortho*) fractions of Aroclor 1260 to the induction of altered hepatic foci in female Sprague–Dawley rats. *Toxicol Appl Pharmacol* **169:** 255–268.

Potrawfke, T., Lohnert, T.-H., Timmis, K.N., and Wittich, R.-M. (1998) Mineralization of low-chlorinated biphenyls by *Burkholderia* sp. strain LB400 and by a two-membered consortium upon directed interspecies transfer of chlorocatechol pathway genes. *Appl Microbiol Biotechnol* **50:** 440–446.

Quensen, J.F., III, Tiedje, J.M., and Boyd, S.A. (1988) Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediments. *Science* **242:** 752–754.

Quensen, J.F., III, Boyd, S.A., and Tiedje, J.M. (1990) Dechlorination of four commercial polychlorinated biphenyl mixtures (Aroclors) by anaerobic microorganisms from sediments. *Appl Environ Microbiol* **56:** 2360–2369.

Safe, S. (1992) Toxicology, structure–function relationship, and human and environmental health impacts of polychlorinated biphenyls: progress and problems. *Environ Health Perspect* **100:** 259–268.

Shields, M.S., Hoper, S.W., and Sayler, G.S. (1985) Plasmid-mediated mineralization of 4-chlorobiphenyl. *J Bacteriol* **163:** 882–889.

Sondossi, M., Sylvestre, M., and Ahmad, D. (1992) Effects of chlorobenzoate transformation on the *Pseudomonas test-*

osteroni biphenyl and chlorobiphenyl degradation pathway. *Appl Environ Microbiol* **58:** 485–495.

Tiedje, J.M., Quensen, J.F., III, Chee-Sanford, J., Schimel, J.P., and Boyd, S.A. (1993) Microbial reductive dechlorination of PCBs. *Bioremediation* **4:** 231–240.

Weber, R., Takasuga, T., Nagai, K., Shiraishi, H., Sakurai, T., Matuda, T., and Hiraoka, M. (2002) Dechlorination and destruction of PCDD, PCDF and PCB on selected fly ash from municipal waste incineration. *Chemosphere* **46:** 1255–1262.