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Trichloroethylene Degradation by Two Independent Aromatic-Degrading Pathways in *Alcaligenes eutrophus* JMP134

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The bacterium *Alcaligenes eutrophus* JMP134(pJP4) degrades trichloroethylene (TCE) by a chromosomal phenol-dependent pathway and by the plasmid-encoded 2,4-dichlorophenoxyacetic acid pathway. The two pathways were independent and exhibited different rates of removal and capacities for quantity of TCE removed. The phenol-dependent pathway was more rapid (0.2 versus 0.06 nmol of TCE removed per min per mg of protein) and consumed all detectable TCE. The 2,4-dichlorophenoxyacetic acid-dependent pathway removed 40 to 60% of detectable TCE.

Trichloroethylene (TCE) is a common pollutant of groundwater throughout the United States. The U.S. Environmental Protection Agency has classified TCE as a priority pollutant on the basis of its ubiquity, suspected carcinogenicity, and propensity to be anaerobically degraded to vinyl chloride in groundwater (15). Vinyl chloride is known to be tumorigenic (7).

Microbial degradation of TCE has been demonstrated under a variety of restrictive conditions. In anaerobic environments TCE is reductively dechlorinated to yield vinyl chloride (13) and eventually ethylene (5). Aerobic consortia have been shown to degrade TCE in the presence of methane, and the soluble methane monooxygenase has been implicated in that mineralization (4, 14, 16, 19). Several single bacterial strains have been shown to degrade TCE when cultured in the presence of specific aromatic substrates (e.g., toluene or phenol) (8, 9, 17, 20). The enzymes toluene dioxygenase (17) and toluene monooxygenase (20) have been implicated in the enzymatic degradation of TCE by toluene-induced cultures.

The 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacterium *Alcaligenes eutrophus* JMP134 expresses two catabolic pathways which degrade phenol and 2,4-dichlorophenol (1, 2, 6). One is encoded on the chromosome; the other is encoded on the plasmid pJP4 (2, 6). In this paper we report the 2,4-D-dependent degradation of TCE by wild-type JMP134 and the phenol-dependent degradation of TCE by an isolate of JMP134 cured of plasmid pJP4.

Chemicals and cultures. TCE, 2,4-D, phenol, and *n*-pentane were purchased from Aldrich Chemical Co. (Milwaukee, Wis.). The bacterial strains used in this study were *A. eutrophus* JMP134 and AEO106. Strain JMP134 carries plasmid pJP4 (1). Strain AEO106 was derived from JMP134 cured of plasmid pJP4 (6). All strains were grown on minimal salts medium (12) supplemented with the appropriate carbon sources (0.05% 2,4-D, 0.02% phenol, and 0.3% Casamino Acids). Cells used in this study were removed from cultures in mid-log phase.

Oxygen uptake assays. Substrate-dependent oxygen uptake was used to demonstrate the independent induction of aromatic-degrading pathways of interest. Harvested bacterial cells were resuspended in minimal mineral medium to an optical density of 1.0. The cell suspension was added to the electrode chamber (Gilson Medical Electronics, Middleton, Wis.) and maintained at 30°C, and the concentration of dissolved oxygen was measured with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). Base-line consumption of oxygen in mineral medium alone was established, and then the carbon substrate to be tested (3 ppm TCE [3 μg/ml], 500 ppm 2,4-D, or 100 ppm phenol or 2,4-dichlorophenol) was injected.

GC. Gas chromatography (GC) analysis was accomplished by using a Hewlett-Packard 5890 gas chromatograph fitted with electron capture and flame ionization detectors. Separation was effected on a 25-m cross-linked methyl silicone gum capillary column (Hewlett-Packard). Operating conditions were as follows: injector temperature, 150°C; electron capture detector temperature, 250°C; oven temperature, 35 to 100°C at 15°C/min postinjection; and nitrogen carrier column flow, 6 ml/min. Analysis of TCE in the micromolar range was performed by direct injection of 1 μl. Headspace analysis of sealed batch cultures was performed courtesy of the Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, Okla.

Biotransformation assays. Cells grown in the appropriate inducing medium were harvested by centrifugation at 10,000 × *g* for 10 min and resuspended at an optical density of approximately 1.0 in fresh medium containing inducing substrate. The cell suspension (20 ml) was placed in a 100-ml serum bottle and sealed with a Teflon-lined stopper. TCE was added to an approximate concentration of 25 μM. The culture was incubated with shaking at 30°C. At regular intervals 0.5-ml samples were removed and injected into a sealed 2-ml serum vial containing 0.5 ml of high-pressure liquid chromatography-grade pentane. The mixture was extracted for 10 min, and 1 μl of the pentane phase was removed for GC analysis.

Experimental results. The ability of JMP134(pJP4) to degrade TCE by a phenol-dependent pathway was first reported by Montgomery et al. (S. O. Montgomery, M. S. Shields, P. J. Chapman, and P. H. Pritchard, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, K-68, p. 256). We observed similar activity in 2,4-D-grown cultures as substrate-dependent oxygen uptake. Cells induced for the expression of the 2,4-D pathway were placed in the electrode chamber, and either 2,4-D (500 ppm) or TCE (3 ppm) was added. Both exhibited an immediate substrate-dependent response. Uninduced cells grown on Casamino Acids did not respond to the addition of 2,4-D or TCE.

JMP134 expresses two monooxygenases which have ac-

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TABLE 1. Induction of substrate-dependent oxygen consumption

Strain	Inducer	Oxygen consumption[a] in the presence of:		
		2,4-D	Phenol	2,4-DCP
AEO106	None	37.5	0.0	16.3
	2,4-D	35.5	0.0	8.8
	Phenol	25.0	338.0	26.3
JMP134	None	38.0	0.0	37.0
	2,4-D	270.0	0.0	313.0
	Phenol	88.0	275.0	42.5

[a] Reported as nanomoles of oxygen consumed per minute, normalized to an optical density of 1.0 at 425 nm. 2,4-DCP, 2,4-Dichlorophenol.

tivities analogous to those of enzymes from other organisms which have been demonstrated to remove TCE. The two JMP134 monooxygenases are the phenol hydroxylase encoded on the chromosome and the 2,4-dichlorophenol hydroxylase encoded as part of the 2,4-D pathway on plasmid pJP4. The response of each pathway to induction and aromatic-substrate activation was unique, indicating the absence of shared regulation or common substrates (Table 1). 2,4-D-induced JMP134(pJP4) exhibited substrate-dependent oxygen responses to 2,4-D and 2,4-dichlorophenol but not to phenol. Phenol-induced AEO106 (plasmid-cured JMP134) exhibited a positive response to phenol but no response to 2,4-D or 2,4-dichlorophenol.

The results of direct measurement of TCE removal are shown in Fig. 1. The initial rate of removal (0.2 nmol per min per mg of protein) for phenol-induced AEO106 is comparable to rates at similar concentrations obtained for *Pseudomonas putida* F1 (17). Phenol-induced AEO106 removed TCE to below detectable levels at a rate higher than that observed for 2,4-D-induced JMP134. 2,4-D-induced JMP134 removed about 60% of the TCE, with apparent cessation of activity at a rate approximately one-third that of the phenol-dependent pathway. This is consistent with the results we obtained in initial batch culture experiments analyzed by headspace GC analysis, in which only 40% of the TCE was consistently removed. Similar observations (17, 18) have been attributed to the action of a toxic intermediate which inactivates the monooxygenase responsible for activity. Others have reported complete removal of TCE as we observed for the phenol-induced chromosomally encoded enzyme (8, 9).

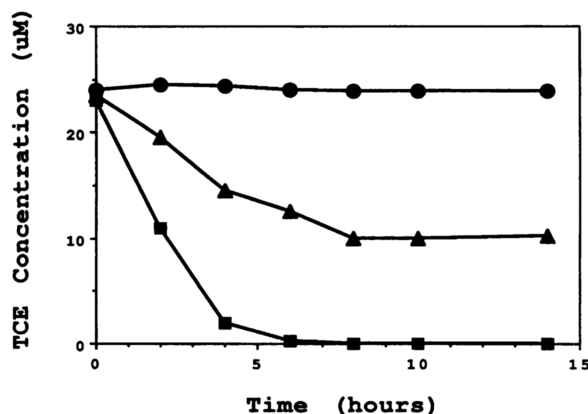

FIG. 1. TCE removal by aromatic-induced *A. eutrophus*, determined by pentane extraction. Symbols: ■, phenol-induced AEO106; ▲, 2,4-D-induced JMP134; ●, Casamino Acids-grown JMP134 (control).

The evidence presented here indicates that the phenol and 2,4-D metabolic pathways in *A. eutrophus* JMP134 are capable of TCE degradation. It is unclear which enzymes in these pathways effect the degradation of TCE, although the phenol hydroxylase and dichlorophenol hydroxylase are likely candidates. Both enzymes are monooxygenases which hydroxylate a substituted aromatic ring. Although the two hydroxylase enzymes differ in many respects from other enzymes already implicated in TCE degradation (e.g., toluene dioxygenase, toluene monooxygenase, and methane monooxygenase), Spain et al. (11) have recently demonstrated hydroxylation of phenol by toluene dioxygenase, suggesting that this alternative mechanism may be operating on TCE. Shields et al. (10) have demonstrated that toluene catabolism in bacterium G4 involves the hydroxylation of toluene to cresols. Winter et al. (20) implicated a similar toluene monooxygenase (converting toluene to *p*-cresol) in the toluene-dependent degradation of TCE in *Pseudomonas mendocina*.

In other experiments in our laboratory, strains of both *Pseudomonas aeruginosa* and *Escherichia coli* expressing activity of the cloned *tfdA* gene (6), which encodes the 2,4-D monooxygenase, were ineffective in TCE removal (data not shown). The 2,4-D monooxygenase is clearly not the agent of TCE mineralization.

The involvement of two distinct enzymes is indicated by TCE removal in the absence of the plasmid (strain AEO106) and by work indicating that induction by 2,4-D did not induce any phenol-related activities. In addition, the two activities exhibited different rates of degradation, with particular differences apparent in the ability to remove all or only a portion of the TCE present. Wackett and Gibson (17) observed similar incomplete removal and hypothesized that the diminution in biodegradation rate over time was due to the formation of toxic intermediates. Wackett and Householder (18) have recently demonstrated the covalent modification of cellular molecules in *P. putida* exposed to labeled TCE. Toluene dioxygenase mutants exhibited neither growth inhibition nor alkylation of cellular molecules. Similar mechanisms may be operating during TCE mineralization by the plasmid-encoded enzyme in *A. eutrophus*. The phenol-dependent pathway did not exhibit such sensitivity. Either an entirely different mechanism of TCE degradation which does not produce toxic intermediates is operational, or the enzyme system is not affected by those intermediates.

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