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Degradation of highly chlorinated PCBs by *Pseudomonas* strain LB400

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SUMMARY

Congeners of polychlorinated biphenyl (PCB) differ in the number and position of chlorine substituents. Although PCBs are degraded, those congoners with five or more chlorines have been considered resistant to bacterial degradation. Metabolism by *Pseudomonas* strain LB400 of PCBs representing a broad spectrum of chlorination patterns and having from two to six chlorines was investigated. Degradation of pure PCB congeners and synthetic congener mixes was measured in resting cell assays with biphenyl- or Luria broth-grown cells. In addition, the appearance of metabolites was followed using HPLC purification, and GC and GC–MS characterization. 2,4,5,2′,4′,5′-[^{14}C]hexachlorobiphenyl was also used to follow the accumulation of ^{14}C-labeled metabolites. Evidence indicates that LB400 aerobically metabolizes representatives of all major structural classes of PCB's including several congeners which lack adjacent unchlorinated carbon atoms. The mechanisms by which many of these congeners are degraded are not fully understood, but it is apparent that aerobic bacteria can degrade a broader spectrum of PCB congeners than previously believed and that this broad spectrum of degradative competence can exist in a single strain.

INTRODUCTION

Polychlorinated biphenyls (PCBs) were widely used in a number of industrial applications for nearly fifty years and, as a consequence, became widely distributed in the environment. Although PCBs can be at least partially degraded by biological, chemical and photo-chemical means, concern has risen over their relative persistence.

The biodegradation of PCBs by eukaryotic organisms has been well characterized. There seems to be preferental oxidation of congeners with relatively few chlorines and vicinal unsubstituted carbon atoms by the mammalian cytochrome *P*-450 system [8,9,10,14]. However, there is also evidence that mammals may degrade some PCB congeners by other mechanisms [11,13]. Metabolites of a PCB congener lacking vicinal, unchlorinated carbon atoms have been isolated following degradation by mammals [13].

Degradation of PCBs by many genera of aerobic bacteria has also been demonstrated. Although most aerobic PCB-degrading bacteria are capable of metabolizing only mono-, di- and trichlorobi-

phenyls [4] pure cultures of *Alcaligenes* Y42 and *Acinetobacter* P6 [5,6] and *Corynebacterium* MB1 and *Alcaligenes eutrophus* H850 [1] have been shown to degrade congeners containing up to five chlorine atoms. Degradation of at least one congener containing six chlorines has also been reported [12].

The principle metabolic pathway for PCB degradation by aerobic procaryotes appears to involve a dioxygenase which acts at unsubstituted 2,3 positions [4]. Recently there have also been reports that a dioxygenase attack may occur at unsubstituted 3,4 positions of some congeners [1,4]. In either case, the organisms appear to require adjacent, unchlorinated carbon atoms. Other mechanisms of PCB degradation by aerobic bacteria have also been proposed [15], but details are lacking.

Preliminary investigations in this laboratory indicated that a new bacterial isolate, *Pseudomonas* strain LB400, could degrade an unusually broad spectrum of PCB congeners. One of these, 2,4,5,2′,4′,5′-hexachlorobiphenyl (2,4,5,2′,4′,5′-CB), lacked adjacent unchlorinated carbon atoms and so seemed especially worthy of more rigorous examination.

MATERIAL AND METHODS

Bacterial strains and culture conditions

Pseudomonas strain LB400 was isolated from a PCB-contaminated site by enrichment in PAS broth containing biphenyl. PAS medium was prepared by combining 78 ml of PA concentrate with 911 ml of distilled water and autoclaving the mixture. Sterile PAS salts (10 ml) and yeast extract (1.0 ml of a 5% wt/vol solution) were then aseptically added. PA concentrate contained, in grams per liter of distilled water: K_2HPO_4, 56.8; KH_2PO_4, 22.0; NH_4Cl, 27.7. PAS salts contained, in grams per liter of distilled water: $MgSO_4$, 19.5; $MnSO_4 \cdot H_2O$, 5.0; $FeSO_4 \cdot 7H_2O$, 1.0; $CaCl_2 \cdot 2H_2O$, 0.3. PAS salts were acidified to pH 2.5 with H_2SO_4 to prevent oxidation of ferrous iron. Both PA concentrate and PAS salts were sterilized by autoclaving. Biphenyl was sterilized by autoclaving in sealed serum bottles and aseptically added to sterile broth while molten. The final concentration was 2.0 ml/l (although this is far more than is soluble). Alternative carbon sources were added to sterile and otherwise complete medium from concentrated stock solutions such that the final concentration was always 0.5% (w/v). Luria (L) broth was prepared as previously described [2]. Cultures were grown at 30°C in a rotary shaker-incubator.

PCB depletion by resting cells

Cells were grown to late log phase, harvested by centrifugation, and washed twice in 1/2 volume each time of sterile 50 mM sodium phosphate buffer (pH 7.5). Excess biphenyl crystals were removed by carefully pipetting the cell suspension away from the biphenyl, which had settled to the bottom. After washing, no residual biphenyl was visible. Cells were then resuspended in sterile phosphate buffer to an A_{600nm} of 1.0 (1×) or 1/10 that volume (10×). Reactions were carried out in glass, 2-dram, vials with foil-lined caps. Each reaction vessel contained 1.0 ml of cell suspension and the appropriate PCBs added from 100× acetone stock solutions. Stock solutions contained one or more PCB congeners. At least one non-degradable congener was added to each reaction as an internal standard. PCBs were usually added to a final concentration of 1 ppm of each congener, although adjustments were made in cases where the response factors in the electron capture detector (ECD) of the gas chromatograph were widely disparate, so that the peak areas in the controls were approximately equivalent. In some experiments, much higher concentrations of PCBs were used, but stock solutions were prepared accordingly so that no more than 20 μl of acetone was ever added to a reaction vessel. Killed cell controls were prepared by adding 20 μl of a 50 mM stock solution of $HgCl_2$ to a standard reaction vessel (final $HgCl_2$ concentration = 1 mM) and incubating for 15 min before the addition of PCBs. Controls were also prepared at time zero by adding 10 μl of concentrated perchloric acid to each reaction vessel immediately following the addition of PCBs. All reaction vessels were incubated in the dark on a rotary shaker at 30°C. Reactions were

stopped by the addition of 10 μl of perchloric acid.

PCBs were extracted in the reaction vessel with four volumes of anhydrous ethyl ether. Contents were mixed for 1 h on a reciprocating shaker at approx. 250 cycles per minute. Vials were then centrifuged at low speed (500 × *g*) for 5 min to separate the phases, and an aliquot of the organic phase was removed for analysis.

Careful analysis of extraction efficiencies indicated that the inclusion of a single internal standard and the use of the appropriate controls allowed accurate quantitation of the data. A detailed analysis of the problems associated with obtaining reliable quantitative data from PCB depletion assays is being published elsewhere (Bedard, et al., unpublished data).

Metabolite analysis

Metabolism of 2,4,5,2′,4′,5′-CB by strain LB400 was followed using both unlabeled and uniformly ^{14}C-labeled 2,4,5,2′,4′,5′-CB, 8.2 mCi/mmol (New England Nuclear, Inc.). Metabolites were extracted with or without acidification. Each sample was extracted three times with two volumes each time of ether. Extracts were concentrated and analyzed directly by gas chromatography (GC) or were derivatized (by silylation) with *N,O*-bis(trimethylsilyl) acetamide (BSA, Pierce Chemical Co.) prior to analysis.

Large quantities of metabolites were prepared by incubating 100 ml of biphenyl-grown resting cells with 10–50 ppm of PCB. Metabolites and residual PCB were extracted three times with one volume each time of ether. Extracts were concentrated on a rotary evaporator at room temperature to final volumes of approximately 500 μl and dried with anhydrous sodium sulfate. Following neutral extraction, the aqueous residue was acidified with 1.0 ml of concentrated perchloric acid and reextracted as described. Replicate samples were also acidified prior to the initial addition of ether and then extracted. These three extraction protocols allowed for the determination of the effect of acid on the metabolites and on their extractability. Concentrated extracts were analyzed by GC, both with and without BSA derivatization.

Metabolites were purified on a Waters high-performance liquid chromatograph (HPLC) equipped with a Waters Nova-Pak C_{18} Radial-Pak cartridge, using a 0–100% water-acetonitrile gradient. The gradient was run linearly over 30 min and then held at 100% acetonitrile for 5 min. Sample detection was by UV absorbance at 254 nm. Fractions were collected on a LKB programmable fraction collector. These were then extracted once with an equal volume of ether, dried with anhydrous sodium sulfate, concentrated by evaporation to approximately 200 μl, and analyzed by GC with and without derivatization.

Gas chromatographic analysis.

All non-derivatized samples were dried with anhydrous sodium sulfate prior to injection. Analyses were performed on a Varian 6000 gas chromatograph equipped with a automatic sampler and an electron capture detector. A glass column packed with 1.5% SP-2250 and 1.95% SP-2401 on 100/120 Supelcoport (Superco, Inc., Bellefonte, PA) was used. Chromatography of congener mixes was done isothermally at 190°C. Metabolite analyses were done using a 150–220°C temperature program run at either 2 or 5°C per minute. Nitrogen was the carrier gas. Depletion of PCBs was measured by calculating the percent decrease in peak area in the experimental sample compared to the nonreacted control and normalizing all data against the internal standards – usually either 2,4,6,4′-CB or 2,4,6,2′,4′-CB – neither of which LB400 could degrade.

Structural analysis of PCBs and metabolites by gas chromatography-mass spectrometry (GC–MS)

PCBs were checked for purity and metabolites were analyzed using a Varian Model 1400 gas chromatograph fitted with a DB-1 capillary column (J and W Scientific) coupled with a ZAB-2F mass spectrometer (VG Analytical). Ionization was by electron impact at 70 eV. Metabolite samples were injected as the ether extracts following derivatization with BSA.

PCBs/solvents

PCBs were obtained as individual congeners

from either Analabs Inc. (North Haven, CT) or Ultra Scientific (Hope, RI) and checked for purity. All solvents and other reagents which might cause artifacts in the analyses were checked for purity by GC.

RESULTS AND DISCUSSION

In each commercial PCB mixture more than 40 individual congeners are present. These are not all resolved, even by capillary GC [3]. In addition, congeners representing some structural classes of interest are absent. Therefore, we have developed synthetic PCB congener mixes which we routinely use in evaluating the PCB-degradative competence of bacteria. (D.L. Bedard, et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, Q61, p. 268). The congeners in each mix are chosen because of both their chlorine substitution patterns and the ability to resolve them by packed-column GC. Analyses are simplified and interpretation of results is unambiguous. Caution must be taken in quantitating data from such assays, however, because many factors other than biodegradation can cause loss of the PCBs. Volatilization, adsorption to glass, absorption by plastic and differential extraction of congeners have all proven to be potential sources of serious error. Consequently, it is necessary to have a non-degradable internal standard present in each assay throughout the incubation period so that data can be properly normalized. Our initial synthetic mixes contained 2,4,5,2′,4′,5′-CB as an internal standard.

Preliminary results with strain LB400 using congener mixes 1 and 2 seemed to indicate that 2,4,5,2′,4′,5′-CB was degraded (data not shown). However, since no other internal standard was present, data could only be normalized against an external control. Therefore, other congeners were sought which this organism could not degrade and which had comparable retention times on the GC column and response factors in the ECD. Two such congeners, 2,4,6,4′-CB and 2,4,6,2′,4′-CB, were found (see Table 2). These were incorporated into congener mixes 1 and 2, thus forming mixes 1B and 2B. Table 1 shows the results of a resting cell assay using these new mixes. Several important observations can be made. First, strain LB400 degrades a broad spectrum of PCB congeners ranging from di- to hexachlorobiphenyls. Second, these congeners are not limited to a single structural class. Congeners with chlorines at the 4 and 4′ positions but at least one unsubstituted 2,3 position (e.g. 4,4′-CB), those at least partially chlorinated in all 2,3 positions but having one or more unsubstituted 3,4 positions (e.g. 2,5,2′,5′-CB), those having at least one 2,3 position and one 3,4 position unsubstituted (e.g. 2,5,4′-CB), and those lacking vicinal unchlorinated carbon atoms (e.g. 2,4,5,2′,4′,5′-CB) are all degraded. Third, the results with mixes 1 and 2 suggesting that 2,4,5,2′,4′,5′-CB is degraded appear valid.

Table 1

Degradation of PCB congener mixes 1B and 2B

PCB resting cell depletion assay. Cells (1 ×) were incubated for 24 h at 30°C with PCB mixtures containing 1 ppm of each congener. Percent degradation was calculated by comparison with killed-cell controls run in parallel and normalization against the internal standard(s).

Congener mix 1B	% Degraded	Congener mix 2B	% Degraded
2,4′	100	2,2′	100
4,4′	47	2,3	100
2,4,4′	98	2,5,2′	100
2,5,2′,5′	100	2,5,4′	100
2,3,2′,5′	100	2,4,2′,4′	100
2,4,6,2′,4′[a]	–	2,4,6,2′,4′[a]	–
2,3,2′,3′	100	2,5,3′,4′	100
2,4,3′,4′	64	2,4,5,2′,5′	100
2,4,5,2′,3′	90	2,3,4,2′,5′	100
3,4,3′,4′	41	2,4,5,2′,4′,5′	45
2,4,5,2′,4′,5′	59		

[a] Non-degradable internal standard.

These results are in striking contrast to those obtained using other bacterial strains. Bacteria for which the degradative competence has been well characterized seem to degrade only a few of the structural classes of PCBs mentioned above. *Acinetobacter* P6 and *Alcaligenes* Y42, for example, have very poor activity against congeners lacking unsubstituted 2,3 positions (e.g. 2,5,2′,5′-CB) and

cannot degrade congeners lacking vicinal, unchlorinated carbon atoms [4,5,6]. *Corynebacterium* MB1 appears to have a 2,3-dioxygenase, but cannot degrade members of the 2,5,2′,5′-CB or 2,4,5,2′,4′,5′-CB classes; and *Alcaligenes eutrophus* H850 has activity against all of the structural classes containing vicinal unchlorinated carbon atoms but has significantly poorer degradative competence against 4,4′-substituted congeners than LB400 and has little or no activity against congeners in the 2,4,5,2′,4′,5′-CB group [1] when tested as part of a complex mixture. However, results indicate that H850 degrades 2,4,5,2′,4′,5′-CB when only that congener and an internal standard are contained in the reaction mixture (Bedard, et al., unpublished data).

To varify the degradation of 2,4,5,2′,4′,5′-CB, resting cell assays were done with just this congener and either of the internal standards. Fig. 1 shows

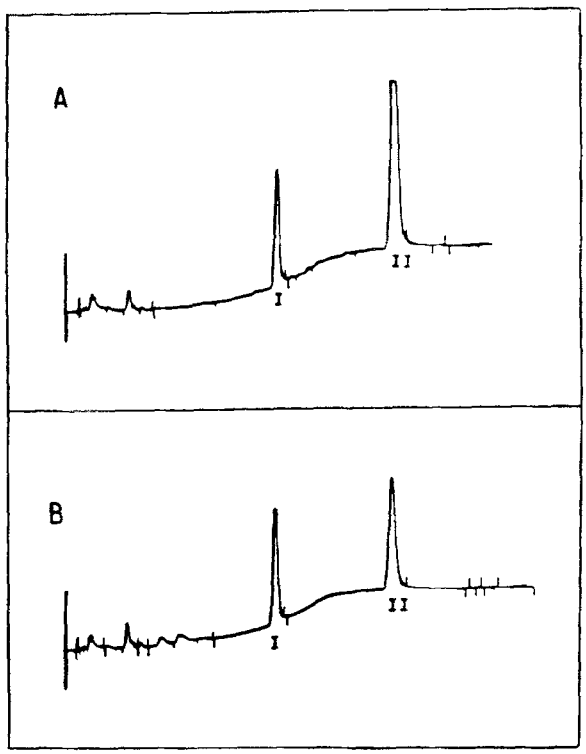

Fig. 1. Degradation of 2,4,5,2′,4′,5′-CB by resting cells. Gas chromatograms of ether extracts from a resting cell assay with 1 × cells, 1 ppm 2,4,6,4′-CB (I) as internal standard, and 5 ppm 2,4,5,2′,4′,5′-CB (II). (A) $HgCl_2$ control. (B) Experimental after 96 h incubation.

a gas chromatogram of the extracts from such an assay. With 5 ppm of starting material, this congener was 58% degraded.

In order to demonstrate conclusively that the depletion of 2,4,5,2′,4′,5′-CB described in the above experiments was the result of biodegradation, an attempt was made to isolate, purify and characterize metabolites of this congener. In experiments such as this, the purity and structure of the starting materials are major considerations. Since some PCB congeners cannot be resolved, even by capillary GC, the peak attributed to 2,4,5,2′,4′,5′-CB could be either a different congener or a mixture of congeners. In either case, depletion might occur even without degradation of 2,4,5,2′,4′,5′-CB. A further complication exists when ^{14}C-labeled material of low specific activity is used. Since only a tiny fraction of the molecules is labeled, it is possible to have an unlabeled but reasonably pure congener contaminated with a small amount of ^{14}C-labeled material of high specific activity. Using such a mixture the contaminant may be degraded and the ^{14}C-labeled products attributed to 2,4,5,2′,4′,5′-CB degradation when no degradation of that compound has actually occurred. Thus, it is necessary to show that the PCB is reasonably pure and that the ^{14}C is associated with it rather than a contaminant. Purity of both ^{14}C-labeled and unlabeled 2,4,5,2′,4′,5′-CB were assessed by GC, HPLC and GC-MS. The PCB was found to be >99% pure, with no single contaminant amounting to more than a small fraction of a percent. The contaminants did not comigrate with 2,4,5,2′,4′,5′-CB in the GC column. Fractionation of the starting material by HPLC showed that the ^{14}C-label was associated with the PCB of interest and not a contaminant. Finally, proton nuclear magnetic resonance spectroscopy (NMR) confirmed that the major component was 2,4,5,2′,4′,5′-CB. Unlabeled 2,4,5,2′,4′,5′-CB spiked with ^{14}C-labeled 2,4,5,2′,4′,5′-CB was degraded by LB400 resting cells, extracted, and the extract fractionated by HPLC (Fig. 2). The starting material was clearly depleted by approximately 50% compared to the control, and there are several peaks which appear in the chromatogram of the degraded sample but

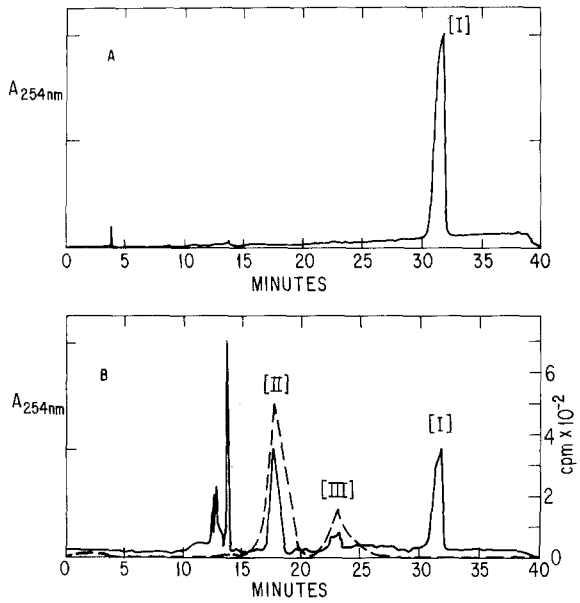

Fig. 2. HPLC purification of metabolites from 2,4,5,2′,4′,5′-CB. Fractionation of a neutral ether extract from a 100 ml resting cell assay using 10× cells and 10 ppm 2,4,5,2′,4′,5′-CB spiked with ^{14}C-labeled 2,4,5,2′,4′,5′-CB. (A) $HgCl_2$ control showing unreacted PCB (I). (B) Experimental after 96 h incubation. Solid line shows 53% PCB depletion (I) and appearance of two metabolite peaks (II and III). Dashed line shows ^{14}C-labeled metabolites extracted from the HPLC fractions. The control was subtracted from the experimental, so that the net cpm in the experimental is shown.

are absent in the control. Fig. 2B shows the ^{14}C content of the fractions collected from the HPLC. Two apparent metabolite peaks at 18 and 23 min contained significant ^{14}C-labeled material which was absent in the control. These fractions were re-extracted, concentrated and analyzed by GC-MS. They were found to contain hydroxylated hexachlorobiphenyls. These could only have been generated as as a result of the biodegradation of 2,4,5,2′,4′,5′-CB. The UV-absorbing materials in HPLC fraction 12–15 are not chlorinated and do not appear to be PCB metabolites. However, the reason for their presence in the experimental sample and absence in the control in unknown.

The absolute structures of the hydroxylated metabolites have not been determined. This will require analysis by NMR, a technique requiring much more material than is presently available. However, some general observations can be made. First, no metabolites with intact aromatic rings and vicinal hydroxyl groups were seen. This can be determined directly from GC–MS data, since such species, upon derivatization, with BSA, show a distinct fragmentation pattern in the MS and can be readily distinguished from species containing non-adjacent hydroxyl groups (R. Unterman, General Electric, personal communication). The lack of such metabolites or a *cis*-dihydrodiol suggests that a dioxygenase is not responsible for the degradation. However, dehydration of dihydrodiols can lead to formation of hydroxylated products such as those seen here, so a dioxygenase cannot be ruled out. Second, a dioxygenase capable of attacking a site at which one carbon atom is chlorinated may be involved. This type of activity has yet to be described, but cannot be excluded. Third, direct hydroxylation of the ring(s) by substitution may have occurred. This has been seen in the bacterial metabolism of chlorobenzoic acids [7], toluene (G.M. Whited et al. 1985. Abstr. Annu. Meet. Am. Soc. Microbiol. K 168, p. 199), naphthalene (B.E. Haigler et al. 1985. Abstr. Annu. Meet. Am. Soc. Microbiol. K165, p. 199) and PCBs [4,6].

Degradation of several other PCB congeners, many lacking vicinal, unchlorinated carbon atoms, was also investigated. The results of these experiments are seen in Table 2. Two congeners,

Table 2

Degradation of recalcitrant PCB congeners

PCB resting cell depletion assays. Cells (10×) were incubated for 24 h at 30°C with approximately 1 ppm of each congener. Congeners were combined in groups of two or three per assay so that the extent of degradation of each one could be independently compared to the others.

Congener	% Degraded
3,5,3′,5′[a]	36
2,4,6,3′,5′[a]	49
2,4,6,2′,4′,6′[a]	29
2,4,6,2′,4′	0
2,4,6,4′	0
2,4,6,3′,4′	19
2,6,2′,6′	0

[a] Congeners lacking vicinal, unchlorinated carbon atoms.

2,4,6,2′,4′,6′-CB and 2,4,6,3′,5′-CB, were of special interest because of the previous observation that 2,4,6,4′-CB and 2,4,6,2′,4′-CB (both of which have unsubstituted 2,3 positions on one ring and chlorines at positions 2,4, and 6 on the other) cannot be metabolized by strain LB400. The ability of this microorganism to metabolize 2,4,6,2′,4′,6′-CB and 2,4,6,3′,5′-CB indicates that chlorination at the 2,4 and 6 positions on one or both rings does not preclude degradation. We have generally found congeners with heavy *ortho-* substitutions to be the most resistant to attack by aerobic bacteria, independent of the availability of other sites for attack (Unterman, R. et al., 1985 Abstr. Annu. Meet. Am. Soc. Microbiol. Q58, p. 267). Consequently, degradation of 2,6,2′,6′-CB was examined (Table 2). Interestingly, this tetrachlorobiphenyl was also resistant to degradation by strain LB400. The reasons for the inability of LB400 to degrade 2,4,6,4′-CB, 2,4,6,2′,4′-CB, and 2,6,2′,6′-CB while still degrading 2,4,6,2′,4′,6′-CB may become apparent with a thorough understanding of the biochemistry involved.

The ability of *Pseudomonas* strain LB400 to degrade PCBs falling into all general structural classes is unusual. Most aerobic bacteria for which the PCB-degradative competence has been accurately determined seem to attack only a few types of congeners. The ability to degrade several congeners lacking vicinal unchlorinated carbon atoms may be especially important, since these are common constituents of some commercial PCB mixtures. Investigations aimed at gaining a more complete understanding of the biochemical mechanisms of PCB degradation in strain LB400 are in progress.

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