Degradation and mineralization of 2-chloro-, 3-chloro- and 4-chlorobiphenyl by a newly characterized natural bacterial strain isolated from an electrical transformer fluid-contaminated soil

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Abstract

A bacterium classified as *Achromobacter xylosoxidans* strain IR08 by phenotypic typing coupled with 16S rRNA gene analysis was isolated from a soil contaminated with electrical transformer fluid for over sixty years using Aroclor 1221 as an enrichment substrate. The substrate utilization profiles revealed that IR08 could grow on all three monochlorobiphenyls (CBs), 2,4'- and 4,4'-dichlorobiphenyl as well as 2-chlorobenzoate (2-CBA), 3-CBA, 4-CBA, and 2,3-dichlorobenzoate. Unusually, growth was poorly sustained on biphenyl and benzoate. In growth experiments, IR08 degraded all CBs (0.27 mmol/L) in less than 96 h with concomitant stoichiometric release of inorganic chloride and growth yields were 2–3 times higher than those observed on biphenyl. In contrast to most of the chlorobiphenyl-degrading strains described in the literature, which are reported to form CBA, no metabolite was identified in the culture broth by HPLC analysis. When co-incubated with respective CBs and biphenyl, strain IR08 preferentially utilized the chlorinated analogues in less than 96 h while it took another 264 h before 90% of the initially supplied biphenyl could be degraded. The promotion of co-metabolic transformation of halogenated substrates by the inclusion of their non-halogenated derivatives may not therefore, result in universal benefits.

Key words: aerobic degradation; chlorobenzoates; mineralization; monochlorobiphenyls; natural strain; polychlorinated biphenyls

Introduction

Polychlorinated biphenyls (PCBs) are among the most stable organic compounds (Erickson, 2001). They are frequently encountered as contaminants in soil and aquatic sediment environments, usually originating from electrical transformer leaks or improper disposal of PCB-containing wastes. In developing countries, particularly Africa, the usage of PCBs is largely under-regulated and spillage, when it occurs is never reported or investigated. The methods of removal of PCBs, including mechanical collection, use of sorbent materials, landfilling, and incineration are expensive. The conventional treatments have undesirable ecological consequences, and have therefore, provided the impetus needed for the examination of alternative technologies, such as bioremediation. Bioremediation is an option that offers the possibility to destroy or render harmless various contaminants using natural microbial catabolic potentials. But the applicability of this approach is often limited by the lack of suitable microorganisms.

Several bacterial species that are able to degrade PCBs have been isolated from contaminated soils, sludges and aquatic environments, and studied extensively. Many of these studies have been devoted to identification of the pathways by which chlorobiphenyls are biodegraded. The pathway involves initial dioxygenation to form metabolites hydroxylated in the 2 and 3 positions of the unsubstituted or less substituted ring which are in turn degraded by *meta* ring cleavage to ultimately give chlorobenzoic acids (CBAs) and 5-carbon aliphatic acid (2-hydroxy-2,4-penta-dienoic acid) (Ahmed and Focht, 1973; Furukawa *et al.*, 1978; Brenner *et al.*, 1994). Although this pathway appears to be the major route for the metabolism of chloro-biphenyls by most bacterial species, there is evidence for the existence of other minor biodegradation pathways in several bacterial species including *Alcaligenes* sp. BM-2 (Yagi and Sudo, 1980), *Achromobacter* sp. B-218, *Bacillus brevis* B-257 (Masse *et al.*, 1984), *Burkholderia xen-ovorans* LB400, and *Alcaligenes* eutrophus H850 (Bopp, 1986; Bedard *et al.*, 1986, 1987; Bedard and Haberl, 1990). Some of the metabolites generated during the metabolism of PCBs might possibly be mutagenic or toxic and thus be a concern for the environment. The generation of toxic metabolites may be particularly severe if the compounds are the sole available carbon and energy source (Camara *et al.*, 2004). For example, CBAs have been reported to be inhibitory to PCB metabolism (Sondossi *et al.*, 1992; Stratford *et al.*, 1996; Vrana *et al.*, 1996) even

though the inhibitory effects is indirect and is actually attributed to chlorocatechols (CCs) which are produced when PCB degraders cometabolically transform CBAs (Parsons *et al.*, 1988; Fava and Marchetti, 1991; Arensdorf and Focht, 1994). A major surprise in PCB catabolism was the finding that released CBA may be transformed to protoanemonin (an antibacterial compound that kills PCB degraders) (Blasco *et al.*, 1997). In this respect, any strain improvement strategy for the biodegradation of PCBs should also aim at preventing the accumulation of the various biotransformation products.

Bacteria that are able to grow on monochlorobiphenyls (CBs) generally derive carbon and energy for growth from 5-carbon aliphatic acid, which is produced from the unchlorinated ring, while accumulating respective CBAs as dead-end metabolites. 4-CB represents the exception to this rule, as at least four naturally-occurring strains have been demonstrated to completely mineralize the chlorinated ring (Shields *et al.*, 1985; Pettigrew *et al.*, 1990; Arensdorf and Focht, 1994; Kim and Picardal, 2000). With the exception of LB400 that was reported to mineralize 3-CB (Potrawfke *et al.*, 1998), no other organism has been demonstrated as capable of mineralizing either 2-CB or 3-CB. Generally, there have been few reports of organisms capable of utilizing all three CBs as growth substrates. Previously, a *Pseudomonas cepacia* P166 was reported to grow on all three CBs, producing CBA intermediates which are further metabolized to CCs (Arensdorf and Focht, 1994). 2-CB and 3-CB were both metabolized to 3-CC, which was *meta*-cleaved to produce a toxic acyl halide. 4-CB was, however, mineralized through 4-CBA and 4-CC. Similarly, Kim and Picardal (2000) described a bacterium classified as SK-3 as capable of growing on all three CBs. While 4-CB was mineralized without accumulating 4-CBA, 2-CB and 3-CB were stoichiometrically transformed to CBAs. More recently, Adebusoye *et al.* (2007a) reported several bacterial species capable of growth with all the CBs producing respective CBAs as primary dead-end products. In the current study, we report for the first time, degradation and mineralization of 2-, 3-, and 4-CB by a bacterial strain isolated from African contaminated system. Unusually, this isolate was shown to grow poorly on non-halogenated aromatic compounds, biphenyl, and benzoate.

1 Materials and methods

1.1 Chemicals

High purity monochlorobiphenyl congeners were supplied by Lancaster Synthesis (Heysham, Lancashire, UK), while other PCB congeners and commercial mixtures were procured from Greyhound Chromatograph and Allied Chemicals (Birkenhead, Merseyside, UK). All chlorobenzoates were obtained from Sigma-Aldrich Corp., USA. All other chemicals used in this study were reagent grade or better.

1.2 Isolation and culture conditions

Soil sample was obtained randomly from the National

Electric Power Authority (now: Power Holding Company of Nigeria (PHCN)) Transformer Workshop, Ijora, Lagos, Nigeria. This site has been heavily contaminated with PCB-containing, electrical transformer fluids for several decades. Chlorobiphenyl-degrading bacterial species indigenous to the soil were initially isolated by traditional enrichment culture methods using batch cultivation. Soil sample was used to inoculate a mineral salts medium (MSM) which contained 100 mg/L of Aroclor 1221 as the primary carbon source. Enrichment cultures were incubated at 30°C on a rotary-platform programmed at 120 r/min for 21 d. Subsequent transfers from this enrichment were made by using the same methods and conditions. After three consecutive transfers, pure cultures were isolated from the enrichment by plating out aliquots of appropriate dilutions onto Luria-Bertani (LB) agar. Colonies were further screened for ability to utilize various PCB congeners. Bacterial isolate selected for this study was maintained in an MSM supplemented with 50 mg/L 4-CB or cultured on inverted MSM agar plates with 4-CB crystals added to the bottom lid.

Enrichments and all degradation experiments were performed in a defined MSM previously described by Kästner *et al.* (1994). The medium which was slightly modified to make it chloride-free contained (pH 7.2, in g/L): Na_2HPO_4 2.13; KH_2PO_4 1.30; $(NH_4)_2SO_4$ 0.50, and $MgSO_4 \cdot 7H_2O$ 0.20 g. Biphenyl, Aroclor 1221, PCB congeners and other insoluble substrates were first dissolved in acetone before distribution into Erlenmeyer flasks. The acetone was vented off and MSM was added and subsequently autoclaved. This method was found to be satisfactory, since loss through volatilization was minimal and recovery of substrates from control tubes was nearly 100%.

1.3 Taxonomic classification of bacterial strain

Partial identification of the bacterial isolate used for this study was undertaken using the taxonomic schemes of Holt *et al.* (1994). Diagnostic properties used included but not limited to Gram-straining, motility, cultural morphology, nitrite and nitrate reduction tests as well as sugar fermentation patterns. A detailed identification study was carried out by sequencing of the 16S rRNA gene of the bacterial genome. Genomic DNA was prepared according to the method described by Ausubel (1997). The 16S rRNA gene fragment of the extracted DNA was amplified by PCR. The PCR reaction contained: 5 μl of DNA sample, 10 μl of 10× reaction buffer (10 mmol/L TrisHCl, 50 mmol/L KCl, pH 8.3), 2.5 mmol/L $MgCl_2$, 200 μmol/L each dNTP, 0.2 μmol/L each primer, and 2.5 U *Taq* polymerase (Boehringer Mannheim, Germany) in a 100-μl reaction volume. Amplification was carried out using a Perkin-Elmer 9600 thermal cycle (PerkinElmer Life and Analytical Sciences, Shelton, CT USA) programmed to perform 35 cycles consisting of an initial hot start step for 5 min at 95°C, denaturing at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension step of 5 min at 72°C. The primers used were: forward primer FD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse primer RP2 (5'-ACG GCT ACC TTG

TTA CGA CTT-3') described by Saiki *et al*. (1988). The negative PCR control was sterile distilled water added to the reaction mix instead of template DNA. The positive control was an *E. coli* DNA. The PCR products were examined on a 0.8% agarose gel electrophoresis containing 0.5 μg/ml ethidium bromide (Sambrook *et al*., 1989) and subsequently purified with a QIA-quick PCR purification kit (QIAGEN GmbH, Germany). The nucleotide sequences of the amplified product was performed on an ABI 3700 sequencer (AP Biotech, UK) and were probed against the GenBank database with the BLAST algorithm.

1.4 Nucleotide sequence accession number

The sequence for the 16S rRNA gene of *Achromobacter xylosoxidans* strain IR08 has been deposited with the GenBank under accession number DQ887776.

1.5 Growth and degradation assays

A single colony of a culture grown on MSM agar with biphenyl as the carbon source was sub-cultured into LB broth and incubated overnight at 30°C. Cells were harvested by centrifugation and washed twice in MSM, and resuspended in the same medium to an optical density (OD_{550}) of 0.5 at 550 nm. This served as our source of inoculum.

For CB degradation studies, the selected CB isomer was added at a concentration of 0.27 mmol/L to 50 ml MSM in 250-ml Erlenmeyer flasks. After inoculation with washed cells, the flasks were crimp-sealed with Teflon-lined rubber stoppers and incubated at 30°C on rotary-platform shaker at 120 r/min. Aliquots were withdrawn periodically from the cultures to evaluate the xenobiotic, chloride, metabolites as well as cell growth. Growth curves were monitored spectrophotometrically at 550 nm (A_{550}). Three replicate samples were analysed at each time point. To evaluate any possible loss of the xenobiotics during the incubation, these analyses were also performed on heat-inactivated controls, which were prepared and incubated under the same conditions of the samples.

1.6 CO$_2$ evolution determinations

The organism was cultivated in MSM as described above in 250-ml Erlenmeyer sidearm flasks (Biometer flask). NaOH solution was introduced into the sidearm to trap any CO_2 released. For CO_2 determinations, the NaOH was withdrawn periodically and analysed with a total organic carbon analyser (Rosemont Dohrmann DC-190, Rosemont Analytical Inc., USA). Control Biometer flasks containing autoclaved cells of the organism were set up and treated similarly. The calibration standard for the carbon analyser was 500 mg/L Na_2CO_3 while the sample injection volume was 100 μl. Samples were analysed in three replicates.

1.7 Extraction of PCBs

For quantification of residual CBs in the broth, ethyl acetate was added to culture broth (1: 1, *V/V*), vortexed for 1 min and thereafter mixed continuously for 20 min before the two phases were allowed to separate. The aqueous phase was re-extracted in the same way three times or more times. The organic extracts collected were then pooled and dried over anhydrous magnesium sulphate before rotary evaporation at 45°C and then reconstituted in ethyl acetate. Extracts were subjected to GC-MS analysis.

1.8 Gas chromatography-mass spectrometry

Ethyl acetate extracts were analysed on an Agilent 5973 Series 7683 GC-MS (Life Sciences & Chemical Analysis Group, UK) of 70 eV in the EI mode using either the selected ion monitoring (SIM) or acquire programmes. Separation was performed with a 30-m Altech Econo-Cap SE54 column (0.25 mm i.d., 0.25 μm film thickness, Altech, UK). Helium was carrier gas at a constant flow rate of 1.6 ml/min. The inlet line temperature and detector was set at 290°C while that of the column was 180°C.

1.9 High-performance liquid chromatography

The presence of metabolites in the culture supernatants was analysed by HPLC (Kontron Instruments, UK) equipped with a programmable absorbance detector (SM 4000, Kontron Instruments, UK). Separation was carried out on a Nucleosil C18 reverse-phase column (Camlab Ltd., UK) fitted with a C18 guard column. A mobile phase of 60% water, 40% acetonitrile, and 0.4% *ortho*-phosphoric acid was used with a flow rate of 1 ml/min. Injection volume was 10 μl; CBAs and CCs were identified in the eluate at a range of 235–275 nmol/L and compared to the peaks obtained with standard solutions.

1.10 Chloride determination

Inorganic chloride was monitored spectrophotometrically (A_{460}) by the method described by Bergman and Sanik (1957). The method was based on the displacement of thiocyanate ion from mercuric thiocyanate by chloride ion in the presence of ferric ion. One of the reaction products, ferric thiocyanate complex produces a strong and stable color, its intensity is proportional to the initial chloride concentration. A standard curve representing correlation between absorbance and chloride concentration was constructed using standard solutions of NaCl at concentration range of 0.005–1.0 mmol/L.

2 Results

2.1 Taxonomic and metabolic characterization of PCB-degrading strain

Batch enrichment of soil sample resulted in the isolation a bacterium that grew on Aroclor 1221 as the primary carbon source. Colonies of the organism designated as IR08 on nutrient agar were generally tiny, circular, entire, convex, and creamy white. Strain IR08 was a motile, aerobic, Gram-negative short rod that was cytochrome oxidase- and catalase-positive, and capable of reducing nitrate to nitrite. It could not produce indole from tryptophan or produce acid, either oxidatively or fermentatively, from sugars with the exception of glucose and xylose. In addition, IR08 was a citrate-utilizer but urea and Voges

Table 1   Substrate diversity of strain IR08

Substrate	Growth (OD_{550})	Substrate	Growth (OD_{550})	Substrate	Growth (OD_{550})
Benzoate	+	2,2'-diCB	−	2-CBA	+++
Biphenyl	+	2,3'-diCB	−	3-CBA	++
Pyrene	++	2,4'-diCB	++	4-CBA	+++
2-CB	+++	4,4'-diCB	+++	2,3-CBA	+++
3-CB	++	2,4',5-triCB	+	Aroclor 1221	++
4-CB	+++			Aroclor 1242	+

+: poor growth (OD_{550}, 0.1–0.2); ++: very good growth (OD_{550}, 0.2–0.3); +++: luxuriant growth (OD_{550} > 0.3); −: no growth. Cultures were incubated for 7 d. All the substrates were supplied at a concentration of 50 mg/L except benzoate (2.5 mmol/L).
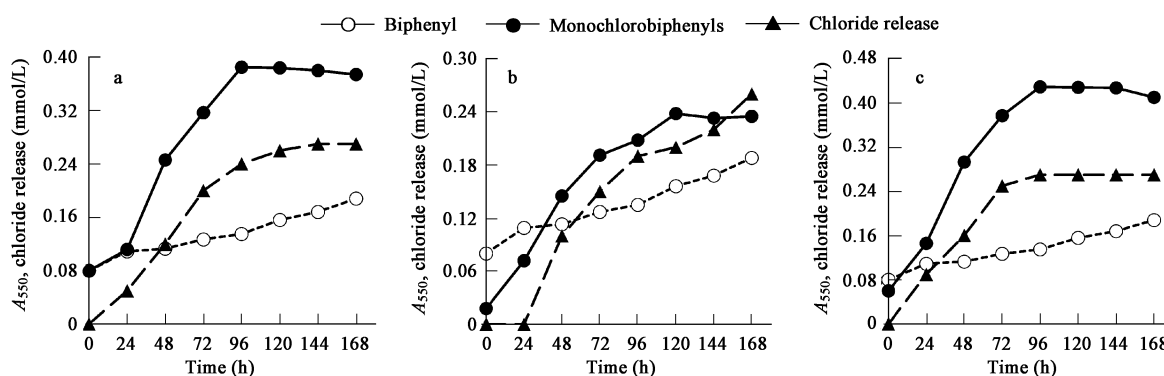
Fig. 1   Comparative growth of strain IR08 on biphenyl and monochlorobiphenyls as measured by A_{550} and chloride release kinetics. (a) 2-CB; (b) 3-CB; (c) 4-CB. All the three CBs were completely utilized in less than 96 h of incubation. No metabolites were recovered from the culture broth during HPLC analysis. Data values represent averages of three replicate determinations.

Proskauer tests were both negative. A tentative taxonomic identity of a strain of *Achromobacter xylosoxidans* was assigned to the organism based on these morphological and physiological characteristics, and the results obtained from the sequencing of the 16S rRNA gene of the genomic DNA.

During our investigation of the degradative competence of strain IR08, we found that this strain was able to grow on a variety of chlorinated and non-chlorinated organic compounds, when presented as sole sources of carbon and energy. Amongst the substrates tested, IR08 utilized quite well 2-, 3-, 4-CB, and 4,4'-dichlorobiphenyl (4,4'-diCB). Other PCB congeners were either sparingly utilized or not degraded at all (Table 1). In addition, further evaluation of the substrate-utilization profile revealed that this organism could grow on 2-CBA, 3-CBA, 4-CBA, 2,3-dichlorobenzoates (2,3-CBA), and pyrene. Interestingly, the growth was very poor on benzoate and biphenyl.

2.2 Mineralization and metabolism of monochlorobiphenyls

The ability of strain IR08 to catabolize CBs as sole carbon and energy sources under growing-cell batch conditions was assayed. All the compounds in growth studies were presented at a concentration of 0.27 mmol/L for CBs and 0.32 mmol/L for biphenyl. No growth occurred in controls lacking CBs, and the CBs were not degraded in incubations lacking cells. In our systems, growth and mineralization were defined as increase in OD (A_{550}) concomitant with substrate disappearance, production of chloride and other metabolites as well as evolution of CO_2. As depicted in Fig.1, it was found that strain IR08 grew logarithmically in the presence of the CB isomers. Degradation was rapid; all three CB substrates were completely utilized in less than 96 h of incubation. Summary of growth kinetics is displayed in Table 2. Growth of the organism peaked at 96 h and appeared to mirror substrate disappearance since this period coincided with when 100% degradation was accomplished for all the CB congeners.

Table 2   Fate of monochlorobiphenyls metabolized by strain IR08 in aerobic batch conditions

PCB substrate	Incubation period (h)	Growth increase	PCB initial conc. (mmol/L)	PCB final conc. (mmol/L)	CBA recovered (mmol/L)	Chloride released (mmol/L)	Depletion (%)	Mineralization[a] (%)	PCB released as CO_2 (%)
2-CB	144	Three-fold	0.27	0	0	0.27	100	100	34.62
3-CB	168	Two-fold	0.27	0	0	0.26	100	96.3	25.32
4-CB	96	Three-fold	0.27	0	0	0.27	100	100	38.46
Biphenyl	168	One-fold	0.32	0.14	NA[b]	NA	56.25	N/A	NA

PCB initial concentration was calculated assuming this molecule is completely soluble in MS medium. The CBA and chloride concentrations reported are those determined in the culture fluid at the end of the experiment. All values presented are means of triplicate determinations. All the CBs were completely utilized in less than 96 h. [a] Expressed as chloride released in the growth medium with respect to that chemically bond in chlorophenyl rings; [b] not applicable.

Interestingly, growth decreased thereafter indicating lack of carbon sources to support further growth. IR08 grew luxuriantly on 4-CB (OD$_{550}$ 0.4). Utilization of this compound was accompanied by more than three-fold increase in cell density over a 168-h period. Early indication of metabolism was the appearance of a transient light-yellow colour, which suggests the presence of a *meta*-cleavage product, within 24 h of incubation. In contrast, growth was least supported by 3-CB (approximately two-fold) (Table 2). Moreover, the culture broth was seen to take on a dense dark colour. Darkening took place after 48 h of incubation but faded with time. No visible colouration was observed in flask containing 2-CB. Comparison of the growth dynamics of IR08 on CBs with that of biphenyl presents an interesting observation. Growth was very poor on biphenyl and the substrate was poorly metabolized. Growth on 2-CB and 4-CB for instance was almost twice that achieved on biphenyl (Table 2). In spite of the extended period of incubation, strain IR08 was only able to degrade 56% of the initial biphenyl substrate supplied.

Analysis of the culture broth by HPLC yielded no metabolites. Neither the potential major metabolites of the upper pathway (i.e., CBAs), nor other metabolites of the lower pathway such as chlorocatechols, were detected throughout the experiment. It is likely that the concentration of these metabolites was too low to permit their identification by this technique since they were rapidly turned over. End-point determination of chloride released into the culture broth revealed that 100% of the original organically bonded chlorine was recovered as inorganic chloride from all incubations. The release of chloride was similar from congener to congener and paralleled growth and was mostly (if not all) released during exponential phase of growth. These results indicate the susceptibility of the chlorinated phenyl rings of all the three CBs to attack and that all of the available substrate was being utilized by this organism.

CO_2 produced by strain IR08 was also assayed throughout the CB mineralization studies. Total amounts of CO_2 evolved from catabolism of the CBs are summarized in Table 2 and Fig.2. Although evolution of CO_2 was the highest in 4-CB incubations, less than 40% of the PCB substrate utilized was released as CO_2. Continued incubation of the culture flasks did not yield significant increase in CO_2 evolution.

2.3 Co-incubation of IR08 with biphenyl and CB isomers

Following the studies summarised in Fig.1 demonstrating that IR08 utilized the CBs better than biphenyl, an investigation was launched to determine which substrate would be preferentially attacked if equimolar biphenyl and CB congeners were supplied as co-substrates to the cells in MSM. Results are summarized in Table 3 and Fig.3. Quite surprisingly, the CB isomers were preferentially utilized and were completely depleted in less than 96 h of incubation similar to when the compounds were presented alone. During this period, less than 50% of the biphenyl co-substrate was utilized. Although cellular growth of IR08 on the CBs was stronger and faster in the presence of biphenyl than when the organism was cultured on CB alone, however, the growth curve was anything but diauxic. By implication, it may be inferred that biphenyl and CBs were degraded concomitantly. Stoichiometric or near-stoichiometric amount of chloride was released into the culture broth in all incubations (Table 3). It is noteworthy that nearly 90% of the biphenyl supplied was degraded when the incubation period was extended to 360 h.

3 Discussion

It is generally accepted that metabolites of the transformation of aromatic compounds can be more toxic than the original substrate, thus constituting another environmental nuisance (Liu *et al.*, 1990; Haugland *et al.*, 1990; Sondossi *et al.*, 1992; Stratford *et al.*, 1996; Vrana *et al.*, 1996; Blasco *et al.*, 1997). Consequently, mineralization rather than biotransformation should be the ultimate goal of bioremediation strategies. In the case of PCBs, most organisms available until now were isolated on the basis of the degradation of the unchlorinated analogue and only cometabolized but did not mineralize the halogenated compounds (Furukawa *et al.*, 1979; Bedard *et al.*, 1987; Fava and Marchetti, 1991; Fava *et al.*, 1994). In this study, however, our enrichment technique enabled us to isolate a bacterium with unique capabilities. Phenotypic typing and sequencing of the 16S rRNA gene classified this bacterium as a strain of *Achromobacter xylosoxidans*. Interestingly, several researchers (Ahmed and Focht, 1973; Masse *et al.*, 1984; Pettigrew *et al.*, 1990; Pieper, 2005) have implicated this genus in the degradation of PCBs. In fact, the first report of microbial degradation of PCBs was observed when a co-culture of two species of *Achromobacter* was cultivated on mono- and dichlorobiphenyls (Ahmed and Focht, 1973).

The results of this investigation have confirmed previous reports (Adebusoye *et al.*, 2007a, 2007b) that natural pure bacterial cultures from contaminated tropical African systems exhibit unique and fascinating PCB catabolic
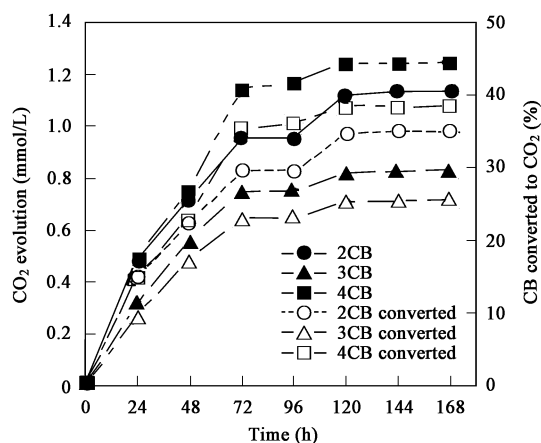
Fig. 2 CO_2 evolution over time during catabolism of 2-CB, 3-CB, 4-CB by IR08 and their corresponding percentage of CBs converted to CO_2. CO_2 values have been calculated with reference to the amount determined from heat-killed inoculated control flasks. Data represent the averages of triplicate determinations.

functions, and perhaps, superior to those generally found in other organisms. IR08 is able to use a wide range of natural and xenobiotic compounds including all CBs and some diCBs as well as some congeners of CBAs (Table 1) as sole sources of carbon and energy, and mineralise these substrates in the process. Surprisingly, the same strain grew poorly with biphenyl and benzoate, and was unable to mineralise them. Furthermore, we observed that CBs were preferentially utilized when supplied simultaneously with biphenyl (Table 3). The observation that strain IR08 could utilize and mineralize CBs in preference to biphenyl is rare and quite unusual. It shows that chlorine substitution rather than impeding growth obviously aided it. It is apparent therefore, that enzymatic reactions other than those widely reported for PCB-degrading organisms are likely to occur in the isolate. Such catabolic anomalies have implications in the evolution of catabolic pathways. The acquisition of simple dehalogenation steps to convert xenobiotic compounds to their non-halogenated derivative may not be the primary mode of evolution of pathways for the biodegradation of such compound as believed by several workers (Janssen *et al.*, 1994; Abraham *et al.*, 2002). Previously, Barton and Crawford (1988) described a *Pseudomonas* sp. MB86 that utilized 4-CB and 4-CBA but not benzoate. This strain was also reported to grow poorly on biphenyl. Similarly, Pettigrew *et al.* (1990) documented an *Achromobacter* sp. strain LBSIC1 that mineralized 4-CB but not biphenyl. It therefore, appears that this property may be a common feature of *Achromobacter* sp. Furthermore, the purified biphenyl dioxygenase of *Burkholderia xenovorans* LB400 was reported to have given higher rates on CBs than biphenyl, presumed to be the natural substrate of the enzyme (Arnett *et al.*, 2000). This phenomenon certainly exist in some bacteria and is worthy of being brought to the fore for further investigation and possible exploitation.

All the CBs were quantitatively utilized without accumulation of intermediates. This metabolic versatility is of special interest because most reports characterizing growth of bacteria on CBs have described CBAs as dead-end products (Furukawa *et al.*, 1979; Bedard *et al.*, 1987; Fava and Marchetti, 1991; Arendsdorf and Focht, 1994; Adebusoye *et al.*, 2007a). However, growth of IR08 on all three CBs resulted in rapid utilization of the substrates without production of respective CBAs. Chloride balances of each degradation experiment as summarized in Table 2 indicate complete mineralization of the chlorobiphenyls. Although mineralization was complete, the growth of IR08 on 3-CB was poor compared to 2- and 4-CB (Table 2). While growth of biphenyl/PCB-degraders are quite frequent on 4-CB (Bedard *et al.*, 1987; Fava and Marchetti, 1991; Arensdorf and Focht, 1994; Potrawfke *et al.*, 1998; Kim and Picardal, 2000; Adebusoye *et al.*, 2007a), it is generally difficult to select 3-CB-degrading strains with strong degrading ability from contaminated environments both under laboratory conditions and under simulated natural conditions (Furukawa *et al.*, 1982; Kong and Sayler, 1983; Fava and Machetti, 1991).

Although the three CBs were degraded and completely mineralized in less than 96 h, without production of detectable metabolites, the early formation of yellow intermediates during catabolism of 4-CB indicates their degradation follows a *meta*-cleavage pathway. On the other hand, the darkening of the culture broth observed when IR08 was grown on 3-CB could be an indication of the presence of chlorocatechol and possibly accounted for the poor growth observed (Fava and Marchetti, 1994; Arensdorf and Focht, 1994).

Table 3   Co-incubation of IR08 with monochlorobiphenyls and biphenyl

Carbon source during growth	Growth increase	PCB conc. (mmol/L)		Biphenyl conc. (mmol/L)		Chloride released (mmol/L)	CBA recovered (mmol/L)	Mineralization[a] (%)
		Initial	Final	Initial	Final			
2-CB + biphenyl	Seven-fold	0.27	0	0.32	0.125	0.23	0	85.18
3-CB + biphenyl	Seven-fold	0.27	0	0.32	0.128	0.21	0	77.78
4-CB + biphenyl	Nine-fold	0.27	0	0.32	0.116	0.27	0	100

Growth occurred at the expense of the CB isomers and all were completely utilized in less than 96 h. [a] Expressed as chloride released in the growth medium with respect to that chemically bond in chlorophenyl rings.
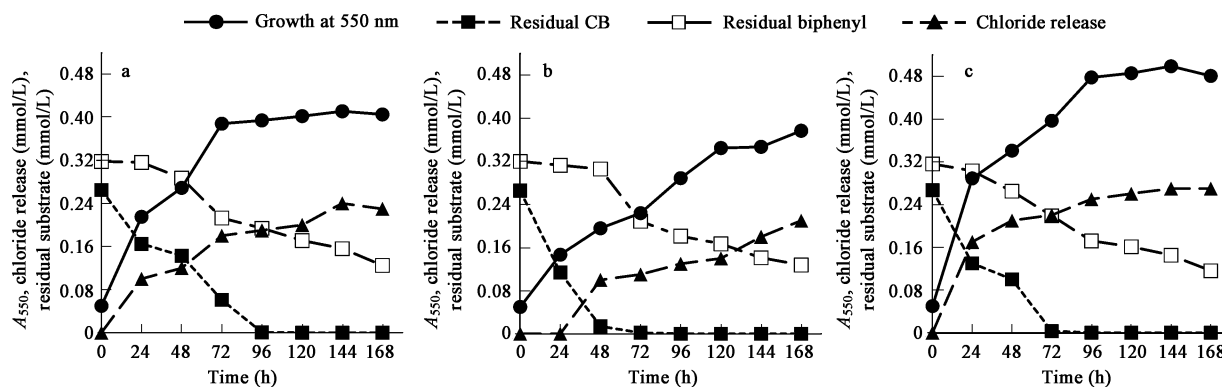
Fig. 3   Simultaneous utilization of biphenyl and 2-CB (a), biphenyl and 3-CB (b), and biphenyl and 4-CB(c). All the CBs were completely utilized in less than 96 h concomitant with stoichiometric elimination of chloride from respective incubations at which time, less than 50% of the biphenyl was degraded. Analysis of culture broth yielded no metabolite.

It is difficult to confirm that the bacterial dioxygenase exclusively attacked the unsubstituted aromatic ring since the CBA intermediate metabolites were not detected. This feature of our isolate makes it stand out from those reported to date in literature. In the case of 3-CB metabolism, there was a slight lag period between 3-CB disappearance and chloride release (Fig.1b), thus implying that the initial attack probably occurred on the unsubstituted phenyl ring. However, further studies will be undertaken to understand the biochemical aspects that underlay the degradation process in IR08 especially to determine if the initial dioxygenase attack is on the chlorinated or unsubstituted ring. If the initial step involved oxygenation of the unsubstituted ring and if this was the rate-limiting step, rapid degradation of the CBA products might explain our lack of CBA detection during the metabolism of the three CBs. Kim and Picardal (2000) described similar observation when a newly characterized isolate, SK-3 grown with 4-CB mineralized the substrate without production of 4-CBA.

4 Conclusions

The isolation and characterization of strain IR08 are significant and unique in its ability to completely mineralize CBs without production of respective CBAs, thus greatly reducing the risks associated with production of many metabolites, which, besides CBAs, could be harmful to the environment. It must be emphasized that this ability to mineralize all three CBs has never been reported for an aerobic pure bacterial strain or consortium of organisms. With the exception of Kim and Picardal (2000), no other isolate has been shown to mineralize CBs without first producing CBA as a central metabolite before subsequent transformation. Since strain IR08 is capable of growth on several compounds including chlorobiphenyls and chlorobenzoates, it may therefore be able to serve as seeds for bioremediation of PCB-contaminated soils for total destruction of PCBs especially in systems that have undergone anaerobic reductive dechlorination stage.

Acknowledgments

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