Aerobic degradation of polychlo-rinated biphenyls by Alcaligenes sp. JB1: metabolites and enzymes

Commandeur, L.C.M.; May, R.J.; Mokross, H.; Bedard, D.L.; Reineke, W.; Govers, H.A.J.; Parsons, J.R.

Publication date
1997

Published in
Biodegradation

Biodegradation **7**: 435–443, 1996.

Aerobic degradation of polychlorinated biphenyls by *Alcaligenes* sp. JB1: metabolites and enzymes

Laetitia C.M. Commandeur[1,*], Ralph J. May[2], Heinrich Mokross[3], Donna L. Bedard[2], Walter Reineke[3], Harrie A.J. Govers[1] & John R. Parsons[1]

[1] *Department of Environmental and Toxicological Chemistry, Amsterdam Research Institute for Substances in Ecosystems, University of Amsterdam, Amsterdam, The Netherlands;* [2] *GE Corporate Research and Development, Environmental Laboratory, General Electric Company, Schenectady, NY, USA;* [3] *Bergische Universität, Gesamthochschule Wuppertal, Chemische Mikrobiologie, Wuppertal, Germany; (*Present address: Wageningen Agricultural University, Department of Environmental Technology, P.O. Box 8129, 6700 EV Wageningen, The Netherlands)*

Accepted 24 October 1996

Key words: aerobic, biodegradation, enzymes, induction, polychlorinated biphenyls, resting-cell assay

Abstract

In contrast to the degradation of penta- and hexachlorobiphenyls in chemostat cultures, the metabolism of PCBs by *Alcaligenes* sp. JB1 was shown to be restricted to PCBs with up to four chlorine substituents in resting-cell assays. Among these, the PCB congeners containing *ortho* chlorine substituents on both phenyl rings were found to be least degraded. Monochloro-benzoates and dichlorobenzoates were detected as metabolites. Resting cell assays with chlorobenzoates showed that JB1 could metabolize all three monochlorobenzoates and dichlorobenzoates containing only *meta* and *para* chlorine substituents, but not dichlorobenzoates possessing an *ortho* chlorine substituent. In enzyme activity assays, *meta* cleaving 2,3-dihydroxybiphenyl 1,2-dioxygenase and catechol 2,3-dioxygenase activities were constitutive, whereas benzoate dioxygenase and *ortho* cleaving catechol 1,2-dioxygenase activities were induced by their substrates. No activity was found for pyrocatechase II, the enzyme that is specific for chlorocatechols. The data suggest that complete mineralization of PCBs with three or more chlorine substituents by *Alcaligenes* sp. JB1 is unlikely.

Abbreviations: PCB – polychlorinated biphenyls, CBA – chlorobenzoate, D – di-, Tr – tri-, Te – tetra-, Pe – penta-, H – hexa

Introduction

Many bacteria that are able to grow aerobically with biphenyl as a sole carbon source can degrade PCBs with fewer than four chlorine substituents (Bedard et al. 1986). Evidence for aerobic co-metabolism of some PCBs with five and six chlorine substituents has been published for several bacteria. During growth on 4-chlorobiphenyl, *Acinetobacter* sp. J111 appeared to co-metabolize some pentachlorinated and hexachlorinated congeners in Aroclor 1254 (Hernandez et al. 1992). In a resting-cell assay, biphenyl-grown cells of *Alcaligenes euthrophus* H850 and *Pseudomonas* sp. LB400 significantly degraded, amongst others 2,2',3',4,5-, 2,2',3,4,5'- and 2,2',4,5,5'-pentachlorobiphenyls and 2,2',4,4'5,5,'-hexachlorobiphenyl (Bedard et al. 1986). In chemostat cultures, *Alcaligenes* sp. JB1 co-metabolized several tetra-, penta- and hexachlorobiphenyls, during growth on 3-methylbenzoate (Commandeur et al. 1995). In these chemostat experiments, it was only possible to monitor the disappearance of PCBs because they were present at extremely low concentrations due to their low water solubility. Consequently, PCB metabolites were not present at high enough concentrations to analyze with our equipment.

Alcaligenes sp. JB1 is often used in studies on the degradation of chlorinated aromatic compounds and, recently, also in genetic recombination studies. Hence,

it is desirable to have a better understanding of PCB metabolism in JB1. In this study, we have investigated the PCB degradation capability of this strain in more detail. We have also used enzyme assays to determine which degradation pathways are operative in this strain. The basic question was, does *Alcaligenes* sp. JB1 have the potential to mineralize PCBs, and in particular PCBs with four or more chlorine substituents, into biomass, CO_2, H_2O and chloride ions?

First, biphenyl-grown cells of *Alcaligenes* sp. JB1 were screened for PCB degradation in resting-cell assays as described by Bedard et al. (1986). These incubations were analyzed for the accumulation of chlorobenzoates, a common intermediate of aerobic PCB metabolism. Second, the degradation of 2,2',3,3'-tetrachlorobiphenyl and 2,2',3,3',6,6'-hexachlorobiphenyl by benzoate-grown cells was investigated. In chemostat cultures, 2,2',3,3'-tetrachlorobiphenyl was cometabolized very rapidly by *Alcaligenes* sp. JB1 (Commandeur et al. 1995). In the same experiment, 2,2',-3,3',6,6'-hexachlorobiphenyl was also degraded significantly, although it was expected to be recalcitrant because all four *ortho* positions are substituted with chlorine atoms. Third, since the degradation of 2,2',3,3',6,6'-hexachlorobiphenyl must be catalyzed by either a 2,3-dioxygenase which exhibits a dehalogenase property or a dioxygenase which acts at the 3,4-position, *Alcaligenes* sp. JB1 was tested for 2,3-dihydroxybiphenyl 1,2-dioxygenase and 3,4-dihydroxybiphenyl dioxygenase activities. The presence of these enzymes would suggest the existence of complete 2,3- and 3,4- dioxygenase pathways.

Two distinct *meta*-cleavage pathways are required for the mineralization of biphenyl. In different bacteria the enzymes of the upper pathway (biphenyl to benzoate) vary considerably in their ability to metabolize chlorinated biphenyls. But generally the enzymes of the lower pathway (benzoate to citric acid cycle intermediates) do not metabolize chlorobenzoates efficiently. Hence, most PCB degrading bacteria accumulate chlorobenzoates as dead-end products (Furukawa 1982; Commandeur & Parsons 1993) This is probably because 3-chlorocatechol, a common intermediate of chlorobenzoate degradation, binds irreversibly to the *meta* cleaving catechol 2,3-dioxygenase (Bartels et al. 1984) leading to the shut-down of the pathway. The *meta* cleaving 2,3-dihydroxy(n-chloro)biphenyl dioxygenase was also shown to be inhibited by 3-chlorocatechol (Strubel et al. 1991). Bacteria that efficiently degrade chlorobenzoates generally exhibit *ortho* cleavage of their chlorocatechols catalyzed by pyrocatechase II (Dorn & Knackmuss 1978). *Alcaligenes* sp. JB1 can co-metabolize chlorobenzoates (Parsons et al. 1988). Therefore, we examined this organism for the activity of both *ortho*- and *meta*-cleaving catechol dioxygenases and for induction by their substrates.

Materials and methods

Bacterial strain *Alcaligenes* sp. JB1 (previously tentatively identified as *Pseudomonas* strain JB1) was isolated as described by Parsons et al. (1988). *Alcaligenes* sp. JB1 was maintained on agar slopes with 0.25 mM 3-methylbenzoate. The purity of this strain was controlled by frequent plating on nutrient agar and API-20NE (API systems S.A., Montalieu, France) tests.

Resting-cell incubations for PCB degradation

Incubations of *Alcaligenes* sp. JB1 with PCB mixtures 1B, 2B and Aroclor 1242 and the PCB analysis were performed as described in detail by Bedard et al. (1986). Culture samples (2 ml), were grown overnight and killed by adding 1 ml 0.2 m NaOH (pH > 11). Ethyl acetate (3 ml), was used to extract the PCBs from the culture samples by shaking overnight on a horizontal platform shaker. Then, the samples were centrifuged at 10 °C, 700 g for 30 minutes. The extracts were transferred to glass vials and rinsed three times with 1 ml anhydrous ethyl ether. The aqueous phase for each sample was set aside. The extracts were concentrated by a gentle N_2-gas stream to 3 ml. The samples were analyzed for PCBs with gas liquid chromatography (GLC) using a DB1 capillary chromatographic column (30m × 0.25 mM I.D. × 0.25 µm, J & W Scientific) and an electron capture detector. The aqueous phases were acidified with 1 ml 50% H_2SO_4/H_2O and the chlorobenzoates were extracted with anhydrous ethyl ether (2 ml) by shaking overnight on a horizontal platform shaker. The organic and aqueous phases were separated by centrifugation at 700 g for 30 minutes at 10 °C. Derivatization of the chlorobenzoates with pentafluorobenzylbromide and analysis by GC-MS were performed according to the method of Flanagan and May (1993).

Assay for 2,2',3,3'-TeCB and 2,2',3,3',6,6'-HCB degradation

Cultures of *Alcaligenes* sp. JB1 were grown in batch at 25°C in an Evans mineral medium as described by Commandeur et al. (1995), with 8.2 mM benzoate as sole carbon source. Before the experiment, these cultures were transferred three times (1:50) to adapt the cells to the growth substrate. The experiment was started by adding 100 µl acetone solutions of 2,2',3,3'-TeCB (9.2 mg/l) and 2,2',3,3',6,6-HCB (15.4 mg/l) to cultures (100 ml) that had been grown overnight. Initial concentrations of 2,2',3,3'-TeCB and 2,2',3,3',6,6'-HCB were 31.6 nM and 43 nM respectively. The bacterial dry weight was approximately 0.42 g dw/l, measured as described by Herbert et al. (1971). Samples (10 ml) were taken at 0, 3, 5 and 24 hours. Clean-up and analysis of the PCBs in these samples was according to Commandeur et al. (1995). Acid-killed sterile controls (4M H_2SO_4, pH < 3) were analyzed to evaluate possible abiotic disappearance.

Resting-cell assays for chlorobenzoate degradation

Cultures of *Alcaligenes* sp. JB1 were grown in batch at 25°C in an Evans mineral medium as described by Commandeur et al. (1995), with 8.2 mM benzoate or 10 mM citrate as sole carbon source. The whole-cell assays were performed with 3 g dw/l JB1 cells in 50 mM phosphate buffer. The initial concentration of chlorobenzoates was 2 mM. Samples (0.5 ml) were taken 7 times during three hours. Samples were centrifuged and chlorobenzoates were measured in the supernatant with reversed phase HPLC as described by Parsons et al. (1988).

Measurement of specific activities of enzymes involved in biphenyl degradation

The specific activities of some key enzymes involved in biphenyl degradation were measured spectrofotometrically with cell-free extracts of *Alcaligenes* sp. JB1. The preparation of cell-free extracts has been described earlier by Schwien et al. (1988).

Catechol 1,2-dioxygenase (pyrocatechase I and II) was measured spectrofotometrically by the absorbency of the *ortho* cleavage product at λ=260 nm, as described by Dorn & Knackmuss (1978). Before assaying catechol 1,2-dioxygenase, catechol 2,3-dioxygenase was destroyed by adding hydrogenperoxide, according to Nakazawa & Yokota (1973). Catechol 2,3-dioxygenase was determined by the absorbency of the *meta* cleavage product at λ=375-380 nm, as described by Nozaki (1970). 2,3-Dihydroxybiphenyl 1,2-dioxygenase was measured at λ=434 nm according to Furukawa & Miyazaki (1986). These activities are expressed as µmoles cleavage-product formed per gram of cell-protein per minute (µmol/g pro * min). Protein was determined according to the method of Bradford (1976). 3,4-Dihydroxybiphenyl dioxygenase was assayed by monitoring the decrease of absorbency at λ=205 nM during incubation of cell-free extracts with 3,4-dihydroxybiphenyl in 50 mM phosphate buffer (pH = 7.4).

Results

PCB degradation in resting-cell assays

When *Alcaligenes* sp. JB1 was exposed to Aroclor 1242 in resting-cell incubations, some congeners were selectively degraded as shown in Figure 1. Peak identification is according to Bedard et al. (1987b). Degradation could be detected of peak 3: 2,4- + 2,5-DCB (100%), peak 4: 2,3'-DCB (100%), peak 5: 2,3- + 2,4'-DCB (90%), peak 9: 2,3,6- + 2,3',6-TrCB (24%), peak 12: 2,3',4-TrCB (100%), peak 14: 2,4,4'- (52%), peak 15: 2,3,3'- + 2',3,4-TrCB + 2,2',5,6'-TeCB (30%) and peak 16: 2,3,4'-TrCB + 2,2', 4,6'-TeCB (81%). The observed degradation of all other peaks was less than 20% and was not considered significant.

More extensive removal of some congeners was observed when they occurred in the artificial mixtures 1B and 2B (Table 1) than when they occurred in Aroclor 1242. The 2,3- + 2,4'-DCB were totally depleted in mixtures 1B and 2B, but these congeners were only reduced by 90% when exposed in an Aroclor mixture (see peak 5). 2,2',5-TrCB was degraded by 24% in mixture 2B but was not degraded in Aroclor 1242. Also 2,4'5-TrCB and 2,2',3,3'-TeCB were degraded by 34% and 28% in mixtures 2B and 1B, respectively, whereas no significant degradation of these congeners was found in Aroclor 1242 (peak 13 and 26, respectively). Furthermore, no degradation of 2,2'-DCB (peak 2) was seen in the Aroclor 1242 incubation, whereas this compound was shown to be degraded by 31% in mixture 2B.

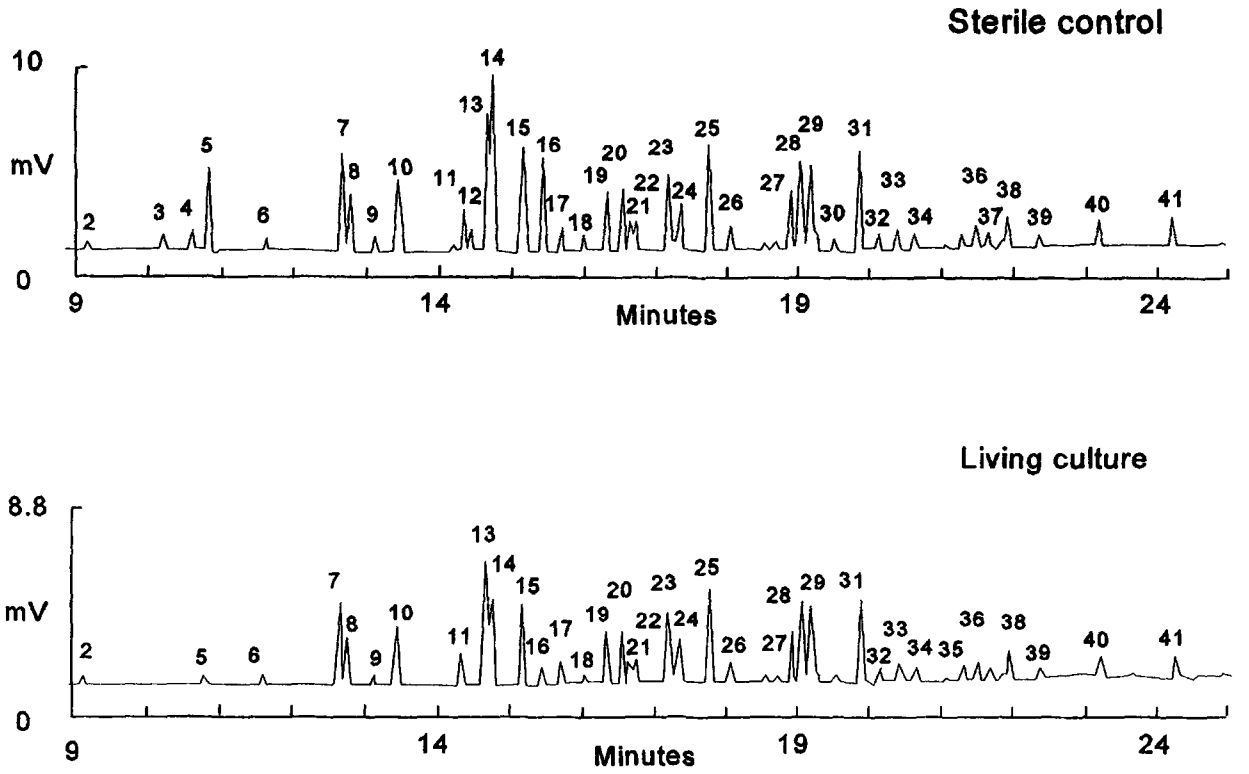

Figure 1. GC-chromatograms *of* Aroclor 1242 (10 µg/ml) incubated with *Alcaligenes* sp. JB1 for 48 hours. Peak assignments: 2= 2,2'- and 2,6-DiCB, 3= 2,4- and 2,5-DiCB, 4= 2,3'-DiCB, 5= 2,3- and 2,4'- DiCB, 6= 2,2',6-TrCB, 7= 4,4'- and 2,2',5-TrCB, 8= 2,2',4-TrCB, 9= 2,3,6- and 2,3',6-TrCB, 10=2,2',3- and 2,4',6-TrCB, 11= 2,3', 5-TrCB, 12= 2,3',4-TrCB, 13= 2,4',5-TrCB, 14= 2,4,4'-TrCB, 15=2,3,3'- and 2',3,4-TrCB and 2,2',5 ,6'-TeCB, 16= 2,3,4'-TrCB and 2,2',4,6'-TeCB, 17= 2,2',3,6-TeCB, 18= 2,2',3,6'-TeCB, 19= 2,2',5,5'-TeCB, 20=2,2',3,5- and 2,2',4,5'-TeCB, 21= 2,2',4,4'- TeCB, 22= 2,2',4,5- and 2,2',4,6-TeCB, 23= 2,2',3,5'-TeCB, 24= 3,4,4'-TrCB and 2,2',3,4'- and 2,3,3',6-TeCB, 25= 2,2',3,4-, 2,3,4',6- and 2,3',4',6-TeCB, 26= 2,2',3,3'-TeCB, 27= 2,4,4',5-TeCB, 28= 2,3',4',5-TeCB, 29= 2,3',4,4'-TeCB, 30= 2,2',3,4',6-PeCB, 31= 2,3,3',4'- and 2,3,4,4'-TeCB, 32= 2,2',3,3',6- and 2,2',3,5,5'-PeCB, 33= 2,2',3,4',5- and 2,2',4,5,5'-PeCB, 34= 2,2',4,4',5-PeCB, 35= 2,2',3',4,5-PeCB, 36= 2,2',3,4 ,5'- and 2,3,4',5,6-PeCB, 37= 2,2'3,4,4'-Pe- CB, 38= Internal Standard = 3,3'4,4'-TeCB and 2,2',- 3,3',6,6'-HCB, 39= 2,2',3,3',4-PeCB, 40= 2,3',4,4- ',5-PeCB and 2,2',3,4',5',6-HCB, 41= 2,3,3',4,4- '-PeCB and 2,2',3,3',4,6'-HCB.

PCB degradation by resting cells of *Alcaligenes* sp. JB1 was limited to PCB congeners with four chlorine substituents or less (Table 1 and Figure 1). Most dichlorobiphenyls were completely removed (2,3-, 2,3'-,2,4-, 2,4'-and 2,5-DCB), although 2,2'-DCB was not. Trichlorobiphenyls with 2,2'-chlorine substituents (2,2',3-, 2,2',4-, 2,2',5- and, 2,2'6-TrCB) were apparently poorer substrates and were not depleted to the same extent as trichlorobiphenyls with only one *ortho* chlorine substituent (2,4,4'-, 2,3,4'-, 2,3',4-, and 2',3,4-TrCB).

In chemostat culture, the co-metabolism of 2,2',3,3'-TeCB and 2,2',3,3',6,6'-HCB by *Alcaligenes* sp. JB1 was relatively fast, with second order rate constants of 13 and 3.17 h^{-1} g dw^{-1} respectively (Commandeur et al. 1995). From these results, complete transformation of these congeners into metabolites may be expected within 24 hours. The 2,2'3,3'-TeCB was degraded in the resting-cell assay, but no significant degradation of 2,2',3,3',6,6'-hexachlorobiphenyl was observed in the same assay (Figure 2). The concentration of 2,2',3,3'-TeCB was similar to that in the chemostat experiment (35nM). However, the half-life measured in the chemostat experiment (0.09 h) is much shorter than that estimated from the resting-cell assay (approximately 3 h).

Chlorobenzoate production from PCB degradation by Alcaligenes sp. JB1

Incubations with PCB mixtures 1B and 2B were screened for chlorobenzoate production. Assuming no dechlorination or chlorine-shift took place, the

Table 1. Degradation of PCB mixtures 1B and 2B by resting cells of *Alcaligenes* sp. JB1. Incubation was for 24 hours. Initial concentrations of all congeners was 5 µm

Mix 1B	Degradation (%)	Metabolites identified
2,4'-DCB	100	2-CBA (0.9 µM),
4,4'-DCB	88	4-CBA (0.7 µM),
2,4,4'-TrCB	84	2,4-CBA (0.9 µM),
2,2',3,3'-TeCB	28	2,3-CBA (0.4 µM),
2,2',3,5'-TeCB	17*	
2,2',5,5'-TeCB	16*	
2,3',4,4'-TeCB	24	
3,3',4,4'-TeCB	7*	
2,2',3',4,5'-PeCB	14*	
2,2',4,4',6'-PeCB	13*	
2,2',4,4',5,5'-HCB**	Internal standard	
Mix 2B	**Degradation (%)**	**Metabolites identified**
2,2'-DCB	31	2-CBA (0.5 µM),
2,3-DCB	100	2,3-DCBA (4 µM),
2,2',5-TrCB	24	2,5-DCBA (0.3 µM),
2,4',5-TeCB	34	
2,2',4,4'-TeCB	19*	
2,3',4',5-TeCB	17*	
2,2',3,4,5'-PeCB	16*	
2,2'3,5,5'-PeCB	14*	
2,2',4,4',6'-PeCB	14*	
2,2',4,4',5,5'-HCB**	Internal standard	

* Observed degradation less than 20% is not considered significant.
** In contrast to Bedard et al. (1987) 2,2'4,4',5,5'-HCB was used as Internal Standard here, as 2,2'4,4'6-PeCB was slightly degrated.

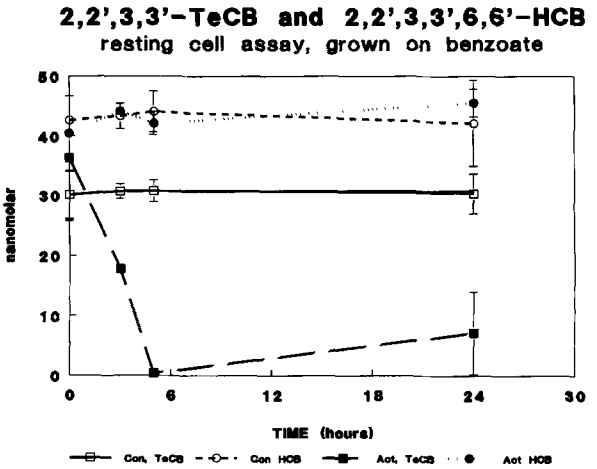

Figure 2. Degradation of 2,2',3,3'-tetrachlorobiphenyl and 2,2',3,3'-,6,6'-hexachlorobiphenyl by resting cells of *Alcaligenes* sp. JB1, grown on benzoate. Con, TeCB = acid killed, sterile control for 2,2',3,3'-TeCB, Con, HCB = acid killed, sterile control for 2,2',3,3',6,6'- HCB, Act, TeCB = live culture assay for 2,2',3,3'-TeCB degradation, Act, HCB = live culture assay for 2,2',3,3',6,6'-HCB degradation.

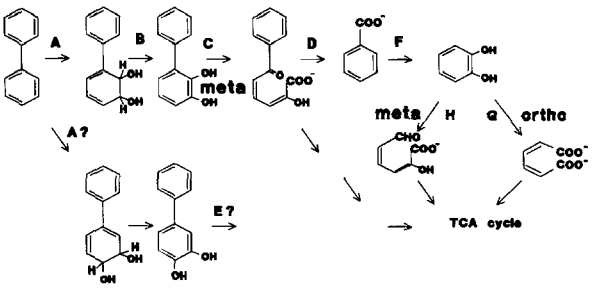

Figure 3. Proposed metabolic pathways of aerobic degradation of biphenyl by *Alcaligenes* sp. JB1. A=biphenyl 2,3-dioxygenase, A?=biphenyl 3,4-dioxygenase, B=biphenyl 2,3-dihydrodiol dehydrogenase, C=2,3-dihydroxybiphenyl 1,2-dioxygenase, D=2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase, E?=3,4-dihydroxybiphenyl n,n-dioxygenase, F=benzoate dioxygenase, G= catechol 1,2-dioxygenase, H= catechol 2,3-dioxygenase.

production of 2-chlorobenzoate (2-CBA) in mixture 1B resulted exclusively from the degradation of 2,4'-DCB. 4-DCBA could have been formed from the degradation of 2,4'-DCB, 4,4'-DCB and 2,4,4'-TrCB. 2,4-Dichlorobenzoate (2,4-DCBA) could have been formed from the degradation of 2,4,4'-T-rCB and 2,3',4,4'-TeCB; and 2,3-DCBA from the degradation of 2,2',3,3'-TeCB. In the case of mixture 2B, 2-CBA could have been formed from 2,2'-DCB and 2,2'5-TrCB degradation. The large amount of 2,3-DCBA produced resulted exclusively from 2,3-DCB degradation and 2,5-DCBA was probably produced from both 2,2',5-TrCB and 2,4',5-TrCB.

Chlorobenzoate degradation by Alcaligenes sp. JB1

Benzoate dioxygenase activity (enzyme F in Figure 3) indicates that *Alcaligenes* sp. JB1 has a preference for *meta* substituted chlorobenzoates. *Ortho-* and *para-*substituted chlorobenzoate showed approximately 5-fold lower degradation rates (Table 2, column 3). No activity was found for *ortho*-substituted dichlorobenzoates (DCBA), whereas those substituted with double *meta* or *meta* and *para* chlorine atoms showed some degradation. Comparison of resting cells of *Alcaligenes* sp. JB1 grown on citrate or benzoate show that the benzoate dioxygenase activity was inducible by its substrate. Incubation of benzoate-grown cells with 3-CBA yielded 70% 4-chlorocatechol and 30% 3-chlorocatechol (data not shown). This means that

Table 2. Specific activities of benzoate dioxygenase in benzoate- and citrate grown resting-cell assays of *Alcaligenes* sp. JB1

Substrate	Specific activity of citrate-grown cells (μmol·g dw^{-1}·h^{-1})	Specific activity of benzoate-grown cells (μmol·g dw^{-1}·h^{-1})
2-chlorobenzoate	0.2	44.4
3-chlorobenzoate	6.4	198.4
4-chlorobenzoate	0.0	35.4
2,3-dichlorobenzoate	n.d.	0.0
2,4-dichlorobenzoate	n.d.	0.0
2,5-dichlorobenzoate	n.d.	0.0
2,6-dichlorobenzoate	n.d.	0.0
3,4-dichlorobenzoate	n.d.	11.6
3,5-dichlorobenzoate	n.d.	14.8

n.d. = not determined.

Table 3. Specific activities of enzymes in crude cell-free extracts of *Alcaligenes* sp. JB1 grown on different growth substrates

Enzyme	Substrate	Growth substrates		
		Citrate (μmol*g pro^{-1}*min^{-1})	Biphenyl (μmol*g pro^{-1}*min^{-1})	Benzoate (μmol*g pro^{-1}*min^{-1})
2,3-dihydroxybiphenyl 1,2-dioxygenase (C)	2,3-dihydroxybiphenyl	2204	1853	1115
3,4-dihydroxybiphenyl dioxygenase[1] (E)	3,4-dihydroxybiphenyl	n.d.	n.a.	n.d.
Catechol 2,3-dioxygenase (H)	catechol	90	120	53
	4-chlorocatechol	n.d.	64	n.d.
Catechol 1,2-dioxygenase (G)				
pyrocatechase I	catechol	5	38	335
pyrocatechase II	3-chlorocatechol	n.a.	n.a.	n.d.

n.a.= no observed enzyme activity for the substrate
n.d.= not determined
[1] measured by the decrease of its absorption spectrum

the benzoate dioxygenase attacks at both 1,2- and 1,6-positions and attack at the 1,6-position is preferred.

Specific activities of enzymes involved in biphenyl degradation

The specific activities of the dihydroxybiphenyl- and catechol-dioxygenases were assayed in cell-free, crude extracts of *Alcaligenes* sp. JB1. The results (Table 3) show activity towards 2,3-dihydroxybiphenyl (enzyme C, Figure 3), but not 3,4-dihydroxybiphenyl (enzyme E). This may indicate that if the biphenyl dioxygenase attacks at the 3,4-positions (enzyme A?) the product may not be degraded further.

2,3-Hydroxybiphenyl 1,2-dioxygenase (enzyme C) and catechol 2,3-dioxygenase (enzyme H) activities were independent of the substrate on which the cells were grown, i.e. they were constitutive. The latter enzyme also showed activity towards 4-chlorocatechol. In contrast, catechol 1,2-dioxygenase (enzyme G) showed low activity after growth on citrate, a non-selective substrate. After growth on biphenyl, the activity was somewhat higher and was highest after growth on benzoate. There was no detectable catechol 1,2-dioxygenase activity for chlorinated catechols after growth on citrate or biphenyl.

Discussion

In contrast to the degradation in chemostat cultures (Commandeur et al. 1995), limited or no degradation of tetrachlorinated to hexachlorinated PCB congeners was detected in resting-cell assays of *Alcaligenes* sp. JB1. Several plausible explanations for this phenomenon are possible. One major difference between the

chemostat experiments and the resting-cell assays was that in the latter case the cells were not growing. It is possible that energy may have to be invested in the first steps of aerobic PCB metabolism. Indeed, Haddock et al. (1995) showed that the biphenyl 2,3-dioxygenase from *Pseudomonas* sp. LB400 required the addition of NAD(P)H and reduced iron (Fe^{2+}). In growing cultures this energy (reducing power) may be delivered by the growth substrate. In non-growing cultures the energy supply may limit the extent of PCB degradation. Stimulation of aerobic PCB degradation during growth was also found by Kohler et al. (1988).

Second, toxic metabolites may inhibit the degradation in batch experiments. As shown by Bartels et al. (1984), Strubel et al. (1991) and Loyd-Jones et al. (1995) the degradation of some halogenated compounds results in inactivation of the dioxygenases. This seems especially true for the *meta* cleaving dioxygenases enzymes C and H (Table 3). It is not clear whether these two enzymes are distinct. For example, during incubation with 3-chlorocatechol both 2,3-hydroxybiphenyl 2,3-dioxygenase and catechol 2,3-dioxygenase are inhibited (Strubel et al. 1991). In chemostat experiments, there is a continuous refreshment of cells, so inactivated enzymes may be dissipated. No growth is possible in a resting-cell assay. Furthermore, the concentration of the PCBs was much lower in the chemostat experiment, thus possibly avoiding toxic effects.

Third, bacteria in chemostats are exposed to the PCB mixture for a longer period of time. Therefore, the phenotype or even the genotype may be adapted to this PCB mixture and differ from that of the cells in the resting-cell assays. However, if this were the case, it might be expected that the degradation rate constant would increase as the experiment continued. This was not true for the chemostat experiment described in Commandeur et al. (1995).

Fourth, the degradation rates of the less chlorinated biphenyls are probably much higher than those of the more highly chlorinated biphenyls. By the time the experiment was stopped the highly chlorinated congeners may not have had time to be metabolized. Bedard et al. (1986) and Parsons et al. (1988) showed that some congeners which were not significantly degraded within 24 hours, were degraded within 72 hours.

PCBs containing *ortho* chlorine substituents on each ring showed less degradation than other congeners. This was particularly clear from the Aroclor 1242 in which several 2,2'- substituted congeners were not degraded. Yet, the fact that 2,2'-DCB (or 2,2',5-TrCB) was degraded to some extent in mixture 2B, was proven unequivocally by the detection of its metabolite 2-CBA. The relative persistence of *ortho* substituted congeners suggests that the biphenyl dioxygenase that attacks 2-chlorophenyl rings in JB1 differs from the enzyme present in strains LB400 and H850. The latter strains both exhibit high activity against 2,2'-DCB (Bedard et al. 1986; Bedard & Haberl, 1990), presumably because they express a biphenyl 2,3-dioxygenases that can attack at the *ortho* chlorinated position (Bedard, 1990; Haddock et al. 1995). From the enzyme assays it is clear that 3,4-dihydroxybiphenyl dioxygenase activity is not present in JB1. Thus, when 2,2',3,3',6,6'-hexachlorobiphenyl was degraded in the chemostat culture experiments it is likely that 3,4-dihydroxy 3,4-dihydro-(2,2',3',5,6,6'-hexachloro)biphenyl was the end product. Accumulation of 3,4- dihydroxy 3,4-dihydro(n-chloro)biphenyls in resting-cell assays has been reported by Nadim et al. (1987) and in enzyme assays by Haddock et al. (1995). Alternatively, 3,4-dihydroxy (2,2',3',5,6,6'-hexachloro)biphenyl might be the end-product of 2,2',3,3',6,6'-HCB degradation in JB1. The latter product might inactivate 2,3-dihydroxybiphenyl 1,2-dioxygenase (Figure 3; enzyme C) as shown by Loyd-Jones et al. (1995). This might explain why no significant degradation of 2,2',3,3',6,6'-hexachlorobiphenyl was detected in the resting-cell assay.

The absence of Pyrocatechase II (Table 3) suggests that JB1 does not completely mineralize most of the PCB congeners. However, Table 3 also shows that catechol 2,3-dioxygenase is constitutive in JB1 and that it degrades 4-chlorocatechol. In addition, the Table 2 shows that JB1 degraded 3-CBA and 4-CBA, and that 3-CBA was degraded predominantly via 4-chlorocatechol. This suggest that those PCBs that are efficiently metabolized via 3- or 4-CBA might be mineralized via meta-fission of 4-chlorocatechol. However, since JB1 does not convert tetrachlorobiphenyls efficiently into chlorobenzoates and has no activity against most dichlorobenzoates, it is not likely that JB1 can mineralize PCBs containing four or more chlorine substituents.

The benzoate dioxygenase assay showed that the induction of this enzyme by its substrates may be an important factor in aerobic PCB degradation. The accumulation of chlorobenzoates in the resting-cell assays for PCB degradation may be the result of a low benzoate dioxygenase activity. Enhanced mineralization of PCBs in soil by inoculation of chlorobenzoate-

degrading bacteria was observed by Hickey et al. (1993).

Catechol 1,2-dioxygenase (enzyme G, Figure 3) generally shows no or only low activity towards chlorinated catechols, so accumulation of chlorinated catechols may block the mineralization of PCBs. Induction of pyrocatechase II (an *ortho* cleaving catechol dioxygenase with high affinity for chlorocatechols) may remove this blockade. However, there is no evidence that this enzyme is present in *Alcaligenes* sp. JB1. Moreover, Table 2 shows that *ortho* substituted dichlorobenzoates can not be metabolized by JB1. Unfortunately, these are the most likely CBA-products because most PCBs in Aroclors 1242 and higher, contain at least one *ortho* chlorine substituent. To accomplish mineralization of PCBs, *Alcaligenes* sp. JB1 genes for the upper pathway (biphenyl to benzoate) could be combined with benzoate dioxygenase and pyrocatechase II genes in other strains. Successful transfer of the biphenyl catabolic genes of JB1 to other bacteria has already been observed (Springael et al., 1996).

Acknowledgment

This work was supported in part by the European Environmental Research Organisation (EERO), P.O. Box 191, NL-6700 AD Wageningen, The Netherlands We gratefully acknowledge J. Lobos for assistance with PCB analysis.

References

Bartels I, Knackmuss H-J & Reineke W (1984) Suicide inactivation of catechol-2,3-dioxygenase from Pseudomonas putida mt-2 by 3-halocatechols. Appl. Environ. Microbiol. 47: 500–505

Bedard, DL (1990) Bacterial transformation of polychlorinated biphenyls. In: D Kamely, A Chakrabarty & GS Omenn (Eds) Biotechnology and Biodegradation. Advances in Biotechnology series, Vol 4 (pp 369–388). Portfolio Pub. Co., The Woodlands, TX, USA

Bedard DL & Haberl ML (1990) Influence of chlorine substitution pattern on the degradation of polychlorinated biphenyls by eight bacterial strains. Microb. Ecol. 20: 87–102

Bedard DL, Haberl ML, May RJ & Brennan MJ (1987) Evidence for novel mechanisms of polychlorinated biphenyl metabolism in Alcaligenes eutrophus H850. Appl. Environ. Microbiol. 53: 1103–1112

Bedard DL, Wagner RE, Brennan MJ, Haberl ML & Brown JF Jr. (1987) Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. 53: 1094–1102

Bedard DL, Unterman R, Bopp LH, Brennan MJ, Haberl ML & Johnson C (1986) Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. 51: 761–768

Bradford MM (1976) Rapid and sensitive method for quantitation of microgram quantities of protein, utilizing principle of protein-dye bond. Analyt. Biochem. 72: 248–254

Commandeur LCM & Parsons JR (1993) Biodegradation of halogenated aromatic compounds. In: Ratledge C (Ed) Biochemistry of Microbial Degradation, Chapter 13 (pp 423–458). Kluwer Publishers, Dordrecht, The Netherlands.

Commandeur LCM, van Eyseren HE, Opmeer MR, Govers HAJ & Parsons JR (1995) Kinetics of biodegradation of highly chlorinated biphenyls by *Alcaligenes* sp. JB1 in an aerobic chemostat culture system. Environ. Sci. Technol. 29: 3038–3043

Dorn E & Knackmuss H-J (1978) Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2-dioxygenation of catechol. Biochem. J. 174: 85–94

Flanagan, WP and May, RJ (1993) Metabolite formation as evidence for in situ aerobic biodegradation of polychlorinated biphenyls. Environ. Sci. Technol. 27: 2207–2212

Furukawa K (1982) Microbial degradation of polychlorinated biphenyls. In: Chakrabarty AM (Ed) Biodegradation and Detoxification of Environmental Pollutants (pp 33–57). CRC Press Inc., Boca Raton, FL, USA

Furukawa K & Miyazaki T (1986) Cloning of a gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes*. J. Bacteriol. 166: 392–398

Gibson DT, Cruden DL, Haddock JD, Zijlstra GJ & Brand JM (1993) Oxidation of polychlorinated biphenyls by *Pseudomonas* sp. strain LB400 and *Pseudomonas pseudoalcaligenes* KF707. J Bacteriol. 175: 4561–4564

Haddock JD, Hornton JR & Gibson DT (1995) Dihydroxylation and dechlorination of chlorinated biphenyls by purified biphenyl 2,3-dioxygenase from *Pseudomonas* sp. strain LB400. J. Bacteriol. 177: 20–26

Hernandez BS, Arensdorf JJ, Focht DD (1992) Characterization of biphenyl-utilizing isolates which co-metabolize PCBs. In: Preprints of the International Symposium 6-9 December 1992, Soil Decontamination using Biological Processes (pp 429–433). Karlsruhe, Germany

Hickey WJ, Searles DB & Focht DD (1993) Enhanced mineralization of polychlorinated biphenyls in soil inoculated with chlorobenzoate-degrading bacteria. Appl. Environ. Microbiol. 59: 1194–1200

Mokross H, Schmidt E & Reineke W (1990) Degradation of 3-chlorobiphenyl by in vivo constructed hybrid pseudomonads. FEMS Microbiol. Lett. 71: 179–186

Nadim LM, Schocken MJ, Higson FK, Gibson DT, Bedard DL, Bopp LH & Mondello FJ (1987) Bacterial oxidation of polychlorinated biphenyls. Proc. US EPA Thirteenth Ann. Res. Symp. on Land Disposal, Remedial Action, Incineration, and Treatment of Hazardous Waste (pp. 395-402)

Nakazawa T & Yokota T (1973) Benzoate metabolism in *Pseudomonas putida* (arvilla) mt-2: demonstration of two benzoate pathways. J. Bacteriol. 115: 262–267

Nozaki M, Kotani S, Ono K & Senoh S (1970) Metapyrocatechase. II. Substrate specificity and mode of ring fission. Biochim. Biophys. Acta 220: 213–223

Parsons JR, Sijm DTHM, van Laar A & Hutzinger O (1988) Biodegradation of chlorinated biphenyls and benzoic acids by a *Pseudomonas* strain. Appl. Microbiol. Biotechnol. 29: 81–84

Schwien U, Schmidt E, Knackmuss H-J & Reineke W (1988) Degradation of chlorosubstituted aromatic compounds by

Pseudomonas sp. strain B13: fate of 3,5-dichlorocatechol. Arch. Microbiol. 150: 78–84

Springael D, van Thor J, Goorissen H, Ryngaert A, de Baere R, Van Hauwe P, Commandeur LCM, Parsons JR, De Wachter R & Mergeay M (1996) RP4::Mu3A mediated in vivo cloning and transfer of a chlorobiphenyl catabolic pathway. Microbiology 142: 3283–3293

Strubel V, Engesser K-H, Fischer P & Knackmuss H-J (1991) 3-(2-Hydroxyphenyl)catechol as substrate for proximal meta ring cleavage in dibenzofuran degradation by *Brevibacterium* sp. strain DPO 1361. J. Bacteriol. 173: 1932–1937