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ORIGINAL PAPER

Influence of chlorobenzoic acids on the growth and degradation potentials of PCB-degrading microorganisms

Sunday Adekunle Adebusoye · Flynn W. Picardal · Matthew O. Ilori · Olukayode O. Amund

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Abstract The biodegradation of polychlorinated biphenyls (PCBs) by diverse bacteria including those utilized in this study is often incomplete, a concomitant accumulation of chlorobenzoic acids (CBAs) are released as dead-end products. The build-up of these metabolites in the growth medium may result in feed-back inhibition and impede PCB biotransformation. In this investigation using GC-ECD and HPLC analyses, we confirmed that CBAs inhibit growth and PCB biodegradation potentials of five tropical bacteria namely, *Pseudomonas aeruginosa* SA-1, *Enterobacter* sp. SA-2, *Ralstonia* sp. SA-3, *Ralstonia* sp. SA-5 and *Pseudomonas* sp. SA-6. Among the four CBAs (2-CBA, 3-CBA, 4-CBA acids and 2,3-diCBA), 3-CBA was the strongest inhibitor followed by 4-CBA. Furthermore, we found that 3-CBA heavily inhibited growth of SA-3 and SA-6 on monochlorobiphenyls by 82–90% while elimination rate was inhibited by 71–88%. In the case of 2,3-diCBA, inhibition was generally less than 60%. However, effects of both acids were stronger in SA-3 than SA-6. We also found that 3-CBA and 2,3-diCBA completely inhibited carbon-chloride cleavage of 2-CB and 3-CB since cultivation in the absence of the acids resulted in recovery of 23–50% chloride in the culture fluids of organisms. These findings may therefore, have practical and ecological significance and are useful for improving the efficiency and the stability of some biological treatment processes.

S. A. Adebusoye (✉) · M. O. Ilori · O. O. Amund
Department of Botany and Microbiology, University of Lagos, Akoka, Lagos, Nigeria
e-mail: sadebusoye@yahoo.com; sadebusoye@unilag.edu.ng

S. A. Adebusoye · F. W. Picardal
Environmental Science Research Center, School of Public and Environmental Affairs, Indiana University, Bloomington, IN 47405, USA

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Introduction

Microbial metabolism of polychlorinated biphenyls (PCBs) by pure or co-cultures of PCB-degrading microorganisms is generally incomplete with few exceptions (Potrawfke et al. 1998; Kim and Picardal 2000, 2001) and results in the formation of isomeric mixtures of chlorinated benzoic acids (CBAs) which differ in substitution and configuration from the original congeners (Masse et al. 1984; Bedard and Haberl 1990; Fava and Marchetti 1991; Maltseva et al. 1999). In fact, the soil bacteria that co-metabolize PCBs tend to accumulate CBAs as dead-end products because they are generally unable to grow on these substrates (Walia et al. 1988; Bedard and Haberl 1990; Sondossi et al. 1992; Kobayashi et al. 1996). Failure to utilize a target compound can arise from the inability of catabolic enzymes to transform the substrate or its metabolites, as well as from the inhibition of these enzymes by metabolites. Such catabolic blocks are particularly problematic for structurally diverse chemicals and are a common occurrence during microbial degradation of PCBs and CBAs (Abramowicz 1990; Fava and Marchetti 1991; Dai et al. 2002). Therefore, aerobic mineralization of PCBs involves the participation of two groups of bacteria, namely those that (co)metabolize PCB congeners to CBAs, and those which utilize CBAs (Hernandez et al. 1995). Interestingly, previous studies have shown that efficient aerobic degradation of lightly substituted biphenyls can occur in defined co-cultures in which PCB-degrading bacteria are closely associated with CBA-degrading bacteria (Adriaens et al. 1989; Fava et al. 1994). In these studies, it was

demonstrated that the rate of CBA removal from the growth media dictated the rate of PCB degradation by the microbial cells. On this basis, many investigators believed that CBAs have an inhibitory effect on the degradation of PCBs and therefore, need to be considered in remediation strategies. Stratford et al. (1996) investigated the effects of CBAs on the utilization of PCBs and CBA mixtures. They reported the utilization of 2-, 3-, and 4-chlorobiphenyl (CB) by *Burkholderia cepacia* JHR22 that was inhibited by the presence of 2,3- or 3,4-dichlorobenzoic acid (diCBA). Similarly, Vrana et al. (1996) confirmed that CBA inhibited PCB degradation. Among mono- and dichlorobenzoates studied, 3-CBA was reported as the greatest inhibitor of PCB degradation (Vrana et al. 1996). Recently, *Burkholderia xenovorans* LB400 which possesses the biphenyl pathway, was engineered to contain the oxygenolytic *ortho* dehalogenation (*ohb*) operon, allowing it to grow on 2-CBA and to completely mineralize 2-CB as a way to circumvent CBA toxicity and inhibition (Rodrigues et al. 2006).

The continuous fear of CBAs inhibition is occasioned by their solubility. Since CBAs are much more water soluble than their parent compounds, they could enter into aqueous phase of polluted sites and water courses where they could interact closely with organisms mediating their production. The aim of this work was to investigate the impact of the presence of CBAs on PCB degradation potentials of five new tropical bacterial strains which were recently reported to possess wider xenobiotic substrate diversity than some previously characterized strains (Adebusoye et al. 2007a, b, c, d). The results would be useful in designing effective bioremediation processes for clean-up of halogenated pollutants especially in African systems where speciation of these pollutants is poorly understood and indiscriminate discharge of haloaromatics continued unabatedly due to lack of enforcement of regulatory acts.

Materials and methods

Strains

Pseudomonas aeruginosa SA-1, *Enterobacter* sp. SA-2, *Ralstonia* sp. SA-3, *Ralstonia* sp. SA-5 and *Pseudomonas* sp. SA-6 capable of utilizing all monochlorobiphenyls (CBs) and some dichlorobiphenyls were isolated from transformer-fluid contaminated soils in Lagos, Nigeria after several months of enrichment cultivation on Askarel fluid and Aroclor 1221 as the sole carbon source. Identification of these strains was based on the sequencing of the full-length 16S rRNA genes coupled with phenotypic characterization using API 20 E system (bioMerieux Vitek, Inc. Hazelwood, MO) as previously described (Adebusoye et al. 2007c). The 16S rRNA gene sequences have since been deposited in the GenBank database under the accession numbers DQ854840, DQ854842, DQ854843, DQ854845, and DQ854841 respectively for strains SA-1, SA-2, SA-3, SA-5, and SA-6.

Chemicals

Analytical grades of high purity (99–100%) 2-chlorobiphenyl (2-CB), 3-chlorobiphenyl (3-CB) and 4-chlrobiphenyl (4-CB) were supplied by AccuStandard Inc. (New Haven, CT, USA), while all standard solutions of CB congeners with 95% purity were obtained from Ultra Scientific (North Kingstown, RI, USA). 2-chlorobenzoic acid (2-CBA), 3-chlorobenzoic acid (3-CBA) and 4-chlorobenzoic acid (4-CBA), 2,3-dichlorobenzoic acid (2,3-diCBA) were procured from Sigma-Aldrich Corp. (St. Louis, MO, USA), and all had a purity of 98%. Sodium benzoate (>99% purity), 2,2,4,4,6,8,8-heptamethylnonane (>99% purity), and culture mineral constituents were supplied by Fisher Scientific Corp. (Springfield, NJ, USA). Analytical grade solvents for GC and HPLC analyses were also purchased from Fisher Scientific.

Inoculum preparation

Organisms were incubated for 24 h in mineral salts (MS) medium with 2.5 mM benzoate at 25°C on a shaker table set at 120 rev/min. The MS was prepared as described by Adebusoye et al. (2007b). Cells were harvested by centrifugation, washed twice in MS medium lacking benzoate and resuspended in the same medium to an optical density of 0.5–0.6 at 600 nm. The inoculum was used for growth and inhibition experiments.

Evaluation of inhibitory effects of CBAs

The five strains were grown in 6 ml MS medium contained in 30-ml Balch tubes. Monochlorobiphenyl isomers (2-CB, 3-CB and 4-CB) were added to a final concentration of 0.3 mM. Each of this tube was further supplied with selected CBA isomers to a final concentration of 0.3 mM. The CBA isomers tested were 2-CBA, 3-CBA, 4-CBA and 2,3-diCBA. Tubes were inoculated with 10^5–10^6 cells/ml, crimp-sealed with Teflon-coated stoppers to prevent abiotic losses and incubated horizontally on a shaker table (120 rev/min) at 25°C. Growth, substrate utilization, and metabolic products were determined by sacrificing a whole set of three tubes by the addition of 5 ml *n*-hexane. Cell density was monitored by acridine orange direct counting method (Kepner et al. 1994). Percent inhibition was resolved from the residual concentration of CB in the presence of CBA divided by concentration utilized in the absence of CBAs. For instance, 0% inhibition = amounts of CB transformed in absence of CBAs.

Analytical procedures

Monochlorinated biphenyls in the n-hexane extracts were analyzed on an HP 5890 Series II GC fitted to an electron capture detector and a 30-m DB-5 megabore capillary column (J & W Scientific, Folson, CA) (Adebusoye et al. 2007a, b). Chlorobenzoic acids were analyzed directly in cell-free growth medium by reverse-phase HPLC using a Waters system equipped with a YMC-Pack ODS-AQ column (4.6 × 250 mm, 5 μm) as previously described (Adebusoye et al. 2007b). Chloride ion concentration was measured with the same HPLC fitted with a conductivity detector using an IonPac AS17 analytical column (4 × 250 mm; Dionex) preceded by an AG17 guard column. Chloride, CBs and CBAs were quantified using four-point calibration curves constructed using standard solutions ranging from 0.05 to 1.0 mM.

Results

The organisms utilized in this study have the natural ability to biodegrade a wide range of natural and xenobiotic pollutants including various congeners of PCBs (Adebusoye et al. 2007a, b, c, d). However, in order to get information on how CBA and their metabolites affect bacterial growth and CB degradation potential, the possibility of the organisms using the test CBAs as carbon and energy source must be eliminated. Substrate profiling of the isolates showed that growth was not sustainable on any monochlorobenzoic acids and dichlorobenzoic acids. A study was subsequently undertaken to establish the influence of CBAs on the elimination rates of all three CBs by the five strains. The bacteria were grown in liquid culture as described with 0.3 mM of respective CBAs in the presence of their parent CB isomers. The PCB degrading ability reduced significantly. Results obtained showed statistical difference at $P < 0.05$ level of significance. The greatest inhibitor was 3-CBA followed by 4-CBA and 2-CBA in that order (Fig. 1). The percent inhibition by 3-CBA ranged between 71.48 and 93.73%. Degradation of 2-CB by strains SA-5 and SA-6 was largely unaffected by the presence of 2-CBA.

Having exhibited the strongest inhibitory effect, further investigation was carried out to evaluate the influence of 3-CBA on the growth and CB transformation dynamics of two of the bacterial strains, *Ralstonia* sp. SA-3 and *Pseudomonas* sp. SA-6. These organisms were also grown in the presence of 2,3-diCBA. The results obtained present a very interesting phenomenon as summarized in Table 1 and Fig. 2. Growth of organisms grown with CB in the absence of CBA differed just significantly (*t*-test) from those obtained in the presence of CBA at $P < 0.05\%$ level of significance. Growth of SA-3 and SA-6 was impeded by more than 80% when incubated with 3-CBA. The growth of the former was most affected on 2-CB (Table 1). In contrast to observations with 3-CBA, addition of 2,3-diCBA to the culture media resulted in less than 60% growth inhibition. However, like 3-CBA, the effect of this acid was more pronounced on the growth of strain SA-3. Additionally, both strains grew exponentially without lag phases in the presence of these acids similar to growth curves observed in their absence (Adebusoye et al. 2007c).

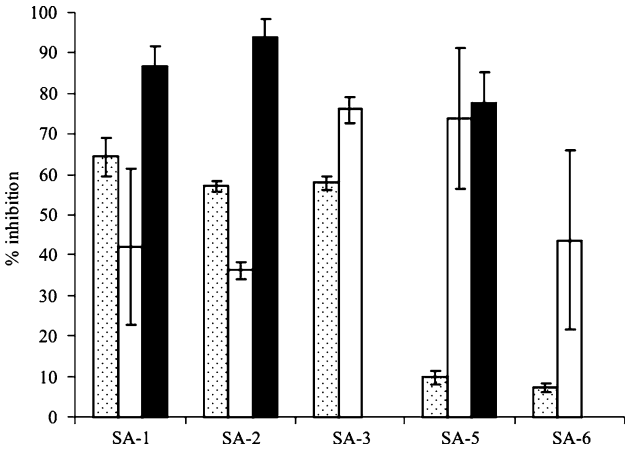

Fig. 1 Effects of chlorobenzoates on the degradation of monochlorobiphenyls by different bacterial strains. ▩, 2-CB; ■, 3-CB; □, 4-CB. Percent inhibition was resolved as described in "Materials and methods." All measurements are means ± standard deviations for three replicate samples. The CB substrate (100 ppm) was incubated with their respective CBA metabolite at a concentration of 0.3 mM for 7 days

The degradation dynamics were not too different from the results obtained from growth experiments. While metabolism of 4-CB by SA-3 was the most severely affected by 3-CBA, it was 3-CB in SA-6 (Fig. 2). In the case of 2,3-diCBA, less than 50% inhibition was obtained across board. Generally, inhibition of CB by 3-CBA was more pronounced on SA-6 eventhough the differences were not significant at 5% confidence limit. Of a significance as depicted in Table 1 is the ability to mineralize 2-CB and 3-CB that was completely impaired in the presence of both acids. In all cases, no chloride was released into the culture fluids whereas both strains released between 23 and 50% chloride from the two isomers when grown alone (Table 1). It is also noteworthy that the results obtained at day 5 of incubation were comparable to those obtained at day 7 and no utilization of the CBAs occurred.

Discussion

Bioremediation is a promising biotechnology for the treatment of PCB-contaminated environments. This technology has been applied successfully for the removal of petroleum and allied pollutants (Bragg et al. 1994; Rosenberg et al.

Table 1 Growth of *Ralstonia* sp. SA-3 and *Pseudomonas* sp. SA-6 on monochlorobiphenyls in the presence of 3-CBA and 2,3-diCBA

Carbon source	*Ralstonia* sp. SA-3						*Pseudomonas* sp. SA-6					
	No addition		+3-CBA		+2,3-diCBA		No addition		+3-CBA		+2,3-diCBA	
	Cell #/ml[a]	Cl^{-b} (mM)	Cell #/ml[a]	Cl^{-b} (mM)	Cell #/ml[a]	Cl^{-b} (mM)	Cell #/ml[a]	Cl^{-b} (mM)	Cell #/ml[a]	Cl^{-b} (mM)	Cell #/ml[a]	Cl^{-b} (mM)
2-CB	5.13×10^8	0.20 ± 0.03	5.20×10^7	0	2.73×10^8	0	8.71×10^8	0.22 ± 0.04	1.29×10^8	0	5.05×10^8	0
3-CB	5.37×10^8	0.10 ± 0.01	6.96×10^7	0	2.45×10^8	0	2.34×10^8	0.10 ± 0.01	4.07×10^7	0	1.26×10^8	0
4-CB	7.24×10^8	0	8.98×10^7	0	2.91×10^8	0	8.91×10^8	0	1.55×10^8	0	4.61×10^8	0

[a] Cell number per ml, data are mean for two replicate determinations

[b] Values are means ± standard deviations for three replicate samples

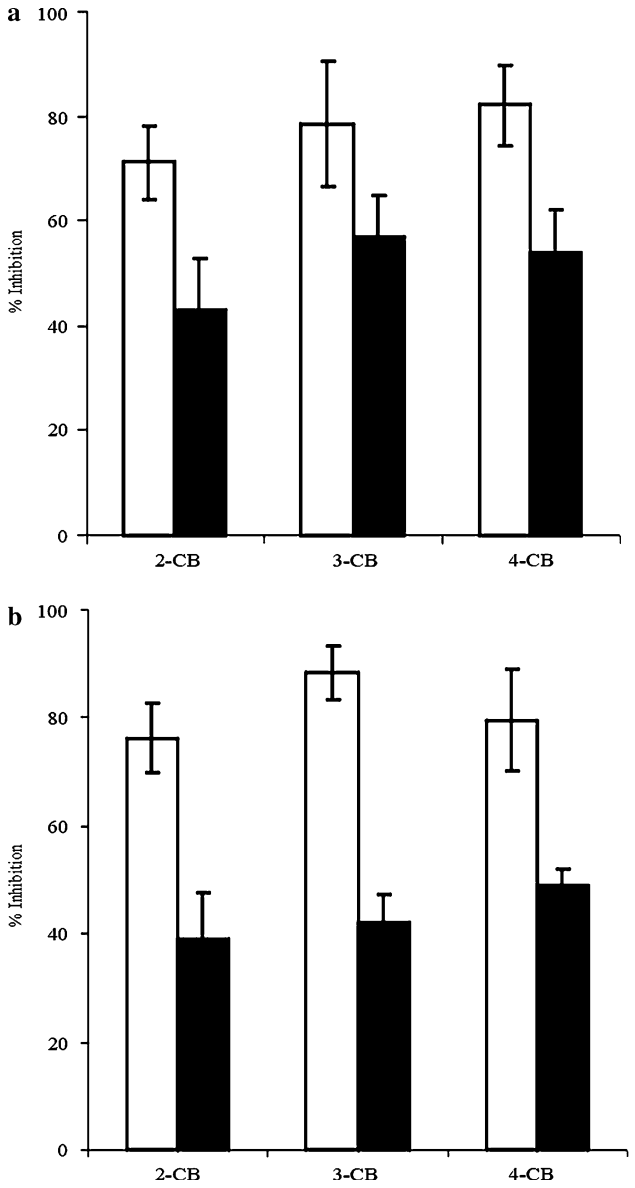

Fig. 2 Effects of 3-CBA, □ and 2,3-diCBA, ■ on the degradation of monochlorobiphenyls by *Ralstonia* sp. SA-3 (**a**) and *Pseudomonas* sp. SA-6 (**b**). Percent inhibition was resolved from the residual concentration of CB in the presence of CBA divided by concentration utilized in the absence of CBAs. 0% inhibition = amounts of CB transformed in absence of CBAs. All measurements are means ± standard deviations for three replicate samples. The CB substrate was incubated with the CBA at a concentration of 0.3 mM for 7 days

1996). The process of PCBs bioremediation is still inefficient and not well established in spite of the existing body of knowledge on microbial biodegradation of these xenobiotics. Most PCB degraders are only able to biotransform these compounds to CBAs as dead-end products (Kobayashi et al. 1996; Kim and Picardal 2001; Rodrigues et al. 2006; Adebusoye et al. 2007b, c). The biodegradation performance of the scavenging organism could be affected by the toxicity of the metabolites (Stratford et al. 1996; Vrana et al. 1996;

Erb et al. 1997). The generation of toxic metabolites may be particularly severe if the compounds are the only available source of carbon and energy. The current study proves that the presence of CBAs affects bacterial growth and therefore, strongly affects their overall degradation performance. Consequently, the toxicity of metabolites generation during microbial metabolism of PCBs may partially explain the recalcitrance to biodegradation of these pollutants, hence, their persistence in the environment.

All the CBAs tested caused significant reduction in growth ($P < 0.05$) and degradation of PCBs. During growth studies on CB congeners however, no apparent inhibition of growth or degradation by the build-up of CBA metabolites as dead-end products was observed (Adebusoye et al. 2007c). The concentration of CBA possibly liberated to the medium by the partial degradation of the congeners is negligible in comparison with the combined CBA concentration added. This would likely be the reason why no apparent toxicity of accumulated CBA products was recorded during growth studies.

When comparing the elimination rates of PCB in the presence of CBAs, the influence of 3-CBA was most pronounced. This acid was previously reported to have the strongest inhibitory effect on PCB degradation (Sondossi et al. 1992). Similar result was also reported by Vrana et al. (1996). Conversely, our results are relatively different from those documented by Guilbeault et al. (1994). These authors observed inhibitory effects of a range of mono- and dichlorobenzoic acids on the capacity of an enhanced population to degrade Aroclor 1242. They suggest that 3,4-diCBA is the potent inhibitor of PCB degradation. More problematic was the discovery from our study that 3-CBA and 2,3-diCBA were very effective at preventing carbon-chloride cleavage of 2-CB and 3-CB. Stratford et al. (1996) reported that 2,3-diCBA caused significant reduction in the ability of *B. cepacia* JHR22 to mineralize CBs but did not completely impaired it as observed in this study.

Inhibition by CBAs of PCBs stem from the observation that some CBAs during PCBs metabolism could be converted to unproductive metabolites, which ultimately reduce flux of PCB catabolic pathways. In the case of 3-CBA, one possible explanation to the striking inhibitory property of this acid was provided by Knackmuss (1981) and Sondossi et al. (1992). They proposed that 3-CBA is degraded via 3-chlorocatechol, the *meta*-cleavage product of this compound is 5-chloroformyl 2-hydroxypenta 2,4-dienoic acid which rapidly inactivates the 2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3-DBPD), the central enzyme involved in PCB metabolism and also 2,3-catechol dioxygenase. Stratford et al. (1996) confirmed 3-chlorocatechol as the metabolite that inhibits catechol 2,3-dioxygenase and 2,3-DBPD. Havel and Reineke (1992) assumed the formation of a toxic compound from 4-CBA by the natural microflora led to a rapid die-off of a biphenyl-metabolizing bacterial strain. We suspect that a similar mechanism may occur for the inhibition of CBs in the SA strains particularly since these organisms readily utilize the *meta* route for dissimilation of benzoic acid and catechol (Adebusoye et al. 2007a). A similar mechanism might also be responsible for the potency of 2,3-diCBA. However, the mechanism by which both 3-CBA and 2,3-diCBA impede carbon-chloride cleavage is not clear. In any case, the nature and character of CBA inhibition will be the focus of our further studies.

To summarize, the effect of the formation of toxic intermediates should be an important consideration when designing remediation strategies. It appears that when CBAs produced from chlorobiphenyls accumulate in the growth medium, they either interact directly with biphenyl/PCB degradation pathway or they are converted into unproductive metabolites that reduce the flux of the chlorobiphenyl degradation pathway. Therefore, in order to establish optimized bioremediation processes for PCBs, the problem of the toxicity of these metabolic intermediates should be approached. Thus, it will be of critical importance to overcome dead-ends in the catabolic process by the requirement for the presence of microorganisms or consortia with diverse CBA utilization abilities during bioremediation of PCBs.

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