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Degradation of Chlorinated Phenols by a Pentachlorophenol-Degrading Bacterium

JOHN G. STEIERT,[1]* JOSEPH J. PIGNATELLO,[2] AND RONALD L. CRAWFORD[3]

Gray Freshwater Biological Institute, University of Minnesota, Navarre, Minnesota 55392[1]; Department of Soil and Water, Connecticut Agricultural Experiment Station, New Haven, Connecticut 06504[2]; and Department of Bacteriology and Biochemistry, University of Idaho, Moscow, Idaho 83843[3]

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A pentachlorophenol (PCP)-degrading *Flavobacterium* sp. was tested for its ability to dechlorinate other chlorinated phenols by using resting cells that had been grown in the presence or absence of PCP. Phenols with chlorine atoms at positions 2 and 6 of the phenol ring were dechlorinated completely by PCP-induced cells. Other chlorinated phenols were not significantly mineralized. When PCP was added to a culture growing on L-glutamate, there was a lag period before the start of PCP degradation. When similar cells were treated with chloramphenicol prior to the addition of PCP, they did not degrade added PCP, even after prolonged incubations. Thus, the enzymes necessary for PCP degradation appeared to be inducible. Suspensions of cells grown in the presence of 2,4,6-trichlorophenol or 2,3,5,6-tetrachlorophenol did not show a lag period for mineralization of PCP, 2,4,6-trichlorophenol, or 2,3,5,6-tetrachlorophenol, indicating that one enzyme system probably was induced for the biodegradation of all three compounds. Nondegradable chlorophenols were toxic toward the *Flavobacterium* sp., probably acting as uncouplers of oxidative phosphorylation.

Chlorinated phenols and their derivatives are used extensively as insecticides, fungicides, and herbicides by industrial and agricultural users throughout the world. These compounds are quite toxic, and their toxicity tends to increase with their degree of chlorination (12). Many chlorophenols tend to persist in the environment, where they may become public health hazards.

The microbial degradation of multichlorinated phenols has been reported by several groups (1–3, 6, 10, 13–17). We recently isolated a *Flavobacterium* sp. that was shown to utilize pentachlorophenol (PCP) as a sole source of carbon and energy (13, 14). In this paper, we report on the ability of this bacterium to mineralize other chlorinated phenols. We also demonstrate that the PCP-degradation enzymes of the bacterium are under the control of an inducible enzyme system and that in addition to PCP, 2,4,6-trichlorophenol and 2,3,5,6-tetrachlorophenol are inducers of the complete PCP degradation pathway.

(This material was presented in part in preliminary form at the 1985 annual meeting of the American Society for Microbiology.)

MATERIALS AND METHODS

Cell growth and preparation and handling of cell suspensions. The *Flavobacterium* sp. was grown in a minimal salts medium of the following composition: deionized water, 1,000 ml; $NaNO_3$, 0.5 g; K_2HPO_4, 0.63 g; KH_2PO_4, 0.19 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; and L-glutamate (monosodium salt), 4.0 g. After autoclaving, 2 ml of sterile 0.01 M $FeSO_4$ stock solution was added. Cells were induced when desired for PCP degradation by the addition of 50 mg of sodium-PCP per liter to a culture in early logarithmic growth. Disappearance of PCP was measured spectrophotometrically at 320 nm (5). Cells were harvested when added PCP had disappeared. Cell growth was monitored by measuring the optical density of cultures at 560 nm. Cells were harvested by centrifugation at 3,300 × *g* for 30 min at 5°C in sterilized centrifuge bottles. The cells were washed with sterile 5 mM potassium phosphate buffer (pH 7.2), pelleted by centrifugation, and suspended in a small volume of the same buffer. Samples of resultant cell suspensions were then added to an Erlenmeyer flask containing 0.2 mM (final concentration) of a chlorinated phenol in 5 mM potassium phosphate buffer (pH 7.2) to give an optical density at 560 nm of 1.0. Chlorophenol solutions were prepared by dissolving the sodium salt of a chlorophenol in a potassium phosphate buffer and were sterilized by filtration through a filter (0.22-μm pore size). At appropriate time intervals, samples were removed from the cell suspensions for chlorophenol analysis. For analysis by gas chromatography, a 100-μl sample was removed and placed in a test tube containing 2 ml of 0.2 N HCl. For chloride analysis, a 10-ml sample was removed and placed in a 65°C water bath for 15 min. This sample was centrifuged at 12,000 × *g* for 10 min to remove cells before analysis (see below).

Quantitative analysis of chlorinated phenols. For gas chromatographic analyses, the chlorinated phenols were extracted from the culture fluid by acidifying the fluid with HCl to a pH of <2 and shaking with ethyl acetate. The ethyl acetate fraction was dried over beads of molecular sieve. The chlorinated phenols in ethyl acetate were derivatized by the addition of diazoethane in hexane. The derivatized extracts were injected into a gas chromatograph (Hewlett-Packard 5790A) equipped with a Hewlett-Packard cross-linked 5% phenyl methyl silicone capillary column and a ^{63}Ni electron capture detector. Chlorophenol concentrations were determined by integration of peak areas. Hexachlorobenzene was used as an internal standard.

Spectrophotometric analyses of aqueous concentrations of PCP and other chlorinated phenols were made by measuring UV absorbances at the maximum wavelength for each chlorophenol.

* Corresponding author.

TABLE 1. Dechlorination and degradation of chlorinated phenols by *Flavobacterium* cells

Substrate	% Response of:			
	PCP-induced cells		Uninduced cells	
	Dechlorination[a]	Substrate remaining[b]	Dechlorination	Substrate remaining
PCP	100	0	100	0
Tetrachlorophenol				
2,3,4,5-	27	56	7	100
2,3,4,6-	100	0	24	65
2,3,5,6-	100	0	100	0
Trichlorophenol				
2,3,4-	10	54	9	78
2,3,5-	10	61	0	100
2,3,6-	100	0	19	50
2,4,5-	0	100	0	100
2,4,6-	100	0	100	0
2,4,5-	0	100	0	100
Dichlorophenol				
2,3-	22	84	0	82
2,4-	10	89	17	ND[c]
2,5-	17	84	12	91
2,6-	100	0	45	ND
3,4-	0	100	15	ND
3,5-	0	100	12	90

[a] After 24 h.
[b] As measured by gas chromatography after 24 h.
[c] ND, Not determined.

Chloride ion analysis. Chloride ion concentrations of culture fluids were determined with an Orion 701A digital pH-millivolt meter equipped with a chloride-specific electrode (Orion 94-17A). Chloride concentration was determined by using a calibration curve plotted from the log of chloride molarity versus millivolts for a series of standard samples.

Oxygen uptake. Oxygen consumption rates of resting-cell suspensions were measured with a Clark oxygen electrode. Endogenous respiration was noted after 10 min of equilibration before a chlorophenol was added. The endogenous rate then was subtracted from the respiration rate measured after chlorophenol addition.

Protein analysis. Protein analysis was measured by the Coomassie brilliant blue method (Bio-Rad protein assay; Bio-Rad Laboratories, Richmond, Calif.).

RESULTS

The ability of the *Flavobacterium* sp. to dechlorinate and mineralize various chlorophenols was related to the chlorine ring substitution patterns of specific compounds (Table 1). Cells that had been induced for PCP degradation dechlorinated and mineralized (parent molecule and all UV absorbance eliminated) 2,3,4,6- and 2,3,5,6-tetrachlorophenols, 2,3,6- and 2,4,6-trichlorophenols, and 2,6-dichlorophenol. Other chlorophenols were either not dechlorinated or only partly dechlorinated after 24 h. Uninduced cells were able to dechlorinate and mineralize PCP, 2,3,5,6-tetrachlorophenol, and 2,4,6-trichlorophenol, but only after a lag phase. These latter two phenols also supported growth of the *Flavobacterium* sp. as its sole sources of carbon and energy.

Oxygen uptake measurements showed stimulation of oxygen consumption by all of the chlorinated phenols tested with *Flavobacterium* cells that had been induced for PCP degradation (Table 2). After 15 min of incubation of substrates with the cells, respiration had returned to endogenous rates. Samples were removed and analyzed by gas chromatography for the added chlorophenol. Results indicated that even though all substrates had stimulated oxygen consumption, in some cases the compound had not been degraded. This difference was obvious for compounds such as 2,4,5- and 3,4,5-trichlorophenols, which increased respiration greatly but were not mineralized. The compounds which did show oxygen consumption with significant mineralization were PCP, 2,3,5,6-tetrachlorophenol, and 2,4,6-trichlorophenol. Uninduced cells showed oxygen consumption without a lag on addition of all the chlorophenols except PCP, 2,3,5,6-tetrachlorophenol, 2,4,6-trichlorophenol, and 2,4- and 2,6-dichlorophenols. Analysis by gas chromatography showed no disappearance of the parent compounds after incubation for 15 min with uninduced cells. Thus, substrates that stimulated oxygen consumption without mineralization by induced cells also stimulated oxygen consumption by uninduced cells.

The addition of chloramphenicol to a culture indicated that key enzymes for the degradation of PCP are under the control of an inducible enzyme system (Fig.1). When 250 mg of chloramphenicol per liter was added to a culture before the addition of PCP, no disappearance of PCP was observed, even after prolonged incubations. When PCP was added to an uninduced culture that had not been exposed to chloramphenicol, PCP was removed completely after 2h, but degradation commenced only after a 0.5 h lag period. After a culture had been induced for PCP degradation, further additions of PCP were followed by rapid removal of the PCP, both in the presence and absence of chloramphenicol.

Flavobacterium cells that had been induced for degradation of 2,3,5,6-tetrachlophenol or 2,4,6-trichlorophenol were examined for their abilities to degrade PCP, 2,4,6-trichlorophenol, and 2,3,5,6-tetrachlorophenol (Fig. 2).

TABLE 2. Oxygen consumption response to chlorinated phenols by *Flavobacterium* cells

Substrate	Response of:			
	PCP-induced cells		Uninduced cells	
	O_2 uptake[a]	% Substrate remaining[b]	O_2 uptake	% Substrate remaining
PCP	142	0	0	100
Tetrachlorophenol				
2,3,4,5-	56.8	90	56.8	100
2,3,4,6-	153	68	ND[c]	100
2,3,5,6-	56.8	29	0	100
Trichlorophenol				
2,3,4-	56.8	91	71	100
2,3,5-	56.8	85	42.6	100
2,3,6-	37	90	60	100
2,4,5-	150	100	164	100
2,4,6-	240	0	0	100
3,4,5-	164	100	164	100
Dichlorophenol				
2,3-	22.4	88	7.5	100
2,4-	44.9	100	0	100
2,5-	15	92	15	100
2,6-	37.4	50	0	100
3,4-	15	100	7.5	100
3,5-	60	100	75	100

[a] Nanomoles of O_2 consumed per minute per milligram of cell protein.
[b] As measured by gas chromatography after 15 min of incubation in oxygraph chamber.
[c] ND, Not determined.

When PCP or 2,4,6-trichlorophenol was added to 2,3,5,6-tetrachlorophenol-induced cells, no lag period was noted for mineralization or dechlorination of either compound. Similar results were observed for the 2,4,6-trichlorophenol-induced cells when PCP or 2,3,5,6-tetrachlorophenol was added to the cell suspensions.

DISCUSSION

Cell suspensions of our PCP-induced *Flavobacterium* sp. were able to dechlorinate and degrade the rings of various di-, tri-, and tetrachlorophenols completely. These cells, however, only partially dechlorinated and mineralized other related chlorophenols. Cell suspensions that were uninduced for PCP degradation were able to dechlorinate and mineralize the same chlorophenols as induced cells, but only after a lag period for enzyme induction. Two chlorophenols that were completely dechlorinated by the *Flavobacterium* sp., 2,4,6-trichlorophenol and 2,3,5,6-tetrachlorophenol, were shown to be, in addition to PCP itself, inducers of the complete PCP degradation pathway.

Substitution patterns of chlorine atoms on the phenol ring appeared to be important regulators of dechlorination and mineralization of the chlorophenol compounds by the *Flavobacterium* sp. Chlorophenols with chlorines substituted in positions 2 and 6 were completely dechlorinated and mineralized. Those phenols that do not contain a chlorine in both positions 2 and 6 were not readily mineralized and appeared to be toxic to the bacterium (unpublished observation). Our observations coincide with those of Liu et al. (12), who reported on the toxicities of various chlorophenols to bacteria. Toxicity increased with the degree of chlorination, but phenols chlorinated in positions 2 and 6 were the least toxic.

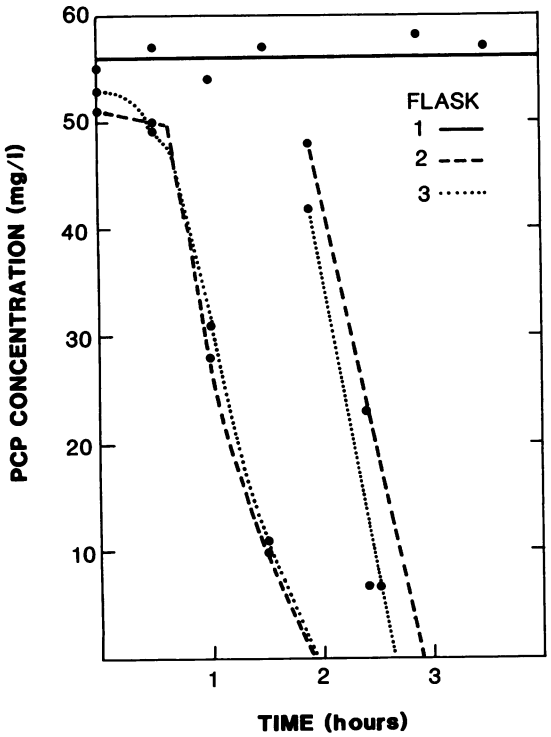

FIG. 1. Effects of chloramphenicol on the induction of PCP degradation pathway. Chloramphenicol (250 mg/liter) was added to flask 1 at time zero. PCP also was added to each flask at time zero. After PCP was removed from flasks 2 and 3, 250 mg of chloramphenicol per liter was added to flask 2, and PCP was added to flasks 2 and 3.

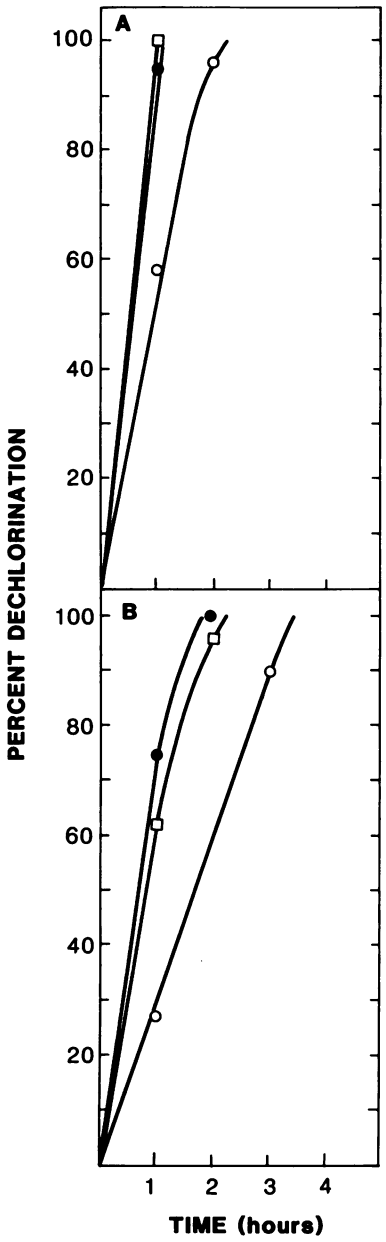

FIG. 2. Percent dechlorination versus time using tetra- or trichlorophenol-induced cells of *Flavobacterium* sp. (A) 2,3,5,6-Tetrachlorophenol-induced cells; (B) 2,4,6-trichlorophenol-induced cells. Curves are for PCP (●), 2,3,5,6-tetrachlorophenol (○), and 2,4,6-trichlorophenol (□).

Other groups have reported on the degradation of chlorophenols by pure cultures. Chu and Kirsch (4) used a PCP-degrading culture designated KC3 for chlorophenol degradation studies and found similar results for ring substitution by chlorine atoms in positions 2 and 6. Karns et al. (9) reported on the degradation and dechlorination of chlorophenols by resting-cell suspensions of 2,4,5-trichloro-

phenoxyacetic acid-degrading *Pseudomonas cepacia* AC1100. Resting-cell suspensions of AC1100 that had been induced for 2,4,5-trichlorophenoxyacetic acid degradation were able to dechlorinate PCP and 2,3,4,6- and 2,3,5,6-tetrachlorophenols. However, degradation patterns observed for the di- and trichlorophenols were quite different with AC1100 and our *Flavobacterium* sp. AC1100 dechlorinated 2,3,4- and 2,4,5-trichlorophenols and 2,3-, 2,4-, and 2,5-dichlorophenols (not dechlorinated by our strain). It poorly dechlorinated 2,6-dichlorophenol and 2,3,6-trichlorophenol and slowly dechlorinated 2,4,6-trichlorophenol, all of which were readily dechlorinated by the *Flavobacterium* sp.

Knackmuss and Hellwig (11) reported on the cooxidation of chlorinated phenols by *Pseudomonas* sp. strain B13 when the bacterium was grown in continuous culture on 4-chlorophenol. The bacterium was able to degrade all the monochlorophenol isomers and dichlorophenol isomers except 2,6-dichlorophenol. Ring cleavage was found to occur by means of a 1,2,-dioxygenase, which would explain the inability of the bacterium to degrade the 2,6-dichlorophenol without prior dechlorination and hydroxylation.

Amy et al. (1) tested the ability of several 2,4-dichlorophenoxyacetic acid-degrading bacteria to degrade chlorinated phenols similar in structure. All isolates tested were able to degrade 2,4-dichlorophenol, and most used 4-chlorophenol. The isolated strains were not able to degrade 2-chlorophenol, which the investigators suggest may be because of the toxicity of the compound. Most of the isolates were able to partially degrade 2,3-dichlorophenol and 2,5-dichlorophenol after incubation for several weeks. Other compounds tested but not used were 2,6- and 3,4-dichlorophenols and PCP.

Our *Flavobacterium* sp. is able to utilize 2,4,6-trichlorophenol and 2,3,5,6-tetrachlorophenol for growth. Chu and Kirsch (4) showed somewhat similar results in that KC3 was able to use 2,4,6-trichlorophenol and 2,3,4,6-tetrachlorophenol but not 2,3,5,6-tetrachlorophenol as growth substrates.

The PCP degradation enzymes were induced when *Flavobacterium* cells were grown in the presence of 2,4,6-trichlorophenol or 2,3,5,6-tetrachlorophenol. Chu and Kirsch (4) reported th KC3 was able to induce some of the enzymes needed for PCP degradation when the culture was grown in the presence of 2,4,6-trichlorophenol. Karns et al. (8) reported that in strain AC1100, chlorophenol degradation enzymes were induced when cells were grown in the presence of either 2,4,5-trichlorophenoxyacetic acid or 2,4,5-trichlorophenol. PCP was not found to be an inducer of either the PCP- or 2,4,5-trichlorophenoxyacetic acid-dechlorinating enzymes. During oxygen uptake studies, we observed that the nondegradable chlorophenols stimulated respiration rates without substrate utilization. This observation may indicate that these compounds are acting as uncouplers of oxidative phosphorylation (7, 18; W. F. Loomis, Fed. Proc., **8**:220, 1949). Our work supports this hypothesis.

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