



Metabolism of chlorinated biphenyls: Use of 3,3'- and 3,5-dichlorobiphenyl as sole sources of carbon by natural species of *Ralstonia* and *Pseudomonas*

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Abstract

Ralstonia sp. SA-3, *Ralstonia* sp. SA-4 and *Pseudomonas* sp. SA-6 are natural strains with a novel capacity to utilize *meta*-substituted dichlorobiphenyls (diCBs) hitherto not known to serve as a sole source of carbon and energy for polychlorobiphenyl-degraders. In growth experiments, axenic cultures of isolates grew logarithmically on 3,3'-diCB with generation times that ranged insignificantly (*t*-test, $P > 0.05$) from 30.4 to 33.8 h. Both 3-chlorobenzoate (3-CBA) and chloride produced as metabolites were recovered in non-stoichiometric quantities. The release of chloride by the cultures lagged substantially, indicating that the initial dioxygenase attack preceded cleavage of carbon–chloride bonds and that chloride must have been released from the chlorinated hydroxypentadienoate. In the case of 3,5-diCB, SA-3 and SA-6 metabolised this substrate primarily to 3,5-CBA. The lack of chloride in the culture media coupled with stoichiometric recovery of 3,5-CBA suggests that growth by these strains occurred predominantly at the expense of the unsubstituted phenyl ring. The unique metabolic properties of these three aerobic isolates point to their potential usefulness as seeds for bioremediation of PCBs polluted environments without the need for repeated inoculation or supplementation by a primary growth substrate such as biphenyl.

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1. Introduction

For nearly 50 years prior to early 1970s, PCBs were perceived as immutable and completely refractile to microbial degradation owing to their super hydrophobicity, thermodynamic stability, structural diversity and toxicity. However, it was the early report of Ahmed and Focht (1973b) and several other investigators (Furukawa et al., 1978, 1979; Bedard et al., 1986; Bopp, 1986) that shattered the dogma of PCB recalcitrance. PCBs are transformed aerobically by the biphenyl pathway, which is found in a wide

variety of soil organisms (Furukawa et al., 1979; Bedard et al., 1986). The degradation of PCBs by microorganisms thus far isolated is initiated by a biphenyl 2,3-dioxygenase. The *cis*-2,3-dihydro-2,3-dihydroxybiphenyl formed by this reaction is dehydrogenated to give 2,3-dihydroxybiphenyl, which is subjected to extradiol cleavage. The 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) thereby formed undergoes hydrolysis, yielding chlorobenzoate (CBA) and hydroxypenta-2,4-dienoate (HPD; commonly called the 5-C fragment) as reaction products. This sequence of reactions forms what is referred to as the upper pathway. Although certain PCBs serve as substrate for biphenyl dioxygenase (Mckay et al., 1997; Arnett et al., 2000; Zielinski et al., 2002), PCB-degrading organisms do not usually use PCBs as an energy source, but rather catabolize these pollutants cometabolically in the presence of easily

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metabolizable substrates such as biphenyl. This report therefore, suggests that there might be other organisms with better PCB-degrading competence.

Several investigators believed that only monochlorobiphenyls (CBs) function as a sole source of carbon and energy for aerobic bacteria. Others were of the opinion that earlier claims of isolation of PCB-mineralizing or dichlorobiphenyl (diCB)-degrading organisms must be viewed as equivocal because the cultures are not available, the media are insufficiently described to be reproduced by others, the purity of PCBs (i.e., absence of biphenyl) was not determined and no accompanying data on growth curves and chloride liberation were given. The report of McCullar et al. (1994) demonstrating growth of *Pseudomonas acidovorans* M3GY, a recombinant bacterium on 3,4'-diCB was proof that some diCBs can at least support growth of aerobic microorganisms. This strain was reported to mineralize 67% of 3,4'-diCB in 29 days. However, Potrawfke et al. (1998) were the first to document unambiguous growth by a natural organism, *Burkholderia* sp. LB 400 (now *Burkholderia xenovorans* LB400) on 2,3'- and 2,4'-diCB. Similarly, Kim and Picardal (2001) described for the first time growth on a *diortho*-substituted chlorobiphenyl (2,2'-diCB) by a novel natural bacterium capable of mineralizing both 4-CB and 2,4'-diCB. It is noteworthy that the substitution pattern of these diCB congeners makes them particularly recalcitrant to aerobic and anaerobic degradation (Abramowicz, 1990; Bedard and Haberl, 1990; Dai et al., 2002).

It is believed that development of effective bioremediation strategies will be aided by microbial sourcing of competent PCB degraders with the ability to utilize various isomers of diCBs in addition to CBs as sole sources of carbon and energy. In keeping with this consideration, we recently documented some bacterial strains with the uncommon capability for both PCB- and chlorobenzene-degradation phenotypes, thus suggesting a link in the metabolic pathways of both classes of environmental pollutants (Adebuseye et al., 2007a,b,c). The isolates obtained after several repeated enrichment on Askarel transformer fluid (a blend of PCBs and chlorobenzenes), utilized as carbon sources 2,3-diCB in addition to those reported by Kim and Picardal (2001). The work described in the present communication describes the metabolism of *meta*-substituted diCBs by axenic cultures of these bacterial strains. Previously, we stated that growth of these isolates was sustainable on 3,3'- and 3,5-diCB (Adebuseye et al., 2007b,c), but we herein provide detail growth dynamics vis-à-vis end-product distribution of the catabolic pathway. To the best of our knowledge, this is the first definitive documentation of any organism capable of utilizing a diCB bearing a chlorine at the *meta* position of both rings.

Specific PCB congeners can be present in an environmental sample as (a) components of commercial Aroclor mixtures, (b) pollutants produced by other processes or sources or (c) results of reductive dechlorination of more chlorinated congeners. The congeners used in these studies,

3,5- and 3,3'-diCB, are not major components of commonly used Aroclors. Albro and Parker (1979), for example, found 3,5-diCB present at 0.35–0.37 mol% in Aroclors 1242 and 1016, and Frame et al. (1996) found that Aroclor 1221 contained only 0.16% 3,3'-diCB.

Significant non-Aroclor sources, however, for 3,3'-diCB have been recently described. King et al. (2002) found that 3,3'-diCB was the predominant congener present in water and suspended particulate matter samples collected near Halifax Harbour. This congener represented up to 45% of all identified PCB congeners in the dissolved samples and up to 10% of all congeners in the suspended particulate fraction. King et al. suggest that the high 3,3'-diCB concentrations arise from commercial utilization of 3,3'-dichlorobenzidine, used in the production of dyes, pigments and plastic resin curing agents. In another study, Litten et al. (2002) similarly found 3,3'-diCB throughout the New York/New Jersey harbor and also attributed its presence to pigment manufacturing processes. The presence of this congener in grey seals from Sable Island, Nova Scotia (Addison et al., 1999), and in striped bass from Long Island Sound (Bush et al., 1990) suggests that non-Aroclor sources of PCBs may be widespread.

Congeners originally not found at high concentrations in Aroclors can also be generated via reductive dechlorination. Berkaw et al. (1996) reported that 56% of the 2,3,5,6-tetrachlorobiphenyl(2,3,5,6-tetraCB) added to Baltimore Harbor sediment incubations was dechlorinated to 3,5-diCB after 66 days. In another study, William (1994) demonstrated the dechlorination of 3,4,5-trichlorobiphenyl(3,4,5-triCB) primarily to 3,5-diCB. Similarly, Zanaroli et al. (2006) reported significant production of 3,3'-diCB following dechlorination of tetra-, penta- and hexa chlorobiphenyls in Venice Lagoon sediment incubations. In addition, 3,3'-diCB was the only PCB congener detected following incubation of 3,4,3',4'-tetraCB in microcosms incubated with Hudson River sediment microorganisms (Rhee et al., 1993). While absent or present at only low concentrations in commercial Aroclor mixtures, 3,5- and 3,3'-diCB can nevertheless be important constituents of some contaminated sediments either as a result of non-Aroclor sources or reductive dechlorination of more highly chlorinated precursors.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The isolation and characterization of *Ralstonia* sp. SA-4 and *Pseudomonas* sp. SA-6 have been previously described (Adebuseye et al., 2007b). *Ralstonia* sp. SA-3 was isolated from a landfill through successive transfers on mineral salts (MS) medium (Adebuseye et al., 2007c) containing Askarel electrical transformer fluid as growth substrate. All three strains were identified by AP 20 E test systems and 16S rRNA gene sequence analysis. The GenBank accession numbers for the sequences are DQ854843, DQ854845 and DQ854841, respectively, for strains SA-3, SA-4 and

SA-6. Strain SA-3 is physiologically different from SA-4 in that it utilized citrate, hydrolysed gelatin and reduced nitrate. All the strains grew readily on biphenyl, benzoate, naphthalene, all three CBs, 2,2', 2,3- and 2,4'-diCB as well as several congeners of di- and trichlorobenzenes (Adebusoje et al., 2007a, 2007b, 2007c).

2.2. Growth and chlorobiphenyl degradation studies

Studies of growth rates and metabolism of PCB congeners were performed as previously described (Adebusoje et al., 2007b). Briefly, organisms were inoculated (10^5 – 10^6 cells/ml) into MS medium (6 ml) containing 85–118 ppm (0.38–0.53 mM) of selected diCB congener in 30-ml Balch tubes. The sparingly soluble (<2 mg/L, Erickson, 1997) congeners were added from stock solutions of 2,2,4,4,6,8,8-heptamethylnonane (HMN), a highly branched alkane not known to be biodegradable. As previously described, the HMN improves mass transfer and provides a reservoir of congener to replace degraded aqueous-phase diCB (Adebusoje et al., 2007b). Tubes were crimp-sealed with Teflon-coated, butyl rubber stoppers to prevent losses due to volatilisation and/or sorption, and incubated horizontally on an orbital shaker at 25 °C. A whole set of three replicate tubes were sacrificed at each time point in time-course experiments. Metabolic reactions were stopped by the addition of 5 ml of hexane. PCB extraction was handled as previously described (Adebusoje et al., 2007b) while the filtered aqueous phase was analysed for both chloride and CBA products. Cell numbers were monitored in additional replicate tubes using an acridine orange direct counting method (Kepner et al., 1994).

2.3. Chemicals

All chemicals used in this study were reagent grade or better. 3,3'- and 3,5-dichlorobiphenyls were purchased from AccuStandard Inc. (New Haven, CT, USA) and do not contain biphenyl as a contaminant. All standards are solutions of PCB purchased from Ultra Scientific (North Kingstown, RI, USA). Chlorobenzoates were from Sigma–Aldrich Corp. (St. Louis, MO, USA) while HMN, sodium benzoate and HPLC reagents were obtained from Fisher Scientific Co. (Springfield, NJ, USA).

3. Analytical methods

3.1. GC analysis

Hexane extracts were analyzed on an HP 5890 Series II GC (Hewlett Packard Co., Palo Alto, CA, USA) fitted with an HP 3396 Series II Integrator, and a ^{63}Ni electron capture detector (ECD) and flame ionization detector (FID). Samples (1 μl) were chromatographed on a 30 m DB-5 megabore fused-silica capillary column (J & W Scientific, Folsom, CA, USA; 0.53 mm id, 2.5 μm film thickness)

coated with 5% phenyl substituted methylpoly siloxane stationary phase to the ECD.

PCBs were quantified using four-point calibration curves constructed using standards bracketing the concentration expected in sample showing no degradation. Typical coefficient of correlation, r for standard curves was 0.95–0.99. GC efficiency and accuracy was constantly ascertained by injections made from standards prior to sample analysis.

3.2. HPLC analysis

CBA metabolites in the filtered culture fluids were analysed using HPLC (Waters Corp. Milford, MA, USA) equipped with a UV, dual absorbance detector (Model 2487). Separation was performed on a YMC-Pack ODS-AQ reversed-phase column (YMC Co. Ltd., Kyoto, Japan). Chlorobenzoates were detected at $\lambda_{238\text{ nm}}$ and identified with reference standard congener by retention time.

Chloride released was similarly measured via the same HPLC but using a conductivity detector (Model 432) and IonPac AS17 analytical column (4 \times 250 mm; Dionex) which was preceded by an AG17 guard column. Calibrated checks standard and blank were run with each sample test.

3.3. Statistical analysis

Mean generation times (T_g) and specific growth rates (μ) were calculated using non-linear regression of growth curves. Regression, correlation and t -test analysis were performed using the Prism version 2.01 software programme (GraphPad Software, San Diego, CA, USA).

4. Results

Ralstonia sp. strain SA-3, *Ralstonia* sp. strains SA-4 and *Pseudomonas* sp. strain SA-6 are the first pure cultures with the capacity to utilize 3,3'- and 3,5-diCB as a sole source of carbon and energy. Washed benzoate-grown cells of these three isolates grew logarithmically on 3,3'-diCB in almost identical fashion (Fig. 1). Generation times ranged insignificantly (t -test; $P < 0.05$) from 30.4 h (1.3 d) to 33.8 h (1.4 d) with the lowest obtained for strain SA-3 (Table 1). Population densities of the three strains increased by more than two hundred-fold concomitant with disappearance of the PCB substrate and appearance of metabolites of the degradative pathway. However, metabolites recovered in culture fluids lagged substantially and did not mirror the amount of substrate catabolized (Fig. 1). This trend readily suggests transient accumulation of intermediate precursors of CBA and (chlorinated)HPD. In the control tubes without inoculation, no apparent decrease of the substrate was observed. This indicates that the depletion of the PCB from the assay media was due to biodegradation rather than to non-specific losses such as compound volatility or adsorption to the glass tubes. Strain SA-3 was able to transform 84% of the initial 0.38 mM concentration of 3,3'-diCB in

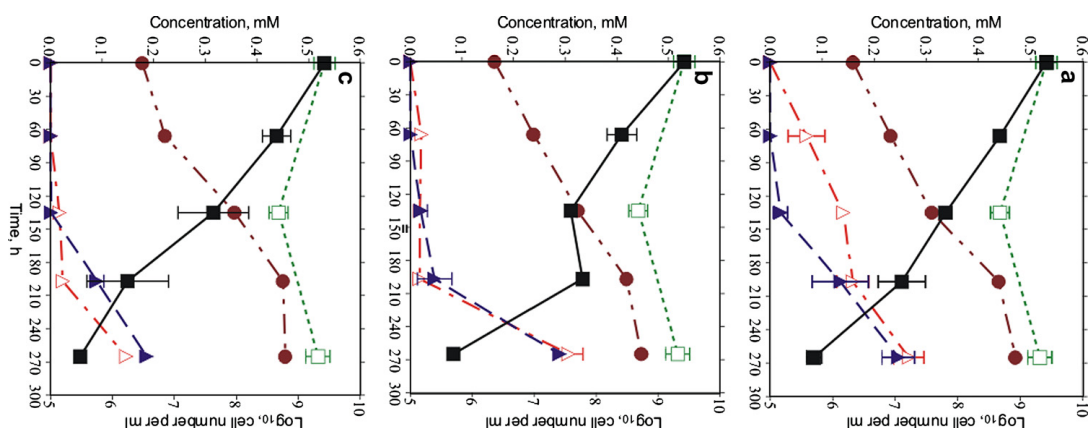


Fig. 1. Transformation of 3,3'-diCB by strains SA-3 (a), SA-4 (b) and SA-6 (c) with accompanying data for cell growth (●), chloride release (▲) and production of 3-CBA (■). 3,3'-diCB concentrations are shown for tubes containing a cell inoculum (■) and control tubes lacking cells (□). All data represent the mean of three replicate tubes and error bars represent standard deviation. Lack of error bars for analytical data indicates that the error bars were smaller than the symbol. Error bars for cell counts were eliminated to improve clarity. The typical standard deviations observed for cell counts were within 20–40% of the mean values shown.

265 h (~11 d) while 0.25 mM 3-CBA and 0.27 mM chloride were recovered as metabolites. However, the 3-CBA

Table 1
Growth kinetics and catabolites formed during metabolism of 3,3'-diCB and 3,5-diCB by natural strains of *Ralstonia* sp. SA-3, *Ralstonia* sp. SA-4 and *Pseudomonas* sp. SA-6

Bacterial strain	3,3'-diCB						3,5-diCB					
	T_g (h)	μ (h^{-1})	% Degradation	3-CBA recovered (mM)	% 3-CBA recovered	Chloride released (mM)	T_g (h)	μ (h^{-1})	% Degradation	3,5-CBA recovered	% 3,5-CBA recovered	Chloride released (mM)
SA-3	30.42	0.023	84.2 ± 0.47	0.25 ± 0.05	54.6 ± 9.86	0.27 ± 0.05	31.1	0.022	84.6 ± 1.71	0.31 ± 0.01	98.4 ± 4.34	0
SA-4	33.33	0.021	84.2 ± 1.31	0.29 ± 0.02	64.1 ± 3.51	0.31 ± 0.04	32.19	0.22	89.5 ± 7.5	0.07 ± 0.01	21.4 ± 3.76	0
SA-6	33.83	0.021	89.0 ± 1.57	0.19 ± 0.02	39.6 ± 3.86	0.15 ± 0.2	32.83	0.021	90.5 ± 2.46	0.34 ± 0.006	100 ± 17.1	0

T_g , mean generation time; μ , specific growth rate. All values are means ± standard deviations for triplicate cultures. Percent degradation values have been calculated with reference to the amount recovered from uninoculated control tubes, while percent mineralization was expressed as chloride released in the medium with respect to that chemically bound in 3-chlorophenyl ring. 3,3'-diCB and 3,5-diCB substrates were supplied at respective concentrations of 0.38 ± 0.03 mM and 0.53 ± 0.04 mM. The dichlorobiphenyl substrates were incubated with respective organisms for 265 h.

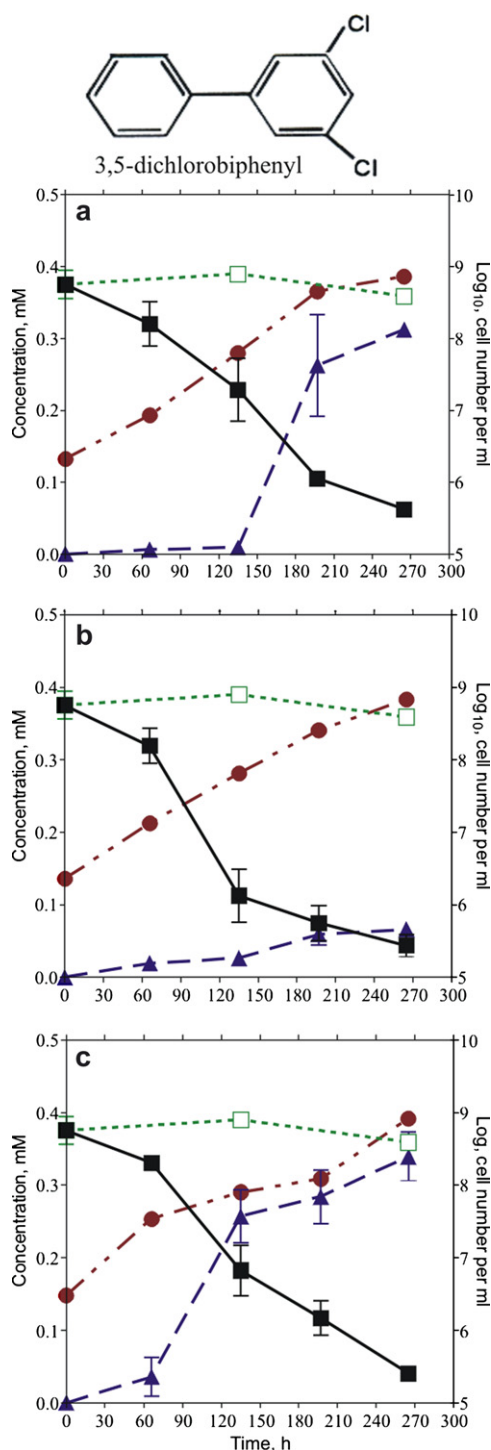


Fig. 2. Transformation of 3,5-diCB by strains SA-3 (a), SA-4 (b) and SA-6 (c) with accompanying data for cell growth (●) and production of 3,5-CBA (▲). 3,5-diCB concentrations are shown for tubes containing a cell inoculum (■) and control tubes lacking cells (□). Growth of the strains did not result in chloride production. All data represent the mean of three replicate tubes and error bars represent standard deviation. Lack of error bars for analytical data indicates that the error bars were smaller than the symbol. Error bars for cell counts were eliminated to improve clarity. The typical standard deviations observed for cell counts were within 20–40% of the mean values shown.

was not detected until after 66 h (~3 d) of cultivation. The amount of chloride released is indicative of 51% minerali-

zation of one of the *meta*-substituted ring (Table 1). In the case of SA-4, a total decrease of the substrate from 0.53 mM to 0.08 mM was observed, giving an overall disappearance rate of $0.32\% \text{ h}^{-1}$. During this period, 64% of the metabolized congener was recovered as 3-CBA, while the amount of chloride dissociated from the biphenyl nucleus was 0.31 mM. It is noteworthy that neither 3-CBA nor chloride production was significant until after 197 h (~8 d) (Fig. 1). The highest degradation value (89%) was obtained in tubes inoculated with SA-6, but the recovery of the expected transformation product, 3-CBA, was relatively low (3-CBA recovered = 39.6%). Similar to observations in SA-3, both chloride (0.15 mM) and 3-CBA lagged significantly (Table 1, Fig. 1).

The kinetics of 3,5-diCB metabolism (initial 0.38 mM) in the three strains is shown in Fig. 2. The growth rate constant of SA-3 in batch cultures was 0.022 h^{-1} ($T_g = 31.1 \text{ h}$). Corresponding values obtained for SA-4 and SA-6 were 0.22 h^{-1} ($T_g = 32.2 \text{ h}$) and 0.021 h^{-1} ($T_g = 32.8 \text{ h}$), respectively. The bacterial strains metabolised 3,5-diCB via oxidation of the unchlorinated ring to yield stoichiometric amounts of 3,5-CBA (with the exception of strain SA-4), but they exhibited varying degradation patterns. Since no chloride was released into the culture fluids and no further reduction of the 3,5-CBA metabolite was observed, the acid was likely produced as a dead-end product. Utilization of 3,5-diCB was gradual in SA-3 and as summarized in Table 1 and Fig. 2, nearly 85% of this congener was completely transformed to 3,5-CBA. However, the product only became noticeable after 135 h (~6 d) of incubation. In the case of SA-4, the congener was slowly metabolised during the first 66 h of incubation. It was much more rapidly and extensively utilized thereafter especially between 66 and 135 h, culminating in approximately 90% degradation. Recovery of 3,5-CBA from the culture media was not stoichiometric. The metabolism of 3,5-diCB by SA-4 was accompanied in less than 48 h (2 d) by the appearance of a transient yellow colour that we have attributed to the accumulation of the *meta*-ring fission product (HOPDA) on the basis of its absorption maximum. This observation suggests that the pathway for dissimilation of PCBs can be rationalized to proceed via HOPDA.

5. Discussion

Only a few of the large number of bacteria isolated that degrade PCBs aerobically can actually grow on CBs or diCBs as sources of carbon and energy. Bacteria capable of growing on diCBs are apparently somewhat rare in nature. However, in the current investigation, we have unequivocally demonstrated that strains SA-3, SA-4 and SA-6 degraded both 3,3'- and 3,5-diCB when supplied as sole substrates, demonstrating that the isolates are able to utilize these congeners as sources of carbon and energy. This is an indication of the presence of efficient and unique metabolic capabilities that may be widespread in the tropical African contaminated systems from where these organ-

isms were isolated. Cometabolism of the diCBs was ruled out since washed benzoate-grown cells were used and congeners used were high purity (99–100%) analytical grades that did not contain biphenyl. The well characterized and widely studied organisms, *Burkholderia xenovorans* LB400, *Alcaligenes eutrophus* H850, *Corynebacterium* sp. MB1, *Rhodococcus* sp. RHA1 and *Alcaligenes* sp. JB1 reported to possess exceptional ability to degrade even larger range of PCB congeners containing up to six chlorine substituents (Bedard et al., 1987; Gibson et al., 1993; Seto et al., 1995; Commandeur et al., 1996; Billingsley et al., 1997; Mondello et al., 1997) have not been reported to definitively utilize PCBs other than CBs as carbon sources for growth with exception of LB400 which Potrawfke et al. (1998) demonstrated to grow on 2,3'- and 2,4'-diCB as sole carbon sources. In addition to the two, *meta*-substituted diCB isomers reported herein, SA-3, SA-4 and SA-6 are able to grow on all three CBs, plus the *ortho*-substituted diCBs, 2,2'-, 2,4'- and 2,3'-diCB (Adebusoje et al., 2007b,c). The range of growth substrates used by our isolates is therefore quite unique and significantly wider than LB400 (Potrawfke et al., 1998), *Burkholderia* sp. SK-3 and *Alcaligenes* sp. SK-4 reported by Kim and Picardal (2001).

Several investigators (Ahmed and Focht, 1973b; Furukawa et al., 1979; Yagi and Sudo, 1980; Masse et al., 1984) have proposed that bacterial PCB degradation is initiated by the attack of a dioxygenase at carbon positions 2,3 (or 5,6) of the unchlorinated or less chlorinated ring. If this same mechanism is operational in our organisms, the degradation of 3,3'-diCB would result in production of chlorinated HPD in addition to 3-CBA (Fig. 3). This pathway represents the most common PCB-degrading metabolic sequence (Furukawa et al., 1979; Fava and Marchetti, 1991; McCullar et al., 1994; Pieper, 2005). This inference is corroborated by the fact that chloride released lagged substantially (Fig. 1), suggesting that the initial degradation step did not result in chloride elimination (see Fig. 3). In the case of 3,5-diCB, the stoichiometric 3,5-CBA production (except in SA-4 incubations) confirmed that the bacterial dioxygenase exclusively attacked the non-chlorinated aromatic ring. This result is in agreement with previous reports on aerobic degradation of PCBs (Furukawa et al., 1979; Abramowicz, 1990). The unsubstituted nucleus was cleaved to give growth substrates, while

the chlorinated ring was excreted into the culture media as an organic chloride end-product. In particular, the capability of these strains to grow on the di-*meta*-substituted 3,3'-diCB is unusual since this congener is sometimes poorly degraded cometabolically in the presence of biphenyl. Ahmed and Focht (1973a), for example, showed that oxygen uptake by CB-grown, resting-cell suspensions of *Achromobacter* pCB was much lower when incubated with 3,3'-diCB than with four other diCBs. Mohn et al. (1997) reported that indigenous microorganisms in a contaminated Arctic soil were unable to substantially degrade 3,3'-diCB present in Aroclor 1221 in the presence of biphenyl, although notable cometabolic degradation was subsequently obtained using two isolates, Sag-50A and Sag-50G, obtained from the soils. In a study comparing the influence of chlorine substitution patterns on the degradation of PCB congeners by eight different bacterial strains, including LB400, H850 and MB1, Bedard and Haberl (1990) reported that 3,3'-diCB was poorly degraded cometabolically or was not degraded at all.

Analysis of catabolic products of 3,3'-diCB metabolism showed that both chloride and 3-CBA were recovered in non-equimolar quantities (Table 1). Apparently, more than 40% of the degraded congener not recovered as CBA in these strains was either arrested at earlier stages of metabolism or that the 3-CBA formed was further transformed to other several chlorinated products thus escaping detection as previously document by Yagi and Sudo (1980). In the latter case, it is possible that the transformation of 3-CBA to 3-chlorocatechol (3-CC) is common to the three strains. The transformation of 3-CB to 3-CBA that was later converted to 3-CC or 4-CC have been demonstrated previously Fava and Marchetti (1991). It is possible that chlorocatechols are able to reduce the activities of both catechol dioxygenase and some enzymes of the upper biphenyl pathway. In fact, Arensdorf and Focht (1994) demonstrated that 3-CC interfered with the utilization of CBs by *Pseudomonas cepacia* P166. This was as a result of suicide inactivation by 3-CC of 2,3-dihydroxybiphenyl dioxygenase, a key enzyme in the upper pathway responsible for the conversion of 2,3-dihydroxybiphenyl to HOPDA. In the former, the result would readily suggest a possible accumulation of the *meta*-fission product of 3,3'-diCB transformation in the culture media. Recent studies using LB400

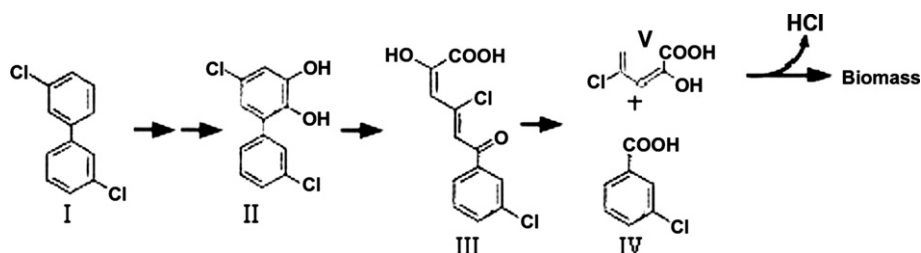


Fig. 3. Hypothetical pathway for metabolism of 3,3'-diCB (I) by SA strains; (II) 2,3-dihydroxy 3,3'-dichlorobiphenyl; (III) 4,9-dichloroHOPDA; (IV) 3-CBA and (V) 4-chloroHPD. 3-CBA production suggests dioxygenase attack at the 2,3-position. The 4-chloroHPD produced is converted to biomass via the TCA cycle, with the eventual release of chloride. Although accumulation of 3-CBA is not unlikely, also further metabolism to chlorinated intermediates is not improbable.

HOPDA hydrolase BphD revealed that this enzyme relatively rapidly transformed HOPDAs bearing chlorine substituents at the phenyl moiety whereas, HOPDAs bearing chlorines on the dienoate moiety were poor substrates, although they act as competitive inhibitors of BphD (Seah et al., 2000; Pieper, 2005). Since dynamics of catabolic products suggest that initial attack of the biphenyl nucleus did not result in chloride elimination, degradation of 3,3'-diCB would certainly produce HOPDA chlorinated at both phenyl and dienoate moieties (4,9-dichloroHOPDA) (Fig. 3) and thus could partly account for the failure to achieve a mass balance. To further provide insight into whether or not the *meta*-fission product of both 3,3'- and 3,5-diCB accumulated in the culture media, a study was conducted to monitor conversion of the congeners into HOPDA by visible spectra scanning of the cell assay mixture according to the methods of Seeger et al. (1995) and Maltseva et al. (1999). Results showed accumulation of HOPDAs from 3,3'-diCB over the 24 h (1 d) monitoring period particularly in strains SA-3 and SA-4 while, with the exception of SA-4, no apparent accumulation of the *meta* product was observed in the 3,5-diCB assay mixture (data not shown). Interestingly, for strain SA-4, metabolism of 3,5-diCB was accompanied by the appearance of a bright yellow *meta*-fission product and the recovery of the expected transformation product, 3,5-CBA, was quite low. In an earlier study, Seeger et al. (1995) observed that 20–25% of 3,3'-diCB was converted to 3-CBA by a recombinant *E. coli* strain containing all the enzymes of the upper pathway. Accumulation of the *meta*-fission product was the reason given by the authors for the obtained results.

If 3-CBA is produced as the primary metabolic product, then growth must have occurred at the expense of chlorinated HPD during metabolism of 3,3'-diCB. However, the deficit in the chloride balance can only be explained by partial mineralization of chlorinated HPD (Fig. 3). Hernandez et al. (1995) reported that this compound does not appear to be metabolized by PCB-degraders. This could be a metabolic bottleneck in the use of this congener and similar others as a source of carbon and energy by PCB-degrading microorganisms.

3,3'-diCB can assume a coplanar configuration but likely has a lower toxicity than more highly chlorinated coplanar PCBs. It may, however, have a synergistic effect on the toxicity of these more highly chlorinated compounds. In a study of the *in vitro* effects of PCBs on human platelets (Raulf and König, 1991), it was shown that the toxic effects of the coplanar 3,3',4,4'-tetraCB were synergistically increased by the presence of 3,3'-diCB. Although not a coplanar PCB, metabolites of 3,5-diCB may belong to the group of compounds that can affect thyroid gland morphology and hormonal status. A recent study examined such effects using biosensor assays for a group of polyhalogenated aromatic compounds including halogenated phenols, halogenated bisphenols, 3,5-diCB and its hydroxylated metabolite, 4-hydroxy-3,5-diCB, formed *in vivo* by the cytochrome P450 mono-oxygenase system (Marchesini

et al., 2006). Although 3,5-diCB itself had a relatively low thyroid disruptor activity, its hydroxylated metabolite had the greatest relative potency of all the compounds tested.

The growth of our isolates on 3,3'- and 3,5-diCB is significant. To our knowledge, organisms able to grow on double *meta*-substituted congeners had not previously been isolated and such a metabolism has not been demonstrated for any PCB-degraders until now. The transformation of these congeners primarily to CBAs may not be a problem in the environment. Since the organisms grow readily on benzoate, it is possible they may be capable of cometabolic degradation of these acids (Janke and Fritsche, 1985; Adriaens and Focht, 1991; Fava et al., 1993), but such studies have not yet been attempted. Alternatively, CBAs produced could be (co)metabolized further by other soil microflora. Further research will be required in more complex microcosms to determine if the CBA products can be degraded under either scenario.

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