Biodegradation (2008) 19:145–159
DOI 10.1007/s10532-007-9122-x

ORIGINAL PAPER

Characterization of multiple novel aerobic polychlorinated biphenyl (PCB)-utilizing bacterial strains indigenous to contaminated tropical African soils

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Received: 29 November 2006 / Accepted: 10 April 2007 / Published online: 30 May 2007

Abstract Contaminated sites in Lagos, Nigeria were screened for the presence of chlorobiphenyl-degrading bacteria. The technique of continual enrichment on Askarel fluid yielded bacterial isolates able to utilize dichlorobiphenyls (diCBs) as growth substrates and six were selected for further studies. Phenotypic typing and 16S rDNA analysis classified these organisms as species of *Enterobacter*, *Ralstonia* and *Pseudomonas*. All the strains readily utilized a broad spectrum of xenobiotics as sole sources of carbon and energy. Growth was observed on all monochlorobiphenyls (CBs), 2,2′-, 2,3-, 2,4′-, 3,3′- and 3,5-diCB as well as di- and trichlorobenzenes Growth was also sustainable on Askarel electrical transformer fluid and Aroclor 1221. Time-course studies using 100 ppm of 2-, 3- or 4-CB resulted in rapid exponential increases in cell numbers and CB transformation to respective chlorobenzoates (CBAs) within 70 h. Significant amounts of chloride were recovered in culture media of cells incubated with 2-CB and 3-CB, suggesting susceptibilities of both 2- and 3-chlorophenyl rings to attack, while the 4-CB was stoichiometrically transformed to 4-CBA. Extensive degradation of most of the congeners in Aroclor 1221 was observed when isolates were cultivated with the mixture as a sole carbon source. Aroclor 1221 was depleted by a minimum of 51% and maximum of 71%. Substantial amounts of chloride eliminated from the mixture ranged between 15 and 43%. These results suggest that some contaminated soils in the tropics may contain exotic micro-organisms whose abilities and potentials are previously unknown. An understanding of these novel strains therefore, may help answer questions about the microbial degradation of polychlorinated biphenyls (PCBs) in natural systems and enhance the potential use of bioremediation as an effective tool for cleanup of PCB-contaminated soils.

Keywords Aerobic biodegradation · Bioremediation · Chlorobenzene · PCBs · Polychlorinated biphenyls

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Introduction

The manufacture, use and disposal of polychlorinated biphenyls (PCBs) have been subject to strict governmental regulations for more than three decades owing

to their environmental and potential carcinogenic effects. Although many developing countries in Africa include PCBs and PCB-waste oils on the list of hazardous substances, the discharge of these pollutants into the environment is often not monitored and regulated. Pollution, when it occurs is never reported or investigated. As a result, data on importation, use, transportation and environmental fate of PCBs are usually unavailable. Leakage from transformers and indiscriminate discharge of the spent PCB insulating fluid (Askarel) are regular occurrences. The safe and economical degradation of PCBs, therefore, is one of the major environmental challenges facing many African countries today.

In spite of the superhydrophobicity and chemical stability, it is amazing that some bacterial strains that can metabolize specific PCB congeners have been described. The isolation of two bacterial strains capable of aerobic degradation of 2-, 3-, 4-monochlorobiphenyl (CB) and 2,2′- and 4,4′-dichlorobiphenyl (diCB) as co-cultures in 1973 (Ahmed and Focht 1973) changed our perception of PCBs as immutable chemicals. Since then, a large number of micro-organisms that can degrade PCBs were isolated from contaminated soils and sludges (Furukawa et al. 1979; Bopp 1986; Bedard et al. 1987a; Kim and Picardal 2000) and studied in greater detail, culminating in elucidation of the PCB biodegradation pathway. One similarity among PCB degrading micro-organisms is that they are all biphenyl utilizers which metabolize PCBs with the same suite of enzymes employed in biphenyl catabolism (Ahmed and Focht 1973; Furukawa et al. 1979; Abramowicz 1990). Aerobic catabolism of PCB usually begins with the attack of biphenyl dioxygenase on an unsubstituted 2,3 position of a non-chlorinated or less chlorinated ring. This is followed by *meta*-cleavage, producing chlorobenzoic acids (CBAs) and a 5-carbon aliphatic acid (2-hydroxy 2,4-pentadienoic acid). None of these steps is linked to energy conservation. If an organism is to grow on the PCB substrate, it must get energy from subsequent metabolism of either the CBA or more commonly, the 5-C fragment, with the resultant accumulation of the former in the culture fluid (Bevinakatti and Ninnekar 1993; Kim and Picardal 2000). Micro-organisms that can utilize PCBs as growth substrates must either utilize non-chlorinated rings, producing CBAs as products, or produce enzymes capable of carbon–chlorine bond cleavage. Ability of micro-organisms to grow on PCBs is generally limited to monochlorophenyls, while congeners containing 2 or more chlorines are usually aerobically degraded via co-metabolism (Furukawa et al. 1979; Bedard et al. 1987b; Abramowicz 1990), which requires biphenyl as a growth substrate and inducer of the requisite enzymes. Biphenyl, however, is toxic, often subject to regulatory restrictions and not easily dispersed in contaminated soils or sludges. Hence, isolation of organisms that grow on environmental PCB mixtures without the need of biphenyl as a primary substrate might obviate this problem.

Isolates, which can utilize 4-CB as a growth substrate are common (Barton and Crawford 1988; Mondello 1989; Ahmad et al. 1990; Arensdorf and Focht 1995). Reports of isolates, which utilize 2- and 3-CB are less common (Potrawfke et al. 1998; Bedard et al. 1987a; Hickey et al. 1992; Kim and Picardal 2000) and, with the exception of the isolates described by Arensdorf and Focht (1994) and Kim and Picardal (2000), no organism has been reported to grow on all 3 monochlorobiphenyl isomers. In addition, most of the reported isolates possess narrow substrate spectra. Furthermore, there is paucity of information on the biodegradation of PCBs by organisms isolated from tropical African environment. In fact, almost, if not all of the micro-organisms characterized to date were obtained from contaminated temperate soils. It is unlikely, however, that identical xenobiotic-degrading bacteria are uniformly distributed around the globe due to differing ambient environmental conditions, soil composition, organic carbon soil inputs and many other factors. In view of the wider geochemical and physical variations in both environments, tropical microbes may exhibit a fascinating metabolic diversity and possess novel catabolic properties for real time degradation of PCBs. In the current study, we isolated a number of organisms from contaminated tropical soils that exponentially grow on all CBs and some diCBs as sole carbon and energy sources under aerobic conditions. These organisms were also able to extensively transform several congeners in commercial mixture of Aroclor 1221 with evidence of chloride elimination. To the best of our knowledge, this is the first report demonstrating the occurrence of competent multiple PCB-degrading micro-organisms in African-contaminated systems.

Materials and methods

Chemicals

Polychlorinated biphenyl congeners (> 99% purity), PCB commercial mixture (Aroclor 1221; > 98.6–100% purity) and chlorobenzene isomers (> 98.6% purity) were purchased from AccuStandard Inc. (New Haven, CT, USA). Since none of the single PCB congeners contained biphenyl as an impurity, they were utilized as received from the manufacturer without further purification. PCB analytical standards were obtained from Ultra Scientific (North Kingston, RI, USA). Biphenyl, naphthalene (99+%), chloroacetic acid (98% purity) and mono- and dichlorobenzoic acids (98% purity) were acquired from Sigma-Aldrich Corp. (St Louis, MD, USA). Sodium benzoate (99+% purity), 2,2,4,4,6,8,8-heptamethylnonane (HMN), and all other organic solvents (HPLC grade) were obtained from Fisher Scientific Co. (Springfield, NJ, USA). Askarel oil (a blend of PCBs and chlorobenzenes) was generously supplied by the NEPA Transformer Workshop (Ijora, Lagos, Nigeria).

Stock solutions and media

The chloride-free mineral salts (MS) medium used in this study was formulated according to Kim and Picardal (2000). Due to limited aqueous solubility, stock solutions of each PCB and chlorobenzene congener were prepared in HMN, a non-degradable carrier, to provide an initial concentration of 100 ppm. The concentration given represents the total mass in both the aqueous and HMN phases, divided by the aqueous volume. The appropriate stock solution was added in 20 µl aliquots to provide a test compound concentration of 100 ppm in the finished medium. Solid MS medium was made by the addition of 1.6% Bacto-agar (Difco Laboratories, Detroit, MI, USA). MS medium was supplemented with test compounds to achieve an experiment-dependent concentration of either 100 ppm or 2.5 mM. Unless otherwise stated, cultures were incubated at 25 °C on a shaker table to improve mass transfer from the HMN-phase into the aqueous phase. In order to establish that the HMN carrier was not a growth substrate for our isolates, preliminary growth studies were performed in MS medium supplemented with HMN as the sole carbon and energy source.

Enrichment of microbial communities

Soil samples were collected randomly from six contaminated sites in Lagos. Three of these sites, namely Ojota; National Electric Power Authority (NEPA) Transformer Workshop, Ijora; and NEPA Thermal Station, Surulere have been heavily polluted for decades, mainly with PCB- and chlorobenzene-containing, electrical transformer fluid (Askarel). Chlorobiphenyl-degrading bacteria were initially isolated by traditional enrichment culture methods: Soil samples (1 g) were used to inoculate MS medium contained in a conical flask (500 ml). The medium was supplemented with Askarel fluid (0.1% v/v) as the primary carbon source. Enrichment cultures were incubated on a gyratory shaker incubator (New Brunswick Scientific Co., Edison NJ, USA) at 120 rpm for 30 days. Subsequent transfers from these enrichments were made (every month) by using the same methods and conditions. A parallel enrichment was also set up in 160-ml serum bottles containing MS medium (40 ml) supplemented with 100 ppm Aroclor 1221. Bottles were inoculated with soil sample (5 g), crimp-sealed with Teflon-coated stoppers and incubated horizontally on a shaker table (Labline Instruments Inc., Melrose Park, IL, USA). After 1 month, enrichment cultures were transferred to fresh medium using a 10% inoculum and continued cultivation under the same conditions. Subsequent transfers were carried out using 1% inoculum and the procedure repeated for six successive times.

Isolation, purification and characterization of pure cultures

Pure cultures from Askarel-enriched media were isolated by directly plating out appropriate aliquots (0.1 ml) of highly enriched cultures onto nutrient agar. Since we desired to use these same enrichments to isolate bacteria growing on chlorobenzenes in the Askarel (Adebusoye et al. 2007), we used nutrient agar to obtain pure cultures rather than MS medium supplemented with biphenyl or benzoate which may have selected against chlorobenzene-degraders unable to utilize those substrates. Pure colonies selected from the nutrient agar plates were subsequently screened for their ability to utilize selected chlorobiphenyls in MS medium as described below.

Organisms in Aroclor 1221 enriched cultures were obtained by a spray plate technique. Desired dilutions (0.1 ml) were spread on MS agar. Immediately after, an ethereal solution of biphenyl was uniformly sprayed onto the surface of the agar. The plates were sealed with paraffin film and incubated for 4–6 weeks. Biphenyl-degrading micro-organisms were identified by compound-cleared zones surrounding individual colony. Such colonies were purified on MS agar containing 2.5 mM benzoate.

Organisms capable of growing on PCB congeners as a sole source of carbon and energy were classified using standard cultural and morphological techniques. The API 20 E test kits (bioMerieux Vitek, Hazelwood, MO, USA) were also employed. Identification of the micro-organisms was achieved on the basis of 16S rRNA gene analysis. Genomic DNA was isolated from overnight cultures of isolates on 2.5 mM benzoate using an ultra clean DNA prep kit (MoBio Laboratories, Solana Beach, CA, USA). Two Eubacterial PCR primers; forward primer 63f and reverse primer 1387r, were used to amplify ~1,300 bp of the 16S rRNA gene. The resulting PCR-generated fragments were gel-purified and cloned into the PCR-TOPO 1 plasmid (Invitrogen Life Technologies, Carlsbard, CA, USA) vector. Recombinant plasmids with correct rDNA inserts were digested with EcoRI and then sequenced (ABI 3700 sequencer; AP Biotech, Buck, UK), and were probed against the GenBank database with the BLAST algorithm. Organism identities so obtained were subsequently verified using the Ribosomal Database Project (Cole et al. 2007) and identical results were obtained.

Growth on different carbon sources and determination of PCB transformation potential

Pure cultures were tested for their ability to grow on a variety of defined carbon sources. The tests were performed in MS medium supplemented with the test compound as the sole carbon source. Substrates were added to 6 ml MS medium in Balch tubes at a concentration of 100 ppm and inoculated with 10^5–10^6 cells/ml of phosphate buffer (pH 7.2)-washed cells pre-grown with 2.5 mM benzoate. Tubes were crimp-sealed with Teflon-coated stoppers to prevent abiotic losses and incubated horizontally on an orbital shaker at 100 rpm. Substrates tested included all mono- and dichlorobenzoic acid congeners, chloroacetic acid, biphenyl, naphthalene and various congeners of chlorobenzenes and chlorobiphenyls. Stock solutions of all substrates were autoclaved prior to use. Growth was evaluated by microscopy and visual monitoring of turbidity in conjunction with periodic GC and HPLC analyses to measure test compound disappearance or appearance of products. In these substrate-screening studies, growth was scored as positive if turbidity was notably greater than in controls lacking the test compound, microscopic examination revealed an increase in cell numbers, and GC or HPLC analysis showed loss of the test compound. Growth tests were conducted in triplicate for each substrate.

In time course experiments, three replicate tubes were sacrificed at each time point. Reactions were stopped by adding 10 ml of hexane, vortexing for 1–2 min and thereafter, mixing continuously on a tube rotator for 12–24 h. The aqueous phase was centrifuged (Hermle Z 180M Labnet) at 13,000 × *g*, and filtered. The hexane extracts and aqueous phases were separately collected for analysis. Growth of the cultures was monitored by a direct counting method using acridine orange staining (Kepner et al. 1994).

Transformation of Aroclor 1221

Experiments to study degradation of the commercial PCB mixture, Aroclor 1221, were similarly conducted in Balch tubes. All tubes were inoculated with respective bacterial cultures, crimp-sealed and incubated horizontally with shaking at room temperature. Transformation reactions were stopped after 12 days by the addition of 10 ml hexane. To assess the dynamics of degradation and release of chloride, the entire contents of triplicate tubes were sacrificed and analysed. Percent degradation was calculated as the decrease in total summation of all ECD (electron capture detector) area counts. The percentage of chloride released from the Aroclor mixture was determined based on percent composition of chlorine in the mixture. For instance, Aroclor 1221 was assumed to contain a chlorine content of 21%.

Analytical methods

GC analysis

Hexane extracts were analysed on an HP 5890 series II gas chromatograph (GC) (Hewlett Packard Co.,

Palo Alto, CA, USA) fitted with an HP 3396 series II Integrator, and an ECD. Hexane extracts (1 µl) injected using a 10-µl Hamilton syringe was carried through 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. The carrier gas was helium, with a linear velocity of 30 cm/s at 100 °C. The injector and detector temperatures were 200 and 275 °C, respectively. The GC was programmed at an initial temperature of 70 °C; this was held for 1 min, then ramped at 30 °C/min to 160 °C, ramped to 200 °C at 2 °C/min for 1 min, and then held for 14 min.

Analytical standards of PCBs were prepared in hexane at concentration range of 0.05–1 mM. Typical coefficients of correlation for standard curves were 0.95–0.99.

HPLC analysis of CBAs and chloride

Chlorobenzoate metabolites in the aqueous phase were analysed by 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) at 30°C by isocratic elution with a mobile phase consisting of a mixture of 25% phosphate buffer (50 mM, pH 2.5), 25 of 50% methanol, and 50% acetonitrile at a flow rate of 0.7 ml/min. Injection volume was 10 µl; chlorobenzoates were monitored by UV detection ($\lambda_{238\ nm}$) and identified with reference standards by retention time.

Chloride was measured using the above HPLC equipped with a conductivity detector (Model 432). Separation was performed on an IonPac AS17 analytical column (4 × 250 mm; Dionex) which was preceded by an AG17 guard column at a flow rate of 1.2 ml/min. Eluent composition was 100% 5 mM NaOH. Injection volume was 10 µl. A calibrated check standard and blank were run with each sample test.

Statistical analysis

All statistical tests were performed using the Prism 2.01 computer software programme (GraphPad Software, San Diego, CA, USA).

Nucletotide sequence accession numbers

The 16S rRNA gene sequences determined in this study have been deposited in the GenBank database under accession numbers DQ854840 through DQ854845 as shown in Table 1.

Results

Taxonomic characteristics of PCB-utilizing isolates

A total of 150 different microbial colonies were selected from nutrient agar plates following initial enrichment on Askarel oil and Aroclor 1221. Upon screening individual isolates for growth on PCBs or CBAs, we obtained six isolates for further study. Initial partial characterization was carried out with API 20 E test kit. A positive identification was established for each isolate genotypically by cloning

Table 1 Genotypic identities of PCB-degrading bacterial isolates obtained from cloned sequences of 16S rDNA fragment of genomic DNA

Bacterial strain	Tentative identity	Origin	Closest relative	Bacteria subdivision	% ID[1]	GenBank accession no.	Length (nt)[2]
SA-1	*Pseudomonas aeruginosa*	Ijora	*Pseudomonas aeruginosa* strain SCD-13	*γ-Proteobacteria*	99	DQ854840	1,304
SA-2	*Enterobacter* sp.	Ijora	*Enterobacter aerogenes*	*γ-Proteobacteria*	98	DQ854842	1,376
SA-3	*Ralstonia* sp.	Ojota	*Ralstonia* sp. AV5BG	*β-Proteobacteria*	99	DQ854843	1,322
SA-4	*Ralstonia* sp.	Ijora	*Ralstonia* sp. BPC3	*β-Proteobacteria*	98	DQ854844	1,364
SA-5	*Ralstonia* sp.	Ijora	*Ralstonia* sp. BPC3	*β-Proteobacteria*	99	DQ854845	1,364
SA-6	*Pseudomonas* sp.	Ojota	*Pseudomonas* sp. Bu34	*γ-Proteobacteria*	99	DQ854841	1,363

[1] *ID* identity, [2] *nt* nucleotides

and sequencing PCR-amplified 16S rRNA gene fragments. A significant proportion of sequences in the libraries were highly similar to those of the bacterial genus *β-proteobacteria* of *Ralstonia* while others were *γ-proteobacteria* of the genera *Enterobacter* or *Pseudomonas* (Table 1). The strains were all Gram-negative, motile short to medium rods, non-H_2S producers, *β*-galactosidase-, lysine decarboxylase-, VP- and oxidase-negative (except SA-1 and SA-6) and were non-sugar fermenters with the exception of SA-1 and SA-6 that fermented arabinose. Isolate SA-1 had a clone sequence type similar to the 16S rRNA gene sequence of species of *Pseudomonas aeruginosa* strain BPC3. In addition, the colonies were flat, spreading and had green colour pigmentation that diffused relatively quickly. Isolate SA-6, to a large extent, showed similar morphological and phenotypic traits as strain SA-1. Unlike SA-1, the cells utilized glucose in addition to arabinose. It also differed from SA-1 in that citrate utilization was positive. Dynamics of nitrate reduction varied markedly. SA-6 reduced nitrate to gaseous nitrogen while SA-1 reduced same to nitrate. SA-2 was 98% homologous to *Enterobacter aerogenes* 16S rRNA gene sequence. The three isolates, SA-3, SA-4 and SA-5 were identical in their cultural (medium, circular, smooth and elevated colonies) and cellular morphologies, but differ in their 16S rRNA gene sequences. Whereas, SA-3 hydrolysed gelatine and utilized citrate, the other two did not. Nitrate reduction differed among the three strains; SA-3 and SA-5 reduced nitrate to nitrite, while only SA-4 reduced it to gaseous nitrogen.

Substrate diversity of bacterial strains

The growth of these isolates on PCB congeners and other environmental pollutants as sole carbon sources is summarized in Table 2. All the strains showed visible signs of growth on non-chlorinated organic compounds, e.g. benzoate, within 12 h of incubation. In addition, they were able to utilize all CB isomers quite rapidly, though growth patterns varied for different isolates. Interestingly, growth was observed on some diCBs including 2,3-, 2,2′-, 2,4′-, 3,3′- and .3,5-diCB. In some instances, the bacterial growth was accompanied by the production of yellow metabolites. Production of this metabolite was occasional and/or persisted in some strains, while in others, it disappeared with time suggesting that the yellow product was further subjected to metabolism. The yellow–green colour observed could be an indication of *meta*-cleavage product—hydroxyl 6-oxo-6-penta 2,4-dienoic acid (HOPDA) in the case of PCBs and 2-hydroxymuconic semialdehyde in the case of benzoate. All di- and trichlorobenzenes tested were good growth substrates while tetrachlorobenzene isomers supported no growth. Similarly, the strains failed to utilize all mono- and dichlorobenzoate congeners tested. Furthermore, the six strains grew heavily on Askarel fluid producing intense turbidity of the culture media. Similar results were also observed when Aroclor 1221 was supplied as the sole source of carbon and energy.

Growth of bacterial strains on CBs

Since PCBs and other sparingly soluble substrates were dissolved in HMN, there was the need to establish that any observed growth was due to the presence of the test substrate rather than the HMN. When HMN alone was added as the only carbon source in preliminary experiments (data not shown), there was a slight to moderate increase in the cell number of some isolates. Over the course of the experiments, this increase was relatively small for SA-4 and SA-6, while no appreciable growth was observed for SA-3 and SA-5. In the case of SA-2, however, an almost tenfold increase in cell numbers was observed at one time-point, possibly due to continued cell division by the inoculum, continued utilization of endogenous substrates, or a transient artefact of our counting method. This increase was obtained only after incubating the cells for more than 120 h, was not observed at the next sampling period, and the overall results indicated no major cell increase on HMN in the absence of CBs. In all cases where growth occurred on the test substrate, cell numbers increased by at least 1.5 orders-of-magnitude more than in tubes containing the HMN carrier alone, clearly demonstrating growth on the test compound.

All three CBs were readily degraded by the six strains and respective CBAs were recovered as primary metabolites. Results are summarized in Table 3. The growth profiles of the strains on CBs showed a logarithmic population increase without exhibiting any lag period. This was of significance, in

Table 2 Substrate utilization spectrum of PCB-degraders

Substrate	SA-1	SA-2	SA-3	SA-4	SA-5	SA-6
Biphenyl	†	†	†	† [2]	†	† [2]
Benzoate	† [1]	† [1]	† [1]	† [2]	† [2]	† [2]
Naphthalene	†	†	†	†	†	† [2]
Aroclor 1221	† [1]	† [1]	† [1]	†	† [1]	†
Aroclor 1242	†	†	†	†	†	†
Askarel fluid	†	†	†	†	†	†
2-CB	†	†	† [1]	† [1]	† [1]	† [2]
3-CB	†	†	†	†[1]	†	† [1]
4-CB	†	†	† [1]	† [2]	† [1]	† [2]
2,3-diCB	NT	†	†	†	†	†
2,4-diCB	†	–	–	–	–	–
2,6-diCB	†	–	–	–	–	–
3,5-diCB	NT	–	†	† [3]	–	†
2,2'-diCB	†	†	†	†	†	† [2]
2,3'-diCB	†	–	–	–	–	–
2,4'-diCB	†	†	†	† [3]	†	† [3]
3,3'-diCB	†	–	†	†	†	†
4,4'-diCB	–	–	–	–	–	–
All monochlorobenzoates	–	–	–	–	–	–
All dichlorobenzoates	–	–	–	–	–	–
1,2-dichlorobenzene	†	†	†	†	†	†
1,3 –dichlorobenzene	†	†	†	†	†	†
1,4-dichlorobenzene	†	†	†	†	†	†
1,2,3 -trichlorobenzene	†	†	†	†	†	†
1,2,4-trichlorobenzene	†	†	†	†	†	†
1,3,5 -trichlorobenzene	†	†	†	†	†	†
1,2,3,4-tetrachlorobenzene	NT	–	–	–	–	–
1,2,3,5 -tetrachlorobenzene	NT	–	–	–	–	–
1,2,4,5 -tetrachlorobenzene	NT	–	–	–	–	–
Chloroacetate	–	–	–	–	–	–

†, Growth; –, no growth; NT, not tested. Culture supernatant fluid turned a yellow colour that was; [1]permanent; [2]disappeared with time and; [3]occasional

All compounds were supplied as sole carbon sources in MS medium at 100 ppm except benzoate presented at a concentration of 2.5 mM. All compounds with the exception of benzoate and chloroacetate were supplied in a HMN carrier.

that organisms were not pre-cultivated on biphenyl that would have induced requisite enzymes of the degradative pathways. All organisms exhibited similar growth dynamics on 2-CB, resulting in 100-fold-cell increase during the 66-h degradation studies while the mean generation times ranged insignificantly ($P < 0.05$) from 8 to 10 h (Table 3). Although nearly all 2-CB was utilized within the incubation period, recovery of 2-CBA was non-stoichiometric (Table 3, Fig. 1). Interestingly, some chloride was dissociated from the *ortho*-chlorinated ring. Between 0.1 and 0.2 mM chloride were released into culture fluids. However, carbon–chlorine cleavage was not detected in cultures of SA-6 growing on 2-CB until after 30 h of incubation (Fig. 1c). Moreover, a reduction in 2-CBA observed in strains SA-2, SA-4 and SA-6 (see Fig. 1) at 50 h suggest that 2-CBA may not be the terminal product of 2-CB metabolism.

Metabolism of 3-CB followed a similar trend as 2-CB. Population densities increased several

Table 3 Degradation of monochlorobiphenyl isomers and production of metabolites by tropical bacterial strains

Bacterial strain	2-CB				3-CB				4-CB			
	Tg (h)	% Degradation	2-CBA recovered (%)	Chloride released (%)	Tg (h)	Degradation (%)	3-CBA recovered (%)	Chloride released (%)	Tg (h)	Degradation (%)	4-CBA recovered (%)	Chloride released (%)
SA-2	8	96 ± 5	34 ± 2.7	27 ± 13.6	7	91 ± 7.5	30 ± 8.2	0	9	88 ± 9.1	98 ± 5.3	0
SA-3	8	99 ± 1.3	49 ± 2.4	43 ± 6.8	9	98 ± 3.6	11 ± 3.8	11 ± 2.9	9	90 ± 2.1	96 ± 36	0
SA-4	10	99 ± 1.2	34 ± 5.7	39 ± 2.3	6	97 ± 1.3	19 ± 2.6	28.6 ± 5.7	9	97 ± 1.31	73 ± 13	0
SA-5	8	88 ± 16	22 ± 16	14 ± 6.8	8	86 ± 9.2	64 ± 19	23 ± 14.3	9	98 ± 1.4	92 ± 52	0
SA-6	10	94 ± 0.99	20 ± 8.8	50 ± 22.7	7	93 ± 7.7	6.4 ± 2	14 ± 22.779	10	90 ± 6.3	85 ± 13	0

Tg, mean generation time. All values are means ± SD for triplicate cultures. Percent degradation values have been calculated with reference to the amount recovered from uninoculated control tubes. 2-CB, 3-CB and 4-CB were supplied at concentrations of 0.44, 0.35 and 0.66 mM, respectively

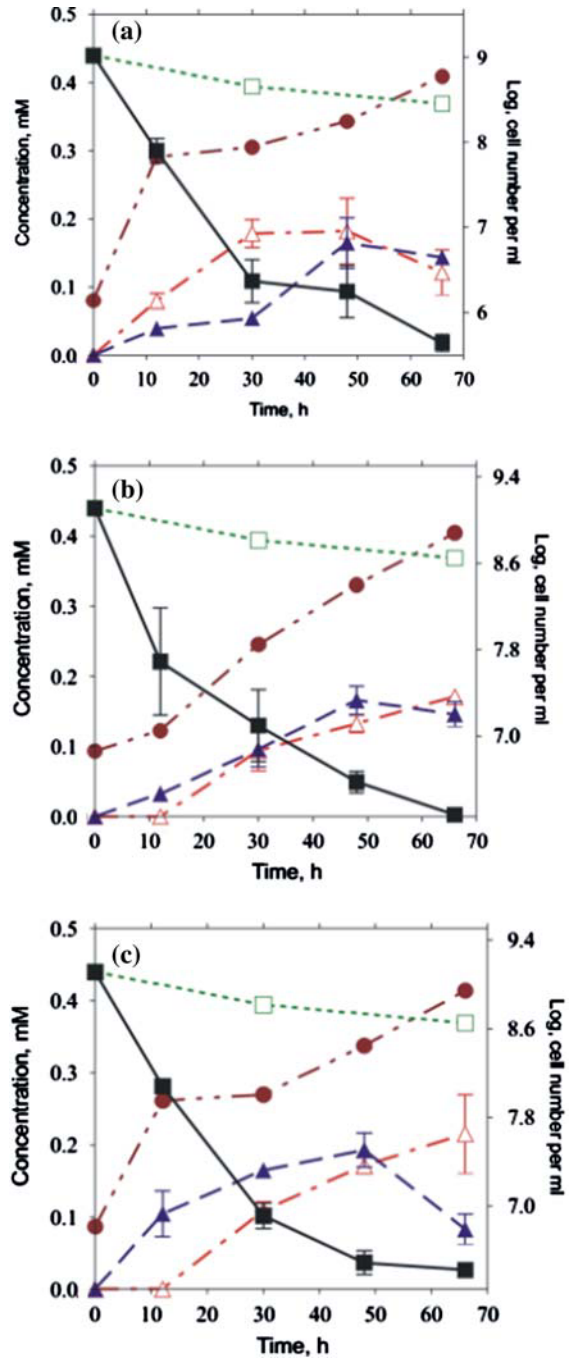

Fig. 1 Time course for 2-CB metabolism and production of benzoate and chloride by strains SA-2 (**a**), SA-4 (**b**) and SA-6 (**c**). ■, 2-CB concentration in experimental tubes; □, CB concentration in non-inoculated controls; ▲, 2-CBA; *open triangular*, Chloride; ●, log cell number. In the controls without cells, CBs were not metabolized and minimal abiotic loss occurred. Data represent the averages and SD of triplicate determinations. Large error *bars* were due to differential response of cells to substrate in replicate tubes

orders-of-magnitude (Fig. 2) with a doubling time in the range of 6 and 9 h (Table 3). More than 90% of the 0.35 mM 3-CB was removed by all cultures after 60 h exposure though, as was observed with 2-CB, non-stoichiometric amounts of 3-CBA (in the range of 6.4–64% of the expected CBA) were produced as the main metabolite. Elimination of minor amounts of chloride was observed in all the strains with the exception of SA-2 (Table 3, Fig. 2). Since the sum of chloride and chlorobenzoate products do not account for all of the 3-CB consumed, this indicated that unmeasured chlorinated metabolites were produced. In the case of SA-5 chloride release became noticeable only after 36 h (Fig. 2b), suggesting slow metabolism of chlorinated intermediates. Nevertheless, partial elimination of chloride presents the possibility that both rings can potentially be metabolized.

In contrast to what was observed on 2-CB and 3-CB, time-course degradation of 4-CB yielded near stoichiometric production of 4-CBA and mirrored the disappearance of substrate in the medium (Table 3, Fig. 3). In the case of SA-3, SA-4, SA-5 and SA-6, an early indication of 4-CB metabolism was a transient change in the colour of the culture media to bright yellow. The lack of chloride production together with near stoichiometric CBA recovery indicates that the extensive growth observed occurred solely on the non-chlorinated ring.

Although we observed differences in patterns of congener degradation and product formation by the various bacteria, all isolates were clearly capable of CB degradation on similar time scales. Analyses of variance showed no significant difference at $P < 0.05$ level of significance in the degradation capabilities of the test organisms.

Degradation of PCB Aroclor 1221

Degradation of Aroclor 1221 was evaluated using washed, benzoate-grown cells. No carbon sources other than the PCB commercial mixture were provided. Growth on this mixture was evidence by intense turbidity of the culture media and significant reduction in concentration of the PCB substrate. Values for net reduction (percent reduction in total ECD area counts) in total PCB content were 51, 53, 54, 68, 71 and 66%, respectively, for SA-1, SA-2, SA-3, SA-4, SA-5 and SA-6 (Fig. 4). Since Aroclor

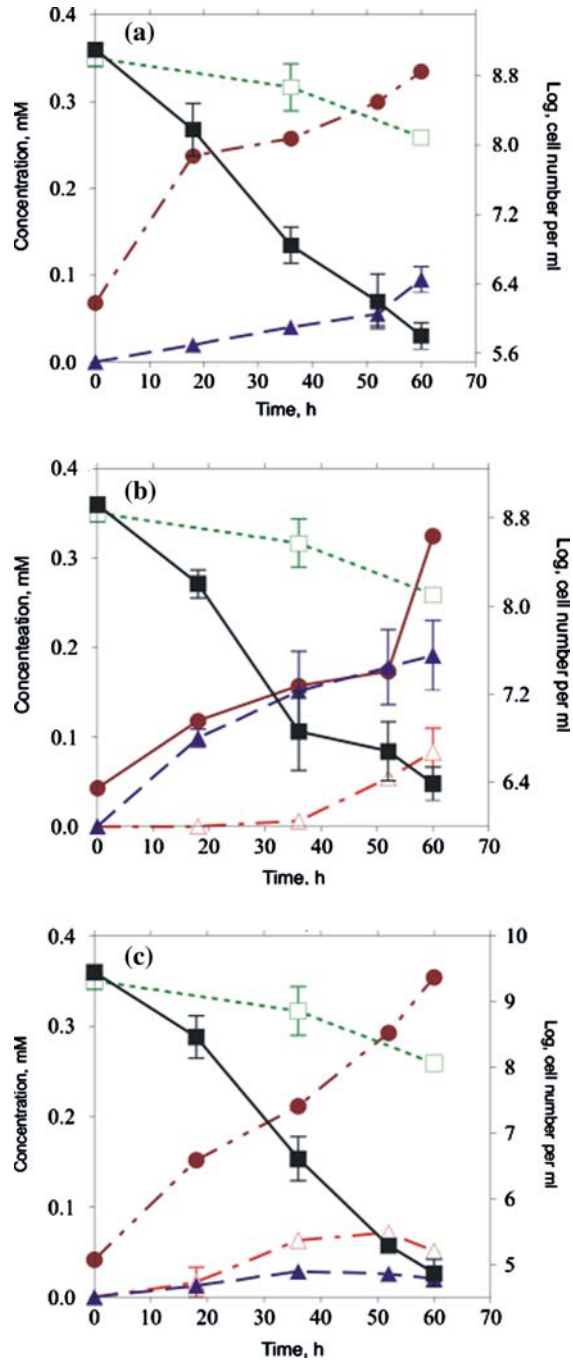

Fig. 2 Time course for 3-CB metabolism and production of benzoate and chloride by strains SA-2 (**a**), SA-5 (**b**) and SA-6 (**c**). ■, 3-CB concentration in experimental tubes; □, CB concentration in non-inoculated controls; ▲, 3-CBA; △, Chloride; ●, log cell number. In the controls without cells, CBs were 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

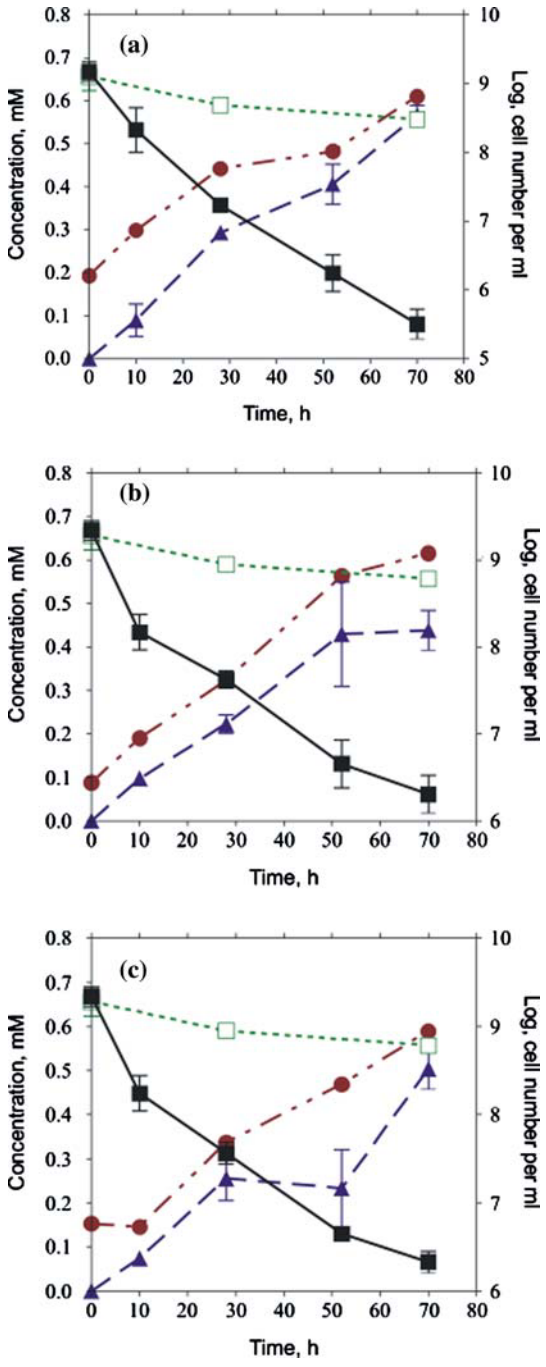

Fig. 3 Time course for 4-CB metabolism and production of benzoate by strains SA-2 (**a**), SA-4 (**b**) and SA-6 (**c**). ■, 4-CB concentration in experimental tubes; □, CB concentration in non-inoculated controls; ▲, 4-CBA; ●, log cell number. In the controls without cells, CBs were not metabolized 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

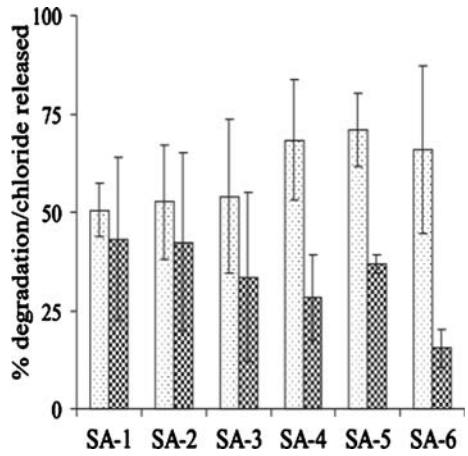

Fig. 4 Transformation of Aroclor 1221, □ and chloride eliminated, ■ Percent degradation represents the net decrease (in ECD area counts) in experimental cultures, compared with that of the non-inoculated controls. No chloride was detected in control tubes. Values presented are means ± SD for triplicate cultures. The PCB mixture was supplied at a concentration of 100 ppm and incubated with each organism for 12 days

1221 contains a significant amount of biphenyl (~15% by weight), some of the observed disappearance may have resulted from cometabolic degradation by cells growing on biphenyl. Our isolates, however, were able to grow on all three CBs and some di-CBs as a sole source of carbon and energy and it is more likely that degradation occurred during growth on the high percentage of CBs and di-CBs (> 80%) present in Aroclor 1221. This inference is further corroborated by preliminary studies that showed extensive depletion of some congeners in Aroclor 1242 (which contains only trace amounts of biphenyl) without the addition of biphenyl (data not shown) and the ability to utilize a broad and unusual spectrum of PCB and chlorobenzenes congeners as growth substrate (Table 2). Chloride produced during Aroclor 1221 degradation ranged from 0.10 to 0.25 mM representing 15–43% of the chloride in Aroclor 1221. There was a colour change from colourless to yellow observed in culture media of SA-1, SA-2, SA-3 and SA-5. It is noteworthy that this *meta*-cleavage product persisted throughout the incubation period (12 days).

The extent of degradation of individual PCB congeners in Aroclor 1221 is tabulated in Table 4. Although unambiguous identification of the individual peaks with the exception of monochlorobiphenyls was

not possible with available instrumentation due to co-elution of some congeners, the degree of chlorine substitution was assigned based on the relative retention times of eluting peaks and comparison with retention times of selected mono- through hexachlorobipenyl standards. Congeners including those assigned to more than three chlorine substituents were extensively attacked. Degradation generally did not follow a particular fashion. One would have expected complete disappearance of congeners containing between one and two chlorine atoms, but quite surprisingly, extensive transformation was accomplished for congeners with more than three chlorine substituents with degradation values higher than lesser chlorinated congeners in some cases. In most cases, strains SA-4, SA-5 and SA-6 depleted most congeners displayed in Table 4 by a minimum of 64.4% and a maximum of 100%.

Discussion

One of the best ways to achieve the isolation of micro-organisms with specific metabolic capabilities is enrichment with substrates contaminated with the target compound. For this approach to be particularly useful, the target compound must serve as a potential carbon source. Acclimation or prior exposure to the compound may enhance the organism's metabolic capabilities. In the present study, microbial communities from contaminated soils were enriched on Askarel as well as Aroclor 1221. In the case of Askarel enrichment, more than 90% of the organisms obtained could not grow on CBs, which likely suggests that the bulk of the isolates may only be marginally effective in stimulating the cometabolism of chlorobiphenyls.

Results of this investigation have confirmed that cultures isolated from contaminated sites in Nigeria exhibited both the chlorobiphenyl and chlorobenzene biodegradation phenotypes. The HMN used as a carrier did not support significant or sustained increases in cell numbers in the absence of CBs or other added substrates. Since we don't expect induction of aromatic ring oxygenases by this highly branched alkane, we believe that HMN functioned as intended, i.e. to facilitate mass transfer of CBs into the aqueous phase. We cannot completely discount, however, the possibility that HMN influenced our results in an unknown manner. As summarized in Table 2, all the bacterial strains isolated possess unique ability to assimilate a diverse range of lightly chlorinated PCB and chlorobenzene congeners when

Table 4 Analysis of the transformation of Aroclor 1221 by microbial strains

Retention time (min)	Peak number	Number of chlorines	Percent degradation					
			SA-1	SA-2	SA-3	SA-4	SA-5	SA-6
7.65	1	1	100 (0)	100 (0)	78.3 (8.5)	96.3 (0.7)	91.9 (0.9)	81.6 (13.4)
8.66	2	1	72.2 (3.5)	61.7 (54.1)	60.0 (23.5)	100 (0)	100 (0)	100 (0)
8.78	3	1	61.8 (10.2)	47.0 (24.0)	66.5 (19.4)	98.3 (2.4)	94.5 (2.1)	80.1 (10.0)
9.48	5	2	59.0 (14.7)	60.9 (9.8)	74.1 (13.5)	77.9 (21.9)	83.1 (6.9)	77.1 (7.7)
10.38	6	2	60.9 (12.6)	72.3 (19.3)	74.9 (13.6)	73.6 (26.9)	85.5 (8.8)	80.1 (12.0)
10.8	8	2	62.0 (11.1)	73.0 (19.4)	76.2 (25.3)	94.2 (5.4)	85.7 (9.3)	80.0 (12.0)
11.05	9	2	59.3 (11.8)	71.8 (19.7)	63.6 (17.6)	95.6 (5.7)	95.8 (0.6)	95.6 (5.7)
13.1	15	2,3	40.2 (10.0)	60.9 (27.4)	–	76.0 (19.5)	74.4 (12.1)	64.4 (7.4)
13.96	18	3	59.8 (6.2)	63.4 (11.8)	54.5 (27.1)	80.4 (19.6)	81.8 (10.0)	80.2 (8.6)
15.48	23	3,4	56.2 (10.6)	63.3 (11.0)	67.7 (21.0)	79.9 (17.7)	78.6 10.0)	79.7 (8.2)
16.04	24	4	73.3 (6.2)	73.7 (6.3)	43.5 (17.8)	87.0 (10.5)	94.5 (6.8)	92.8 (7.9)
16.49	25	>3	56.9 (11.1)	74.2 (15.4)	64.0 (22.3)	72.8 (16.0)	83.3 (5.7)	81.1 (7.1)

Percent degradation represents the net decrease (in ECD area counts) in experimental cultures, compared with that of the non-inoculated controls. Values presented are means of triplicate samples, while those in parentheses represent percent standard deviations. The PCB commercial mixture was supplied at a concentration of 100 ppm and incubated with each organism for 12 days. Chlorine number assignment was based on retention time on a DB-5 megabore capillary column. Blanks are where results could not be ascertained

supplied as sole carbon and energy sources. The ability of our isolates to utilize both chlorobiphenyls and chlorobenzenes is quite rare and unusual. Since Askarel was the enrichment substrate for isolation (with the exception of SA-1 that was enriched using Aroclor 1221), the technique may have selected for those organisms with capacity for both PCB and chlorobenzene phenotypes. In addition, it has been noted that enzymes of PCB pathways may not only transform PCBs and their metabolites, but also other related compounds, such as monocyclic aromatics (Pellizari et al. 1996; Suenaga et al. 2001) and vice versa. Suenaga et al. (2001) engineered the *bphA1* gene of *Pseudomonas pseudoalcaligenes* KF707 and obtained some novel biphenyl dioxygenase that exhibited multifunctional oxygenase activity not only for PCBs but also for several xenobiotics including polyaromatic hydrocarbons (PAHs). In an earlier study, Pellizari et al. (1996) reported that several bacterial species isolated by naphthalene enrichment have PCB-metabolizing ability and concluded that the former (and perhaps other aromatic co-contaminants) may be marginally effective in stimulating the cometabolism of PCBs. Generally, this substrate overlap means that other pollutants in a site may act as co-substrates that can influence the competitiveness and activity of PCB-metabolizing communities.

Of significance is the substrate diversity of *Enterobacter* sp. strain SA-2. The ability of an enteric organism to grow with either PCBs or chlorobenzenes or both is an uncommon phenomenon, even though dechlorination of DDT in pure systems of *E. aerogenes*, *E. cloacae* and *Escherichia coli* has been described (Aislabie et al. 1997; Juhasz and Naidu 2000). The well characterized and widely studied organisms, *Alcaligenes eutrophus* H850, *Pseudomonas testosteroni* H430, *Corynebacterium* sp. MB1 and *Burkholderia* sp. LB400, have been reported to possess an exceptional ability to degrade even a larger range of congeners including penta- and hexachlorobiphenyl co-metabolically (Bopp 1986; Bedard et al. 1987a, b; Commandeur et al. 1996; Potrawfke et al. 1998). These other micro-organisms, however, have not been shown to utilize the range of xenobiotics reported for SA-2 (or any of the SA strains) in this investigation for growth as sole carbon sources (McCullar et al. 1994; Kim and Picardal 2001; Rodrigues et al. 2006).

It is possible that CBA-degrading bacteria are rare to all the six contaminated soils examined, since no growth was possible on all CBAs tested. This may have been a result of the fact that we did not utilize CBAs as enrichment substrates. It may also reflect an observation by Knackmuss (1984) that the frequency of CBA-utilizers to benzoate-utilizers was about one per million. In another report, only three out of 42 bacterial strains isolated by enrichment on biphenyl could grow on 3-CBA (Hernandez et al. 1995). In addition, the investigators detected no CBA-utilizers in all three soils analysed by direct-plating of diluted soil samples onto MS agar supplemented with monochlorobenzoates. In spite the fact that CBA-degrading micro-organisms have been well-studied, they may be relatively scarce in the environment. It is interesting to note that the 3-CBA degrader reported by Dorn et al. (1974) required several months of adaptation to the substrate. Similarly, Marks et al. (1984) documented an adaptive period of 4 months before a 4-CBA-degrader could be isolated.

Unlike LB400 and H850 (Bedard et al. 1987a; Potrawfke et al. 1998), our data clearly established that all three CBs, 2-, 3- and 4-CB were rapidly degraded by all the isolates on the basis of growth studies and disappearance of substrates and appearance of respective metabolites. The layout of growth patterns indicated exponential increase in cell density (Figs. 1, 2, 3) that was similar to previous report by Kim and Picardal (2000). Having pre-grown the organisms on benzoate rather than biphenyl, the observation suggests constitutive expression of the enzymes of the upper biphenyl degradative pathway. Growth of isolates on 2-CB and 3-CB resulted in non-stoichiometric production of 2-CBA and 3-CBA, respectively, concomitant with substantial release of chloride from the substituted rings of these isomers. On the contrary, the bacterial strains stoichiometrically transformed 4-CB to 4-CBA, with no detectable chloride (Table 3). The production of 4-CBA indicates that the bacterial dioxygenase attacked the unsubstituted aromatic ring. Early indication of utilization of the PCB substrates was a change in colour of the culture media to yellow (Table 2), suggesting the operation of a *meta*-cleavage pathway for the degradation of CBs similar to other PCB-degrading organisms. Similar results have been reported by Commandeur and Parsons (1990) and Bevinakatti and Ninnekar (1993). In the case of

4-CB, the yellow metabolite was apparently produced by the *meta*-cleavage of 2,3-dihydroxy 4-chlorobiphenyl which was further transformed into 4-CBA and 5-C fragment by enzymatic hydrolysis (Kobayashi et al. 1996; Omori et al. 1986; Ahmed and Focht 1973). Since the strains could neither grow on nor oxidize 4-CBA, therefore, the growth on 4-CB seemed to depend solely on utilizing the 5-C fragment as the carbon source. Furthermore, the absence of chloride in the reaction tube strongly suggests that the failure of these strains to mineralize 4-CB may be due to the lack of or inhibition of enzymes effecting dehalogenation.

Several workers have shown that isolates able to grow on CBs as sole carbon and energy sources usually metabolise the non-chlorinated ring to produce a CBA or other chlorinated compound as a product (Furukawa and Chakrabarty 1982; Barton and Crawford 1988; Kobayashi et al. 1996). The exceptions to this rule were the reports of Potrawfke et al. (1998), Kim and Picardal (2000) and the results obtained in the present study during growth on 2-CB and 3-CB. Besides significant quantities of chloride released during metabolism of these isomers, especially during growth of SA-6 on 2-CB (Table 3), the incomplete recovery of 2-CBA and 3-CBA observed in all strains implied susceptibilities of both 2- and 3-chlorophenyl rings to attack. CBAs may, after all, not be the final product of chlorobiphenyl metabolism by the organisms.

Since the sum of chloride and chlorobenzoate products do not account for all of the 2-CB and 3-CB consumed, our data suggests accumulation of unknown, chlorinated metabolites. Indeed, incomplete degradation of PCBs is frequently observed with the formation and accumulation of different intermediates besides CBAs (Bruhlmann and Chen 1999; Arensdorf and Focht 1994; Fava and Marchetti 1991). It is also possible that the CBAs were transformed to other chlorinated compounds such as chlorocatechol, thus escaping detection. This inference is further supported by the fact that limited production of CBAs, e.g. production of 3-CBA in Fig. 2, did not always yield a corresponding increase in chloride concentration. Conversion of 3-CB to 3-chlorocatechol (3-CC) and 4-CC was previously reported by Fava and Marchetti (1991). It is possible that chlorocatechols are able to reduce the catechol dioxygenase activity resulting in suicide inactivation of the entire pathway. In fact, other reports show that the formation of chlorocatechol in the medium can be the cause of the incomplete degradation of 3-CBA (Haller and Finn 1979). Arensdorf and Focht (1994) also documented that 3-CC interfered with the utilization of CBs as a carbon by *Pseudomonas cepacia* P166 by arresting biphenyl transformation. The authors proposed a likely mechanism for this phenomenon as the inactivation by 3-CC of 2,3-dihydroxybiphenyl 1,2-dioxygenase.

Data from transformation of Aroclor 1221 further illustrate the notable catabolic properties displayed by our isolates. Since congeners assigned to more than 3 substituents were sometimes degraded more extensively than those assigned to 1 or 2 chlorine substituents, it is possible that the extent of chlorine substitution, by itself, may not determine the effectiveness of oxidative attack by the bacterial enzyme system. Further work, however, is necessary to determine the relative importance of chlorination extent and ring position on CB degradation by our isolates. More importantly, degradation of Aroclor 1221 and components by *Enterobacter* sp. strain SA-2, an enteric organism is novel. To our knowledge, this is the first substantial report of degradation of PCB mixture by this organism unknown to have xenobiotic degradative capability. Generally, there is paucity of information on the release of chloride during metabolic transformation of PCB mixtures by biphenyl-oxidizing bacteria, presumably because they are unable to dehalogenate the chlorinated products. In our own case, we were able to recover significant amounts of chloride. This would mean that some congeners were either completely mineralized or transformed to CBAs or other products with concomitant chloride release.

In summary, results of our investigation have shown the occurrence of multiple bacterial strains with PCB catabolic competence in Nigerian contaminated soils. This conclusion is supported by several lines of evidence. First, isolates were capable of growth on CBs and diCBs, particularly *ortho*-substituted congeners known for their recalcitrance to aerobic and anaerobic biodegradation. Second, Aroclor 1221 was extensively degraded and possibly partially mineralized. It is noteworthy that organisms were pre-grown on benzoate prior to inoculation into PCB supplemented MS medium and that CB degradation occurred without a notable lag period, thus

suggesting either that the enzymes of the upper biphenyl/PCB catabolic route are constitutively expressed or that the biphenyl pathway is co-induced with the benzoate pathway. The catabolic properties of these strains with wider substrate spectrum could be an important step in studying the specificity of aromatic oxygenases, constructing new hybrids with unique abilities, and developing effective PCB bioremediation strategies.

Acknowledgements Authors 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. We thank Nathan Grindle for assistance with 16S rRNA gene sequence analysis and the anonymous reviewers for helpful comments that improved the manuscript.

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