APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1993, p. 2746–2749
0099-2240/93/082746-04$02.00/0

Vol. 59, No. 8

Degradation of Trichloroethylene by *Pseudomonas cepacia* G4 and the Constitutive Mutant Strain G4 5223 PR1 in Aquifer Microcosms

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***Pseudomonas cepacia* G4 degrades trichloroethylene (TCE) via a degradation pathway for aromatic compounds which is induced by substrates such as phenol and tryptophan. *P. cepacia* G4 5223 PR1 (PR1) is a Tn*5* insertion mutant which constitutively expresses the toluene *ortho*-monooxygenase responsible for TCE degradation. In groundwater microcosms, phenol-induced strain G4 and noninduced strain PR1 degraded TCE (20 and 50 μM) to nondetectable levels (<0.1 μM) within 24 h at densities of 10^8 cells per ml; at lower densities, degradation of TCE was not observed after 48 h. In aquifer sediment microcosms, TCE was reduced from 60 to <0.1 μM within 24 h at 5×10^8 PR1 organisms per g (wet weight) of sediment and from 60 to 26 μM over a period of 10 weeks at 5×10^7 PR1 organisms per g. Viable G4 and PR1 cells decreased from approximately 10^7 to 10^4 per g over the 10-week period.**

Trichloroethylene (TCE) is a common priority pollutant of groundwater in the United States (10), having toxic and possibly carcinogenic properties (9). Remediation of groundwater aquifers containing TCE requires extensive treatment of contaminated material and often relies on a pump and treat strategy that is inefficient for TCE removal (1, 16). In situ bioremediation is an alternative strategy by which degradation of contaminants can be enhanced either by stimulation of degradative processes in native microflora or by the addition of pollutant-degrading microorganisms (11, 20, 21). Although the efficiency of in situ bioremediation is relatively unproven, it is a potentially useful technique for remediating aquifers contaminated with TCE (7).

Various aerobic bacteria degrade TCE (2, 3, 5, 8, 9, 12 17–19) and can be considered potential in situ bioremediation agents. One bacterium, *Pseudomonas cepacia* G4 (12), cometabolically degrades TCE to CO_2 and nonvolatile products via a catabolic pathway that is induced by pathway substrates, including phenol. Phenol is a toxic compound, which may preclude its environmental use for induction of TCE degradation by strain G4. However, other means of using G4 in situ without a toxic inducer have been identified. Nelson and Borquin identified tryptophan as a nontoxic inducer of TCE-degrading activity in bacteria, including G4, that cometabolize TCE via catabolic pathways for aromatic compounds (7). In addition, Shields and Reagin (13) developed a stable, transposon Tn*5*-derived mutant of G4 that constitutively produces the toluene *ortho*-monooxygenase responsible for oxidation of TCE. This strain, *P. cepacia* G4 5223 PR1 (PR1), could potentially effect in situ degradation of TCE without the use of an inducer. In this report, degradation of TCE by phenol- and trytophan-induced G4 and by PR1 was assessed in groundwater and aquifer sediment microcosms.

Microorganisms and culture conditions. G4 and PR1 were routinely grown on M9 medium (6) containing lactate (20 mM) as the sole substrate. The medium for PR1 contained kanamycin sulfate (50 μg/ml); resistance to the antibiotic is encoded on the Tn*5* insertion element (13). A spontaneous rifampin-resistant mutant of G4 was isolated for use in survival experiments. G4 and PR1 were selectively enumerated on antibiotic medium 3 (Difco) containing rifampin or kanamycin (50 μg/ml), respectively. The aquifer microflora did not contain detectable densities of bacteria resistant to either rifampin or kanamycin.

Microcosms. Aquifer groundwater and sediment were obtained in July 1991 from site F347 of the U.S. Geological Survey Groundwater Contamination Study Site (Cape Cod, Mass.). Samples were taken 6.7 to 7.2 m below the surface, a depth located within a contaminant plume arising from sand beds receiving treated municipal sewage. Samples were stored at 4°C. The temperature of the aquifer was 11°C. The characteristics of the aquifer and the sampling procedures have been described elsewhere (4, 14).

Groundwater microcosms. Groundwater microcosms consisted of groundwater (20 ml) in 100-ml serum bottles. Degradation of TCE by introduced G4 and PR1 was measured in response to (i) various conditions of pathway induction in G4 and PR1 and (ii) different densities of introduced cells. Control microcosms did not receive bacterial inocula.

(i) **Pathway induction.** G4 was grown for 16 h in lactate medium with either phenol (2 mM) or tryptophan (4 mM) as the inducer. Concentrations of phenol exceeding 2 mM inhibit the growth of G4; phenol was therefore given to cells in two stages, with additional phenol (2 mM) added to the culture after 12 h of growth. PR1 was grown for 16 h in lactate medium and in M9 medium with phenol (2 mM) as the sole substrate. G4 and PR1 were collected by centrifugation, resuspended in groundwater, and added to microcosms at a density of 10^8 bacteria per ml.

(ii) **Bacterial density.** G4 and PR1 were grown to late log phase in M9 medium with phenol (2 mM) as the sole substrate. Cells were collected by centrifugation, resus-

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pended in groundwater, and added to microcosms at densities ranging from 10^6 to 10^9 bacteria per ml.

TCE (10 mM stock in methanol) was added to the microcosms to a concentration of either 20 or 50 μM, assuming that all TCE was present in the aqueous phase. Bottles were sealed with Teflon-lined silicon septa, inverted, and incubated (11°C) in the dark with shaking (50 rpm). At appropriate intervals, samples of groundwater (0.8 ml) were removed from previously unsampled microcosms to 1.8-ml crimp vials containing pentane (0.8 ml). The vials were shaken for 15 s, after which the pentane was removed to clean vials for gas chromatographic (GC) analysis. Dibromomethane (10 μM) was present in the pentane as an internal control. TCE concentrations are given as averages for either two or three microcosms.

Reported concentrations of TCE in groundwater were apparently lower than setup concentrations because of partitioning of TCE into the gaseous headspace of the serum bottles. However, TCE reenters groundwater from which it is depleted, and thus degradation was presumably complete when the concentration of TCE in the groundwater samples was below detection (<0.1 μM).

Sediment microcosms. Sediment microcosms consisted of aquifer sediment in 60-ml serum bottles in which (i) survival of G4 and (ii) survival of and degradation of TCE by PR1 were monitored.

(i) Survival of G4. G4 was grown to late log phase, collected by centrifugation, diluted in groundwater (5 ml), and mixed into microcosm sediment (20 g) to a density of 10^7 cells per g (dry weight) of sediment (dws). The microcosms were stoppered and incubated as described above. The bacteria were extracted at various times by adding buffer (20 ml of 0.1% $Na_4P_2O_7$ [pH 7.0]) to the microcosms and agitating them for 30 min; extracts were diluted in buffer and plated onto selective medium. Bacteria were enumerated as CFU per g dws. The detection limit with this method was 10^3 CFU per g dws.

(ii) Survival of PR1 and TCE degradation. Microcosms contained aquifer sediment (5 g), which had been autoclaved on 2 successive days, plus filter-sterilized groundwater (5 ml); PR1 was added at densities ranging from 10^6 to 10^8 cells per g (wet weight) of sediment (wws). TCE was added to a concentration of 50 μM, assuming that all TCE was present in the aqueous phase. Serum bottles were stoppered and incubated as described above. TCE concentrations were measured at various times by pentane extraction of the entire contents of randomly chosen microcosms. Microcosms and pentane (containing 10 μM dibromomethane) were initially cooled on ice for 15 min. Pentane (5.0 ml) was added to the microcosms, which were placed on ice and shaken (30 min, 75 rpm). Aliquots of pentane (1 ml) were removed to 1.8-ml crimp vials for GC analysis. The efficiency of extraction was predetermined to be 100% for TCE at various concentrations (5 to 100 μM) analyzed from quadruplicate microcosms. Control microcosms did not receive bacterial inocula. All data are given as averages ± SD for three microcosms.

Analysis of TCE concentrations. Pentane extracts were analyzed with a Hewlett Packard model 5890 GC equipped with an electron capture detector, an on-column injector, and a WCOT fused silica column (25 m; 0.32 mm inner diameter). Operating conditions were: electron capture detector, 325°C; oven, 40°C for 4 min, increase from 40 to 120°C at a rate of 30°C/min, and 120°C for 3 min. The injector temperature was 3°C above the oven temperature. Hydrogen was the carrier gas (40 cm/s). Pentane extracts (0.5 ml) were injected with an automatic sampler equipped with a cooling tray (5°C). TCE concentrations were calculated by an internal-standard method with the Hewlett Packard system.

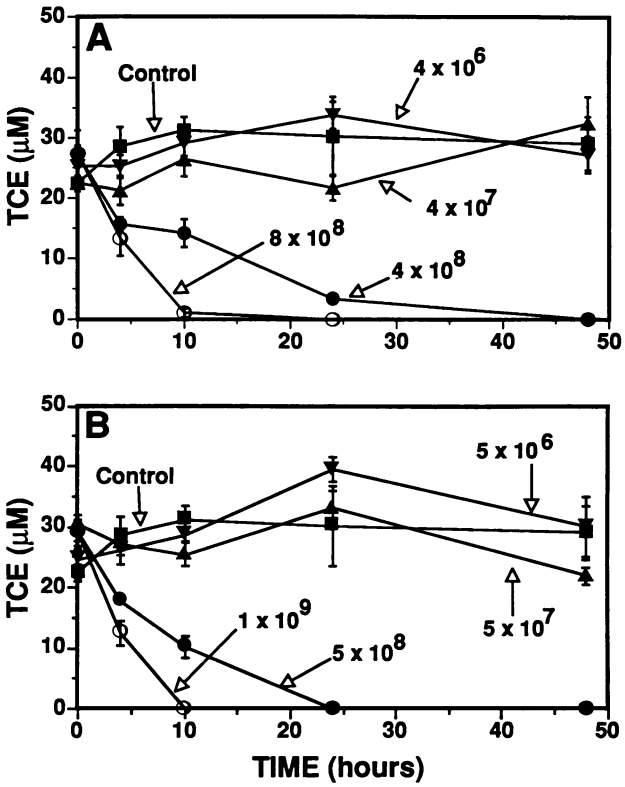

FIG. 1. Degradation of TCE by PR1 (A) and G4 (B) at different cell densities in groundwater microcosms. Inocula (cells per milliliter) are indicated. TCE concentrations are also given for uninoculated control microcosms (■). Values are averages ± SD for two or three microcosms.

Survival of G4 and PR1 in sediment microcosms. Viable G4 and PR1 cells were recovered from sediment microcosms 10 weeks after their introduction; bacterial densities decreased, respectively, from 10^7 to 10^4 CFU and from 4.0×10^6 to 1.5×10^3 CFU per g of sediment. Densities for G4 are from microcosms made with nonsterile sediment and ground water. Densities for PR1 are from microcosms which contained sterilized sediment and groundwater plus TCE (see below) so that removal of TCE could be attributed to the presence of PR1 alone. The efficiency of recovery at time zero was approximately 100% (e.g., recovery of PR1 = $6.1 \times 10^6 \pm 2.1 \times 10^5$).

Degradation of TCE in groundwater microcosms. TCE-degrading activity was induced in G4 with either phenol or tryptophan. Degradation was relatively faster in microcosms receiving phenol-induced G4 (10^8 cells per ml); the concentration of TCE was reduced from 6.5 to <0.1 μM within 24 h. In comparison, tryptophan-induced cells reduced the concentration of TCE from 8.5 to 5 μM in 5 days. PR1 (10^8 cells per ml), when grown on phenol or lactate, reduced the concentration of TCE from 25 to <0.1 μM in 9 h.

Comparative experiments demonstrated that relatively high densities of PR1 and G4 were necessary for significant degradation of TCE to be observed in groundwater microcosms. Approximately 10^9 cells of PR1 (Fig. 1A) and phenol-

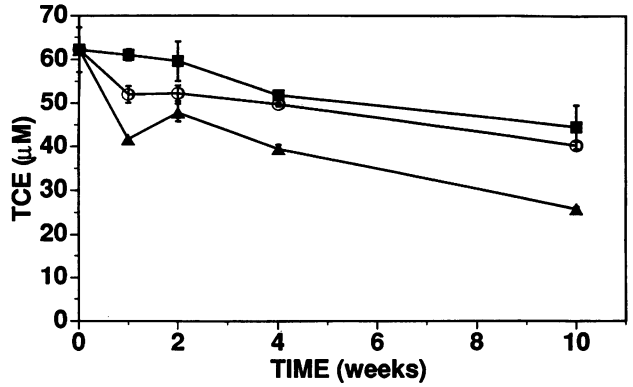

FIG. 2. Degradation of TCE by PR1 in sediment microcosms at densities of 4×10^6 (○) and 4×10^7 (▲) cells per g dws over a period of 10 weeks. TCE concentrations are also given for uninoculated control microcosms (■). Values are averages ± SD for two or three microcosms.

induced G4 (Fig. 1B) per ml reduced the observed concentration of TCE from approximately 30 to <0.1 μM within 10 h. In contrast, significant removal of TCE was not observed in microcosms inoculated with PR1 and G4 at densities of 5×10^7 or 5×10^6 cells per ml (Fig. 1A and B).

Degradation of TCE in sediment microcosms. The TCE degradation experiments in sediments were made with PR1 because of its ability to degrade TCE without the presence of an inducer, a trait deemed important for in situ applications. In sediment microcosms inoculated with 5×10^8 cells of PR1 per g wws, the concentration of TCE was reduced from 60 to <0.1 μM within 24 h, a reduction consistent with that observed in groundwater microcosms. At 5×10^7 cells of PR1 per g wws, degradation of TCE was markedly slower; after 5 days, the concentration of TCE was reduced from 60 to 50 μM. However, degradative activity occurred for an extended period of time, and by 10 weeks, the concentration of TCE was further reduced to 26 μM (Fig. 2). A lower density of PR1 (5×10^6 per g wws) did not significantly decrease the concentration of TCE from that observed in control microcosms.

Microorganisms which are introduced into contaminated environments to act as agents for in situ bioremediation will be required to survive and express their biodegradative capacities (15, 20). In this study, both G4 and PR1 survived introduction into groundwater microcosms and degraded TCE, which suggests that the bacteria may be useful agents for in situ bioremediation of TCE-contaminated aquifers. In addition to our observations, a recent report described degradation of TCE in a simulated aquifer by G4 with continuous application of tryptophan as an inducer (7). Although the results from this study are encouraging, if the native microflora of a contaminated aquifer degrades tryptophan, delivery of concentrations adequate for induction could be difficult and expensive. Also, oxygen is required as a cosubstrate for toluene *ortho*-monooxygenase (19). Thus, in polluted aquifers, where the oxygen supply is often limiting, a decrease in available oxygen caused by the degradation of tryptophan could inhibit oxidative degradation of TCE by G4.

Bioremediation of TCE-contaminated aquifers by an organism that degrades TCE without chemical induction is therefore an attractive alternative. It was noteworthy that PR1 degraded significant amounts of TCE in a relatively short time in groundwater and sediment microcosms without addition of nutrients and inducers. Removal of TCE equaled that obtained with phenol-induced G4 and was faster than with tryptophan-induced G4. It was also noteworthy that PR1 apparently degraded TCE for an extended period of some weeks in sediment microcosms.

We thank Malcolm S. Shields for providing strains G4 and PR1 and for reviewing the manuscript, Richard L. Smith for providing aquifer samples, and Sabine Witt for advice on TCE analyses.

This research was supported by the German Ministry of Research and Technology (BMFT Vorhaben 0319-433A) and the Fonds der Chemische Industrie.

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