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Induction of Toluene Oxidation Activity in *Pseudomonas mendocina* KR1 and *Pseudomonas* sp. Strain ENVPC5 by Chlorinated Solvents and Alkanes

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Toluene oxidation activity in *Pseudomonas mendocina* KR1 and *Pseudomonas* sp. strain ENVPC5 was induced by trichloroethylene (TCE), and induction was followed by the degradation of TCE. Higher levels of toluene oxidation activity were achieved in the presence of a supplemental growth substrate such as glutamate, with levels of activity of up to 86% of that observed with toluene-induced cells. Activity in *P. mendocina* KR1 was also induced by *cis*-1,2-dichloroethylene, perchloroethylene, chloroethane, hexane, pentane, and octane, but not by *trans*-1,2-dichloroethylene. Toluene oxidation was not induced by TCE in *Burkholderia* (*Pseudomonas*) *cepacia* G4, *P. putida* F1, *Pseudomonas* sp. strain ENV110, or *Pseudomonas* sp. strain ENV113.

Chlorinated solvents such as trichloroethylene (TCE), *cis*- and *trans*-1,2-dichloroethylene (*c*- and *t*-DCE, respectively), and vinyl chloride are some of the most abundant and problematic contaminants of groundwaters in the United States (19). Although these compounds are relatively soluble in water, they adsorb to aquifer sediments (2, 3), making them difficult to remove by simply pumping and treating the groundwater. It has been estimated that it will require many years or decades to completely treat some moderately contaminated aquifers by using pump-and-treat technology (11, 12). This limitation has led to an evaluation of alternative treatment strategies that can destroy the contaminants in place, such as in situ biological remediation.

Although many bacteria are known to aerobically degrade chlorinated solvents (1, 5, 10, 13, 15–18), none has been demonstrated to use them as a sole source of carbon and energy. Biological degradation reportedly occurs via cometabolic mechanisms whereby enzymes that have evolved to degrade other substrates fortuitously destroy the chlorinated solvent. Furthermore, the natural enzyme substrate (e.g., toluene or phenol) is normally required for induction of the degradative genes and production of the degradative enzymes. Only recently has it been observed that TCE-degrading activity (toluene dioxygenase) can be induced by TCE (8). In this study we evaluated the induction of chlorinated solvent-degrading activity by chlorinated solvents and alkanes in several known TCE-degrading organisms, as well as three toluene-oxidizing strains isolated by enrichment culturing, to evaluate the potential for aerobic in situ remediation of chlorinated solvents in the absence of toxic aromatic cosubstrates.

Bacterial strains. The strains evaluated in this study included *Pseudomonas mendocina* KR1 (20), *Burkholderia (Pseudomonas) cepacia* G4 (13), *P. putida* F1 (18), *Pseudomonas* sp. strain ENVPC5, *Pseudomonas* sp. strain ENV110, and *Pseudomonas* sp. strain ENV113. *Pseudomonas* sp. strains ENVPC5, ENV110, and ENV113 were isolated from sequential enrichment cultures with toluene as an initial carbon source followed by enrichment with either 20 mM *p*-cresol or 20 mM *o*-cresol. ENVPC5 is capable of growth on *p*-cresol, and ENV110 and ENV113 are able to grow on *o*-cresol as a sole carbon source. Each strain was determined to be a *Pseudomonas* sp. by fatty acid analysis (Acculabs Inc., Newark, Del.).

Assay for measuring induction and toluene oxidation activity. Toluene oxidation activity was measured by a rapid [^{14}C]toluene assay adapted from the method of Ensley et al. (6) that measures the formation of nonvolatile oxidation products from [^{14}C]toluene. The assay was performed by suspending cells in basal salts medium (BSM) (7) to an optical density at 550 nm of 0.5. A 0.5-ml subsample of cells was added to a 15-ml glass test tube, and the reactions were initiated by adding 0.1 μCi of ring-labeled [^{14}C]toluene (1.8 μmol, 56 mCi/mmol, ≥99% purity; DuPont New England Nuclear, Boston, Mass.) in 5 μl of dimethyl formamide (DMF). The reaction mixture was incubated at 25°C for exactly 5 min, and the reaction was terminated by spreading 20 μl of the cell suspension evenly onto a thin-layer chromatography sheet (1.5 by 2.0 cm, PE SIL G/UV [catalog no. 4410 222]; Whatman Ltd., Kent, England). Volatile species were removed by blowing air (25°C) over the chromatography sheets for exactly 20 min, and the activity of the nonvolatile ^{14}C remaining on the silica gel was measured by liquid scintillation counting. One unit of activity was defined as the amount required to convert 1 nmol of toluene per min per mg of total cell protein to nonvolatile products under the exact conditions described above. Protein concentrations in the reaction mixtures were determined with a bicinchoninic acid protein assay system (Pierce, Rockford, Ill.) with bovine serum albumin as a standard.

To measure the induction of toluene oxidation activity by chlorinated solvents, stock solutions of the solvents (>97% purity) were prepared in DMF. Cells were prepared by growing them overnight to early stationary phase in BSM containing 0.4% sodium glutamate (0.4%). Cells were washed with BSM and suspended in BSM to a final cell density (optical density) of 1.0 at 550 nm. Aliquots (5 or 10 ml) of the cell suspension were added to 20-ml serum vials (total volume, 27 ml), and the vials were sealed with Teflon-lined septa and crimp tops. Chlorinated solvents were added by injecting them in 5 or 10 μl of DMF through the septa to give an appropriate final concentration. Solvent concentrations were calculated on the basis of the amount of liquid in the vials, without regard to the partitioning of the solvent into the vapor phase.

Samples containing the potentially inducing substrates were shaken on a rotary shaker (100 rpm) at 25°C. Subsamples of

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TABLE 1. Induction of toluene oxidation activity in *Pseudomonas* sp. strain ENVPC5 and *P. mendocina* KR1

Strain and treatment	[^{14}C]toluene activity (U) at time (h)[a]:			
	1	3	4.5	6.5
ENVPC5				
2 μM TCE	0.1 (0.01)	0.2 (0.01)	1.0 (0.1)	0.6 (0.07)
8 μM TCE	0.1 (0.02)	0.8 (0.03)	1.2 (0.03)	0.6 (0.04)
KR1				
2 μM TCE	0.1 (0.03)	0.2 (0.02)	1.2 (0.1)	0.5 (0.02)
8 μM TCE	0.1 (0.01)	1.2 (0.01)	1.1 (0.1)	0.7 (0.01)
8 μM TCE + glutamate[b]	4.3 (0.3)	10.0 (0.5)	15.4 (0.4)	6.3 (0.4)

[a] Cells were grown overnight in BSM plus 0.4% sodium glutamate. At 0 h, all cells showed 0 U of [^{14}C]toluene activity. For each strain, sets of cells receiving 10 μl of DMF and no TCE were analyzed; none of these cells showed [^{14}C]toluene activity at any time point assayed. Values represent means of three samples. Standard errors are shown in parentheses.

[b] In addition to TCE, 0.04% sodium glutamate was added as an energy source.

the cell suspension (250 μl) were removed at timed intervals, and [^{14}C]toluene assays were immediately performed. Cell suspensions receiving only DMF, or containing no cells or acid-killed cells, served as control samples. The levels of background activity in control samples were usually <100 dpm. Degradation of the substrate was measured by injecting samples of gas from the headspace onto a gas chromatograph equipped with an electron capture detector and comparing the response to a similarly prepared standard curve of substrate.

Induction of toluene oxidation genes by chlorinated solvents and alkanes. Toluene oxidation activity was induced by TCE in both *P. mendocina* KR1 and *Pseudomonas* sp. strain ENVPC5 (Table 1), but not in *B. cepacia* G4, *P. putida* F1, *Pseudomonas* sp. strain ENV110, or *Pseudomonas* sp. strain ENV113. In the presence of an added energy-generating cosubstrate such as glutamate, the level of induction of toluene oxidation activity reached 86% of that observed in toluene-induced cells (Tables 1 and 2). The induction of toluene oxidation activity was followed by the degradation of TCE, but TCE degradation often was not complete in the absence of an energy-generating cosubstrate (Table 3). Toluene oxidation activity in *P. mendocina* KR1 was also induced by *c*-DCE, PCE, and chloroethane (Table 2), but not by *t*-DCE, 1,2-dichloroethane, 1,1,1-trichloroethane, 1,1,2-trichloroethane, or carbon tetrachloride. Similar induction patterns were observed with *Pseudomonas* sp. strain ENVPC5 (data not shown). Toluene oxidation activity in *P. mendocina* KR1 was also induced during growth on alkanes (Table 4), but *Pseudomonas* sp. strain ENVPC5 was not capable of growth on these substrates.

TABLE 2. Induction of toluene oxidation activity in *P. mendocina* KR1

Substrate	[^{14}C]toluene activity (U) at time (h)[a]:	
	2	4
c-DCE	1.0 (0.09)	2.2 (0.1)
Perchloroethylene	0.4 (0.04)	1.4 (0.2)
Chloroethane[b]	0.9 (0.02)	2.3 (0.2)
TCE	7.0 (0.6)	16 (0.2)
Toluene	12 (0.2)	19 (0.4)

[a] Substrates were added in 5 μl of DMF to a final concentration of 20 μM. Each sample also contained 0.04% sodium glutamate. At 0 h, cells on all substrates showed 0 U of [^{14}C]toluene activity. Cells with DMF only and those with *t*-DCE also failed to show any activity at 2 and 4 h. Values represent means of three samples. Standard errors are shown in parentheses.

[b] Added as a gas (5 ml).

TABLE 3. TCE degradation by TCE-induced *Pseudomonas* sp. strain ENVPC5 and *P. mendocina* KR1

Strain and treatment	TCE concn (μM) at time (h)[a]:		
	3	4.5	6.5
ENVPC5			
8 μM TCE	5.1 (0.2)	5.1 (0.2)	5.1 (0.2)
KR1			
8 μM TCE	5.1 (0.5)	5.0 (0.1)	5.0 (0.2)
8 μM TCE + glutamate[b]	2.4 (0.08)	0.1 (0.1)	0

[a] At 0 h, the TCE concentration for all cells was 8.0 μM. Values represent means of three samples. Standard errors are shown in parentheses.

[b] Contained 0.04% sodium glutamate.

The results of this study demonstrate that chlorinated solvents can induce genes responsible for their degradation and that degradation activity can be enhanced in the presence of an additional energy-generating cosubstrate. Although we have not been able to conclusively demonstrate that the genes causing the observed toluene oxidation by *P. mendocina* KR1 are those for toluene-4-monooxygenase (T4MO) (21) we have evidence to suggest this relationship. We have constructed an insertion mutation in cloned T4MO genes and have used the mutated genes, via marker exchange, to generate a *tmo* mutant of *P. mendocina* KR1. The mutant no longer grows on toluene, and toluene oxidation activity is no longer induced by TCE. The strain, however, has retained its ability to grow on alkanes, but after growth on alkanes it does not have toluene oxidation activity (data not presented). Further studies are clearly needed to understand the mechanism of induction of toluene oxidation activity by chlorinated solvents in these strains and to understand the relationship between toluene oxidation and alkane degradation in *P. mendocina* KR1.

The activity observed in this study and in that of Heald and Jenkins (8) suggests a novel form of cometabolism where a cosubstrate (energy source) that is not a substrate for the degradative enzyme is required to support degradation, and the target substrate induces the degradative genes. For years it has been thought that TCE could not be aerobically degraded in the environment in the absence of inducing substrates that were also competitive substrates for the degradative enzyme. Efforts to remediate chlorinated solvent-contaminated aquifers by stimulating the indigenous bacteria have required the injection of methane (14) or phenol (9) or the presence of anaerobic conditions that promoted dehalogenation (4). Our results suggest that if the proper organisms and energy-generating growth substrates are present, aerobic natural attenuation of chlorinated solvents might occur. Furthermore, the observation that the T4MO genes of *P. mendocina* KR1 are

TABLE 4. Toluene oxidation activity in alkane-grown *P. mendocina* KR1[a]

Substrate	T4MO activity (U)	% Activity
Toluene	30 (0.05)	100
Hexane	19 (0.04)	63
Pentane	13 (0.04)	43

[a] Cells were grown overnight on either toluene, hexane, or pentane vapors or on 0.4% sodium glutamate. Sodium glutamate failed to induce T4MO activity. Values represent means of three samples. Standard errors are shown in parentheses.

transmissible and possibly maintained on a transposon (22) suggests that this activity may be naturally disseminated in the environment. Further molecular analysis of the toluene oxidation genes of *Pseudomonas* sp. strain ENVPC5 and analysis of other indigenous toluene-degrading bacteria may provide evidence of such gene dispersion and provide an assessment of the potential for aerobic intrinsic remediation of chlorinated solvent-contaminated environments.

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