APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1996, p. 825–833 Vol. 62, No. 3
0099-2240/96/$04.00+0

Comparison of Factors Influencing Trichloroethylene Degradation by Toluene-Oxidizing Bacteria

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Received 21 November 1995/Accepted 6 December 1995

The degradation of trichloroethylene (TCE) by toluene-oxidizing bacteria has been extensively studied, and yet the influence of environmental conditions and physiological characteristics of individual strains has received little attention. To consider these effects, the levels of TCE degradation by strains distinguishable on the basis of toluene and nitrate metabolism were compared under aerobic or hypoxic conditions in the presence and absence of nitrate and an exogenous electron donor, lactate. Under aerobic conditions with toluene-induced cells, strains expressing toluene dioxygenases (*Pseudomonas putida* F1, *Pseudomonas* sp. strain JS150, *Pseudomonas fluorescens* CFS215, and *Pseudomonas* sp. strain W31) degraded TCE at low rates, with less than 12% of the TCE removed in 18 h. In contrast, strains expressing toluene monooxygenases (*Burkholderia cepacia* G4, *Burkholderia pickettii* PKO1, and *Pseudomonas mendocina* KR1) degraded 36 to 67% of the TCE over the same period. Under hypoxic conditions (1.7 mg of dissolved oxygen per liter) or when lactate was added as an electron donor, the extent of TCE degradation by toluene-induced cells was generally lower. In the presence of lactate, degradation of TCE by denitrifying strain PKO1 was enhanced by nitrate under conditions in which dissimilatory nitrate reduction was observed. The results of experiments performed with strains F1, G4, PKO1, and KR1 suggested that TCE or an oxidation product induces toluene degradation and that TCE induces its own degradation in the monooxygenase strains. The role of TCE as an inducer of toluene oxygenase activity in PKO1 was confirmed by performing a promoter probe analysis, in which we found that TCE activates transcription from the PKO1 3-monooxygenase operon promoter.

Trichloroethylene (TCE), a suspected carcinogen (43) and U.S. Environmental Protection Agency priority pollutant (60), is the most commonly reported volatile organic contaminant of groundwater (51). TCE can be degraded by means of a cooxidation reaction which has been shown to be catalyzed by both toluene dioxygenase (9, 21, 68) and monooxygenase (29, 55, 66) enzyme systems found in toluene-oxidizing bacteria. Linkage of TCE degradation with the metabolism of aromatic hydrocarbons has been established for indigenous microbial populations in soil (11, 13, 42) and groundwater (22–24), in which TCE removal has been shown to be stimulated by the addition of toluene or phenol. Workers have proposed strategies for bioremediation of TCE-contaminated aquifers which involve the addition of these compounds to induce aromatic pathways in toluene- or phenol-utilizing bacteria (22–24) and possibly the direct introduction of specific strains, such as *Pseudomonas* (*Burkholderia*) *cepacia* G4 (44), or constitutive TCE degraders, such as the strain G4 derivative strain PR1 (30, 56).

Oxygen is required for aerobic cooxidation of TCE by bacteria, both as a cosubstrate for the oxygenase and as a terminal electron acceptor for cellular respiration. An exogenous electron donor, typically a carbon source which can serve as the primary growth substrate and as an inducer, is also needed to supply NAD(P)H for the oxygenase reaction (7, 10). The addition of a readily utilizable carbon source to TCE-contaminated groundwater in conjunction with bioremediation efforts would, however, be expected to result in the depletion of dissolved oxygen (30), a phenomenon reported by Hopkins and co-workers (22–24) for both phenol- and toluene-amended groundwater microcosms and for shallow aquifers injected with phenol. Rates of TCE degradation may become limited by insufficient oxygen, as has been shown for other organic pollutants (2, 15, 32). The results of a study of Fan and Scow (11) show that TCE degradation in soil solutions containing toluene was limited by oxygen availability. Although TCE degradation in situ can be maintained by the addition of oxygen to groundwater (22–24), pure oxygen is both expensive and hazardous to use, and sparging with air can yield a maximum of only 8 to 12 mg of dissolved oxygen per liter (32). Hydrogen peroxide has been shown to be an effective source of oxygen for TCE biodegradation, but limitations to its use include its cost, its toxicity to microorganisms, and the potential for blockage of soil pores, which can occur as a result of peroxide decomposition or gas bubble formation (22, 41).

The use of nitrate as a supplementary electron acceptor is attractive because the aqueous solubility of nitrate (660 g/liter) is higher than that of oxygen (3). Under hypoxic conditions (i.e., conditions in which oxygen is present but at low concentrations) nitrate can conceivably serve as an alternative electron acceptor for the degradation of TCE by denitrifying, toluene-oxidizing bacteria that use other carbon sources as electron donors. To our knowledge, however, this has not been confirmed experimentally in any previously published report. On the other hand, there is evidence which indicates that denitrifying bacteria degrade hydrocarbons in groundwater under hypoxic conditions (1, 4, 14, 25, 34, 39), which is the basis for nitrate-based biorestoration of contaminated aquifers (26). In addition, we have recently described evidence for the coupling of hydrocarbon degradation with denitrification by pure cultures (49). The denitrifying, toluene-oxidizing bacteria *Pseudomonas* (*Burkholderia*) *pickettii*, *Pseudomonas* sp. strain W31, and *Pseudomonas fluorescens* CFS215 have been shown to degrade two to seven times more toluene in the presence of nitrate than the previously well-characterized toluene-oxidiz-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
P. putida F1	Strict aerobe; toluene 2,3-dioxygenase pathway[b]	D. T. Gibson
B. cepacia G4	Nitrate respirer; toluene 2-monooxygenase pathway[c]	P. H. Pritchard
Pseudomonas sp. strain JS150	Nitrate respirer; toluene 2,3-dioxygenase pathway[d]	J. C. Spain
B. pickettii PKO1	Denitrifier; toluene 3-monooxygenase pathway[e]	17
P. mendocina KR1	Denitrifier; toluene 4-monooxygenase pathway[f]	D. T. Gibson
Pseudomonas sp. strain W31	Denitrifier; toluene 2,3-dioxygenase pathway[g]	37
P. fluorescens CFS215	Denitrifier; toluene 2,3-dioxygenase pathway[h]	36
Plasmids		
pKRZ1	Ap^r Km^r; broad-host-range promoter probe vector containing *lacZ* as a reporter gene	A. M. Chakrabarty
pKRZ1::352-bp *Xho*I/*Sma*I	Ap^r Km^r; PKO1 3-monooxygenase (*tbuA1UBVA2C*) operon promoter fragment controlling *lacZ* on pKRZ1	6

[a] Nitrate respirers are defined as strains which reduce nitrate to nitrite but do not denitrify.
[b] Data from references 16 and 67.
[c] Data from reference 54.
[d] Data from reference 19. Genes encoding toluene monooxygenases have been cloned from *Pseudomonas* sp. strain JS150 (27), but preliminary evidence indicates that these genes are not induced by toluene in the native strain (28).
[e] Data from reference 48.
[f] Data from reference 65.
[g] Data from reference 37.
[h] Data from reference 38.

ing strains *Pseudomonas putida* F1, *Pseudomonas* sp. strain JS150, and *Pseudomonas* (*Burkholderia*) *cepacia* G4, which are nondenitrifiers.

The purpose of this study was to compare these strains and another denitrifying strain, *Pseudomonas mendocina* KR1, for the ability to degrade TCE under aerobic and hypoxic conditions in the presence and absence of an electron donor, lactate, and a supplementary electron acceptor, nitrate. TCE degradation by *P. putida* F1 (63), *Pseudomonas* sp. strain JS150 (18), *B. cepacia* G4 (12, 45), *B. pickettii* PKO1 (29), and *P. mendocina* KR1 (66) has been described previously, but TCE degradation has not been studied previously for *Pseudomonas* sp. strain W31 or *P. fluorescens* CFS215. Moreover, with the exception of one investigation by Nelson et al. (46) performed with low concentrations of TCE (<3 μM), the relative TCE-degrading abilities of toluene-oxidizing bacteria have not been directly compared.

An additional objective of this study was to confirm and expand upon the recent findings that Heald and Jenkins (21) obtained with *P. putida* and that McClay et al. (35) obtained with *P. mendocina* KR1 and *Pseudomonas* sp. strain ENVPC5 which showed that TCE can act as an inducer of toluene-oxidizing activity. In this study, we obtained evidence that TCE or its products induce toluene-degradative activity in strains which degrade toluene by each of the four toluene ring hydroxylation pathways and also show that *B. cepacia* G4, *B. pickettii* PKO1, and *P. mendocina* KR1 are able to degrade TCE when they are grown in the presence of TCE but in the absence of aromatic inducers.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains and plasmids used in this study are listed in Table 1. All strains were routinely grown in a basal salts medium (BM), which was modified from media formulated by Stanier et al. (59) and Page and Sadoff (50) and contained (per liter of distilled water) 2.49 g of Na_2HPO_4, 3.05 g of KH_2PO_4, 0.20 g of $MgSO_4$, 0.05 mg of $FeSO_4$, 0.25 mg of $NaMoO_4$, 1.0 g of $(NH_4)_2SO_4$, 1.0 g of KNO_3, and 20 ml of Hutner's stock salts solution (8). Cobalt sulfate was substituted for cobalt nitrate as a component of the stock salts solution. Nitrate-free BM was prepared in the same way, except that KNO_3 was omitted from the medium. Plate counts were done on TNA plates (47) as described previously (39).

Cells were typically grown from an initial optical density at 425 nm (OD_{425}) of 0.1 in 500-ml Erlenmeyer flasks containing 100 ml of BM amended with either sodium DL-lactate (17.8 mM), lactate and toluene (2.35 mM), or lactate and TCE (2.35 mM), depending on the experiment. Cultures were incubated for 18 h at 27°C with rotary shaking (250 rpm). Cells were harvested by centrifugation and resuspended in potassium phosphate buffer (40 mM, pH 6.8) to 1/10th the original volume.

Toxicity experiments. The toxicity of TCE to toluene-oxidizing bacteria, as measured by inhibition of the growth rate, was studied by using methods modified from those described by Wackett and Householder (64). Cells grown on lactate and toluene were used to inoculate 500-ml Erlenmeyer flasks containing 100 ml of BM amended with 17.8 mM sodium DL-lactate, with TCE supplied in the vapor phase. Parallel cultures that lacked TCE served as controls. Cultures were incubated at 27°C with shaking at 250 rpm, and growth was measured by determining the OD_{425} at hourly intervals for 8 h. The calculated liquid phase TCE concentration (approximately 40 μM) was maintained by replacing the TCE that was presumed lost from the culture headspace during sampling. The mean growth rate was calculated from the exponential phases of the growth curves generated from duplicate cultures.

Measurement of TCE degradation. TCE degradation was assayed in 160-ml serum bottles that were crimp sealed with Teflon-lined butyl rubber stoppers. Cells were resuspended to an OD_{425} of 0.05 (approximately 1×10^8 to 2×10^8 CFU/ml) in 150 ml of BM. Sodium DL-lactate was added as an electron donor to a concentration of 17.8 mM when appropriate. For experiments conducted under hypoxic conditions, serum bottles containing BM were sparged with an anaerobic gas mixture (85% nitrogen, 10% hydrogen, 5% carbon dioxide) for approximately 3 min to achieve an initial dissolved oxygen content of approximately 23% oxygen saturation (1.7 mg/liter). TCE (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added from a 1/20 stock solution in *N'N'*-dimethylformamide so that the final nominal concentration was 40 μM. Experiments were conducted at 27°C. Samples for dissolved oxygen and TCE content determinations were removed from the solutions at 0, 4, and 18 h. The samples were removed under positive pressure (argon gas) with a 22-gauge hypodermic needle fitted to Tygon tubing. The dissolved oxygen content was measured in-line by using a model FL600 flow cell (Instech Laboratories, Plymouth Meeting, Pa.) and a Clark style polarographic electrode (model 125/05; Instech). The 3-ml samples used for TCE analysis were removed with a 5-ml syringe and extracted with 1 ml of pentane. The percentage of TCE degradation was calculated from the mean TCE concentrations in two or three serum bottles by comparison with the mean concentrations in an equal number of uninoculated controls, in which the amount of TCE lost never exceeded 5%. The statistical significance of TCE degradation was assessed on the basis of the differences between the solution TCE concentrations in inoculated and uninoculated serum bottles. The increase in headspace volume associated with sampling had no significant effect (level of significance, $\alpha > 0.4$) on the concentration of TCE in solution, as judged by the loss of TCE in abiotic controls (2.32% ± 3.46%). Accordingly, we did not correct for this effect when we calculated the extent of TCE degradation in these experiments.

With the exception of strain F1, the TCE degradation rate assays were performed by using procedures identical to those described above, except that the

TABLE 2. Inhibition of growth of toluene-oxidizing bacteria grown on sodium lactate (0.2%) by TCE (nominal concentration, 40 μM)

Strain	Growth rate (h^{-1}) on:		% Inhibition by TCE
	Lactate	Lactate + TCE	
Monooxygenase strains			
G4	0.17	0.14	20.8
PKO1	0.17	0.13	22.8
KR1	0.42	0.33	23.5
Dioxygenase strains			
JS150	0.26	0.23	10.9
F1	0.42	0.36	12.7
CFS215	0.40	0.34	14.1
W31	0.36	0.30	15.4

sampling method and intervals differed and dissolved oxygen concentrations were not measured. Samples (1 ml) were withdrawn through a 16-gauge needle by using a 1-ml syringe fitted with a 22-gauge needle. Samples were removed at 15-min intervals for 2 h and extracted with 0.33 ml of pentane. The rates of degradation in duplicate serum bottles were averaged by performing a regression analysis of the linear portion of the progress curve data. The TCE losses due to sampling (1.92% ± 3.79%) were insignificant ($\alpha > 0.5$) and were neglected for the purposes of rate calculations. For strain F1, TCE degradation rate assays were done in the same way, except that cells were suspended to an OD_{425} of 0.5 rather than 0.05.

Measurement of toluene degradation. In general, assays of toluene-degradative activity were performed by using the procedure described above for TCE assays, except that 1-ml samples were removed at 1-min intervals for 10 min and mixed with 0.5-ml portions of methanol for quantitation by high-performance liquid chromatography (HPLC). Experiments were initiated by adding toluene as a 0.5% stock solution in *N′N′*-dimethylformamide to yield an initial solution concentration of 100 μM. The rates of degradation in duplicate serum bottles were averaged by performing a linear regression analysis of progress curve data. The degradation rates determined by this method did not differ significantly ($\alpha > 0.4$) from the rates measured by using a vial assay, in which toluene disappearance was monitored in 10-ml cell suspensions in 14.3-ml vials shaken at 250 rpm (unpublished data). Our results demonstrated the validity of the bottle assay and confirmed that mass transfer limitations were not encountered with this method.

Gas chromatography. The amounts of TCE in pentane extracts were determined by using a model GC-8A gas chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with a type Rtx-624 fused silica capillary column (length, 30 m; inside diameter, 0.53 mm; thickness of stationary phase, 3.0 μm; Restek Corp., Bellefonte, Pa.) and a flame ionization detector. The operating conditions were as follows: sample volume, 1 μl; carrier gas, nitrogen; injector temperature, 170°C; column temperature, 65°C; and detector temperature, 170°C. Dibromomethane was added to pentane as an internal standard at a final concentration of 150 μM. The detection limit for TCE was 2.4 μM when the methods described above were used.

β-Galactosidase assays. The induction of β-galactosidase expression in cells containing pKRZ1-based (52) promoter-probe plasmids was measured by using the assay method of Miller (40). *B. pickettii* PKO1 containing either the pKRZ1::352-bp *Xho*I/*Sma*I construction or pKRZ1 as a control was grown under the conditions described previously in BM amended with kanamycin sulfate (100 μg/ml; Sigma Chemical Co., St. Louis, Mo.) and either sodium DL-lactate (17.8 mM), lactate plus toluene (2.35 mM), or lactate plus TCE (2.35 mM). The average β-galactosidase activities were determined from the results of two independent experiments performed with triplicate 0.5-ml aliquots of cells.

Other analytical methods. The amount of toluene was determined by HPLC by using methods described previously (36). Cellular protein was measured by using a protein assay kit (Sigma Chemical Co.) following alkaline digestion of cell pellets at 90°C (20). The production of nitrite was tested by the method of Smibert and Krieg (method 2) (57).

Statistical methods. A statistical analysis of the data was performed by using the SAS System for Personal Computers, release 6.04 (53). An analysis of variance was done by the PROC GLM procedure. Specific differences among the data were identified by performing a post hoc comparison with the Student-Newman-Keuls test for equal sample sizes or the Tukey-Kramer test for unequal sample sizes (58). The level of significance used was $\alpha = 0.05$ except where indicated otherwise.

RESULTS

Bacteria which cooxidize TCE when their toluene metabolism pathway is induced were tested with respect to several physiological parameters, including susceptibility to TCE toxicity, TCE degradation, oxygen consumption, toluene-degradative activity, growth, dissimilatory nitrate reduction, and, in the case of *B. pickettii* PKO1, transcription from the promoter which controls the expression of the toluene 3-monooxygenase. Strain characteristics relevant to this study are summarized in Table 1. Depending on the experiment, cells were grown in either the presence or the absence of specific inducing compounds (toluene, TCE). The experimental conditions used were variable, reflecting the initial concentration of dissolved oxygen and the presence or absence of an electron donor, lactate, and a supplementary electron acceptor, nitrate.

Toxicity experiments. Other workers have shown previously (64) that TCE may be inhibitory to the growth and metabolism of bacterial strains capable of TCE degradation. Accordingly, before we compared other environmental factors, we determined the sensitivity of both toluene dioxygenase and monooxygenase strains to growth inhibition by TCE. The results of these experiments are shown in Table 2. We observed significant inhibition with all of the strains studied. Strains G4, PKO1, and KR1 were inhibited by TCE more than dioxygenase strains F1, JS150, CFS215, and W31 were. Moreover, the percentages of inhibition did not vary greatly among the monooxygenase strains (20 to 23%) or among the dioxygenase strains (11 to 14%). The identification of these two groups which correlated with enzyme mechanisms suggested to us that if growth inhibition was associated with the rate and extent of TCE transformation, then either greater or less TCE degradation by the members of one of the groups may result in greater or less cytotoxicity and growth inhibition.

TCE degradation by toluene-induced cells. The levels of TCE degradation by bacteria induced by prior growth with toluene were compared to ascertain the extents of variation which occur among strains and between the two enzyme mechanisms (Fig. 1). When these experiments were done under aerobic conditions, the results (Fig. 1A) clearly showed that there was more TCE transformation by monooxygenase strains G4, PKO1, and KR1 than by the dioxygenase strains. The amounts of TCE removed by the dioxygenase strains in 18 h were less than 12%, whereas the levels of degradation after 4 and 18 h by monooxygenase strains ranged from 19 to 23% and from 36 to 67%, respectively. The levels of TCE degradation by monooxygenase strains G4, PKO1, and KR1 were not significantly different in the first 4 h, but varied greatly after 18 h, with G4 degrading the most TCE, followed by PKO1 and KR1. KR1 exhibited a decline in the apparent rate of degradation of TCE over time, degrading 19% of the TCE in the first 4 h but only an additional 16% over the next 14 h. Oxygen consumption by the strains during the sampling interval varied independently of TCE transformation (Fig. 1B), but the measured dissolved oxygen concentrations exceeded 35% of saturation in all cases after 18 h of incubation.

Since many subsurface environments are hypoxic (i.e., have low oxygen contents), we considered the possibility that strains may also vary in TCE-degrading activity as a function of the avidity of relevant enzymes for oxygen, a cosubstrate. This possibility was supported in part by our previous observation that the K_m values for another oxygen-requiring step in toluene degradation (catechol dioxygenase) were significantly lower for some toluene-degrading strains than for others (31). In this study we performed experiments as described above, but the initial dissolved oxygen content was 23% of saturation. The results of these experiments (Fig. 1C) revealed similar relationships among the strains studied, but in all cases the amount of TCE degraded was less than the amount degraded when more oxygen was present. The oxygen was never completely

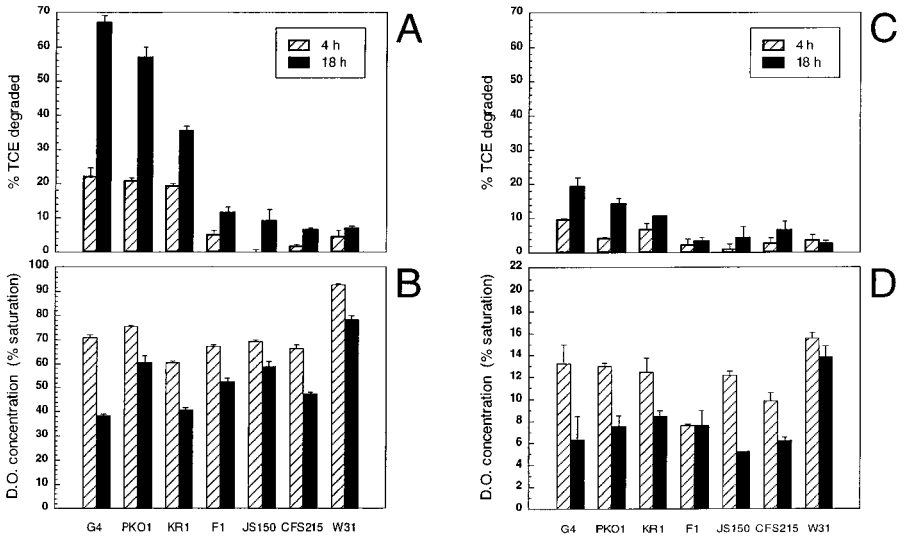

FIG. 1. TCE degradation (A and C) and oxygen consumption (B and D) by toluene-oxidizing bacteria under initially aerobic (A and B) and hypoxic (C and D) conditions in the absence of an exogenous electron donor. Cells were grown on BM containing lactate and toluene, washed, and resuspended as described in the text. TCE, added at an initial nominal concentration of 40 μM, was extracted with pentane, and the amount of TCE was determined by gas chromatography. Dissolved oxygen (D.O.) was measured with a polarographic electrode. The error bars represent the standard deviations for three samples.

depleted in these experiments, and the concentrations remained above 5% of saturation in all cases (Fig. 1D).

Toluene- and TCE-degradative activities of toluene-induced cells. In view of our observations which suggested that there are distinct differences in the abilities of toluene mono- and dioxygenase strains to degrade TCE, we sought to clarify the extent to which these differences were manifested in rates of TCE degradation by using strains G4, PKO1, KR1, and F1 as representative strains for comparison. In these experiments, TCE degradation was measured at 15-min intervals for 2 h. Following a lag period of 0 to 45 min for the monooxygenase strains and 1 h for strain F1, TCE was degraded at essentially a linear rate ($r = 0.8548$ to 0.9851). The rates of TCE degradation (Table 3) varied approximately proportionally with the extents of TCE degradation by these strains (Fig. 1A); i.e., the rates were significantly higher for the monooxygenase strains than for strain F1.

In order to compare rates of TCE degradation with rates of toluene degradation in the same strains and to determine whether toluene-degradative activity was maintained during the 18-h incubation period, the toluene-degradative activities of the four representative strains (i.e., strains F1, G4, PKO1, and KR1) were measured at zero time and after 18 h in the presence and absence of TCE. Our results (Table 3) confirmed that toluene-degradative activity was induced in all strains, but also showed that the rates of TCE degradation were not correlated with the rates of toluene degradation. Strains G4 and PKO1 degraded TCE at a higher rate and to a greater extent than strain F1 but degraded toluene at rates several times lower than the strain F1 rate. Moreover, strain KR1, which degraded TCE at a rate similar to that of PKO1, degraded toluene at a rate more than six times higher than the strain PKO1 rate. These results also show that toluene-degradative activity was maintained in F1, G4, and PKO1 throughout the course of the 18-h incubation and that toluene-degradative activity was not diminished because of the presence of TCE during this period. Strain KR1, in contrast, exhibited nearly a complete loss of activity in the absence of TCE. The activity of KR1 was maintained in the presence of TCE, although it declined by approximately 50% during the period of incubation.

Influence of an electron donor and electron acceptors on TCE degradation under aerobic conditions. In the experiments described above, resting cell suspensions were used. No exogenous electron donors were added. Accordingly, we were interested in what possible effects the presence of electron donors and acceptors might have on TCE degradation. For these experiments we used the strains which displayed the highest TCE degradation rates, strains G4, PKO1, and KR1. Initially, cells induced with toluene during prior growth were compared for their abilities to degrade TCE in the presence of an electron donor, lactate (17.8 mM). Under initially aerobic conditions and in the presence of nitrate (Fig. 2A), degradation of TCE by all three strains was significant, but the extents of

TABLE 3. Specific TCE- and toluene-degradative activities of toluene-induced F1, G4, PKO1, and KR1 cells and the effect on toluene degradation of incubating cells for 18 h in the presence and absence of TCE

Strain	Specific degradative activity (nmol/mg/min)[a]			
	Toluene[b]			TCE[c]
	Initial	After 18 h of incubation		
		In the absence of TCE[d]	In the presence of TCE[d]	
F1	65.2 ± 0.9	66.1 ± 5.3	67.0 ± 3.5	0.5 ± 0.1
G4	7.6 ± 0.0	6.9 ± 0.0	8.7 ± 0.5	3.0 ± 0.3
PKO1	11.3 ± 1.4	10.6 ± 0.3	12.3 ± 0.5	2.4 ± 0.2
KR1	71.2 ± 3.2	3.3 ± 2.3	31.8 ± 1.2	2.4 ± 0.1

[a] Mean ± standard deviation.
[b] Rates of toluene degradation were determined over a period of 10 min by using procedures described in Materials and Methods.
[c] Rates of TCE degradation were determined over a period of 2 h by using procedures described in Materials and Methods.
[d] Serum bottles were sparged with air for 20 min immediately prior to the initiation of toluene degradation assays in order to reestablish fully aerobic conditions and to remove residual TCE and volatile TCE oxidation products.

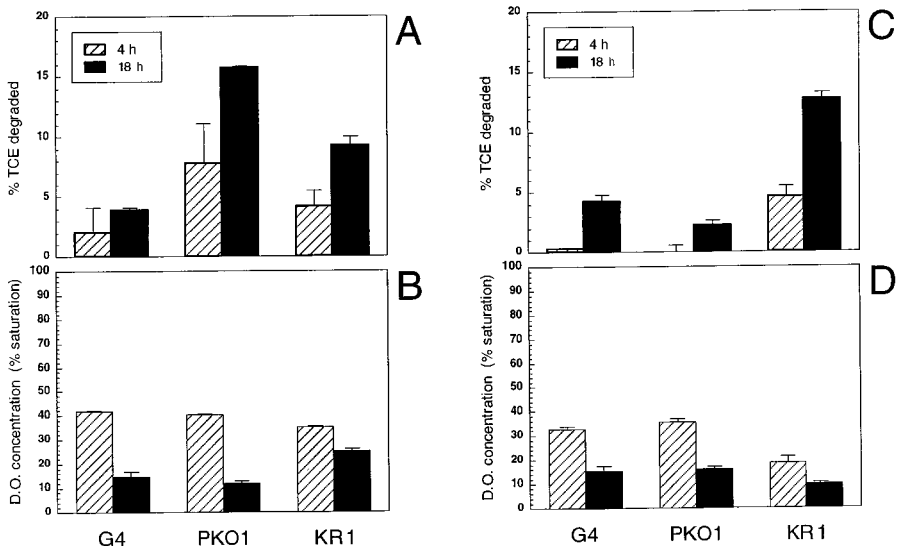

FIG. 2. TCE degradation (A and C) and oxygen consumption (B and D) by toluene monooxygenase strains under initially aerobic conditions in the presence (A and B) and absence (C and D) of nitrate. For the methods used see the legend to Fig. 1. The error bars represent the standard deviations for three samples. D.O., dissolved oxygen.

degradation were considerably less than the extents of degradation observed in experiments conducted in the absence of an electron donor (Fig. 1A). The differences among the strains were significant, with PKO1 degrading the most TCE (16%), followed by KR1 (9%) and G4 (4%).

Growth and nitrate reduction were also measured in the experiments conducted with lactate in order to assess the physiological state of the cells used for these experiments. Growth was exhibited by strains PKO1 and G4, with the OD_{425} of cell suspensions increasing from 0.05 initially to 0.42 (PKO1) and 0.23 (G4) after 18 h. Strain KR1 did not grow under these conditions within 18 h. Denitrification was observed with PKO1, as determined by the presence of nitrite after 4 h of incubation and the presence of gas bubbles in the sampling line after 18 h. Strain G4 reduced nitrate to nitrite, but there was no evidence of nitrate reduction by KR1. More oxygen was consumed under these conditions by all strains (Fig. 2B) in the presence of lactate.

In experiments in which lactate was used in the absence of nitrate (Fig. 2C), the level of TCE degradation in 18 h was significant for each of the three strains, with KR1 degrading more TCE than either PKO1 or G4. When we compared the data obtained in these experiments with results obtained in experiments conducted with lactate and nitrate, we found that the absence of nitrate was associated with a marked decrease in TCE degradation by PKO1 and a slight increase in degradation by KR1, while no significant effect was observed with G4. The growth of PKO1 and G4 decreased by 77 and 44%, respectively, compared with the growth in experiments conducted with nitrate present. No growth was observed with KR1. The levels of oxygen consumption by the strains (Fig. 2D) were similar to those seen in the nitrate experiments (Fig. 2B). Collectively, the results of these experiments suggest that nitrate may stimulate TCE degradation by strains G4 and PKO1 in the presence of an electron donor, lactate, but the presence of an electron donor and conditions permissive for growth resulted in less TCE degradation than the degradation observed with resting cells previously.

Influence of an electron donor and electron acceptors on TCE degradation under hypoxic conditions. We also compared TCE degradation by G4, PKO1, and KR1 under hypoxic conditions in the presence of lactate and nitrate (Fig. 3A). The levels of degradation by all three strains were significant; during the 18 h in which data were collected, strain KR1 degraded the most TCE (11%, the same value obtained under initially aerobic conditions), followed by PKO1 (7%) and G4 (<3%). Unexpectedly, the extent of TCE degradation by PKO1 was less than the extent of degradation obtained in the earlier experiments performed under hypoxic conditions without lactate or under aerobic conditions with lactate. Perhaps this reflected the utilization of electron acceptors for lactate metabolism concurrent with diminished transformation of TCE and also the preferential partition of metabolism toward growth which was observed in the earlier experiments with this strain. In contrast, KR1 was not significantly affected by either initial oxygen concentrations ($\alpha > 0.30$) or the addition of lactate ($\alpha > 0.80$). TCE degradation by G4 was diminished so much in the presence of lactate and nitrate that the effect of oxygen concentration could not be assessed. Denitrification by PKO1 and nitrate reduction to nitrite by G4 occurred within 4 h. KR1 did not grow or reduce nitrate under these conditions, whereas strains PKO1 and G4 grew when nitrate was present. The growth of G4, but not the growth of PKO1, was less than the growth obtained in experiments conducted under aerobic conditions. Oxygen was consumed to similar extents by PKO1 and G4; the final dissolved oxygen concentrations were 4.7 and 7.6%, respectively (Fig. 3B). Oxygen consumption by KR1 was greatly reduced under these conditions, and the dissolved oxygen concentrations were more than 19% after 18 h of incubation.

Under hypoxic conditions in the absence of nitrate, KR1 was the only strain which degraded TCE to a significant extent (Fig. 3C). The amount degraded (10%) did not differ significantly ($\alpha > 0.4$) from the amount degraded by KR1 in the presence of nitrate (11%), although little TCE degradation occurred during the first 4 h. In addition, little (G4 and PKO1) or no (KR1) growth was observed under these conditions. The levels of oxygen consumption by the strains (Fig. 3D) were similar to those observed in nitrate experiments, and the final dissolved

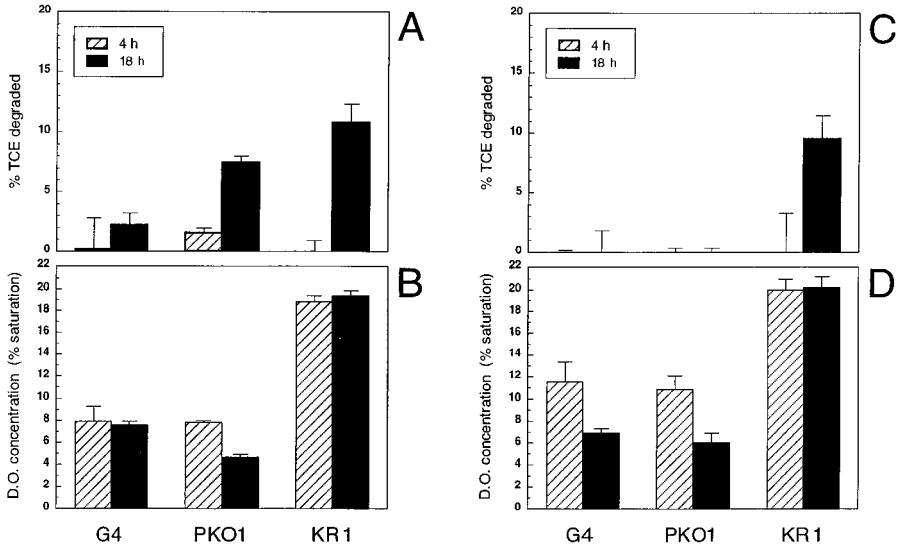

FIG. 3. TCE degradation (A and C) and oxygen consumption (B and D) by toluene monooxygenase strains under hypoxic conditions in the presence (A and B) and absence (C and D) of nitrate. For the methods used see the legend to Fig. 1. The error bars represent standard deviations for three samples. D.O., dissolved oxygen.

oxygen concentrations were 6.1, 6.9, and 20.2% for PKO1, G4, and KR1, respectively.

TCE induction of TCE and toluene degradation. In view of the previous reports of other workers that TCE induced its own degradation (35), strains F1, G4, PKO1, and KR1 were compared to determine their abilities to degrade TCE when they were grown on lactate or on lactate plus TCE, but in the absence of toluene. TCE degradation under aerobic conditions was measured after 18 h (Fig. 4). Significant levels of TCE degradation by cells grown on lactate plus TCE were observed with G4, PKO1, and KR1, but not with F1. PKO1 degraded more than twice as much TCE as either G4 or KR1 under these conditions. When the organisms were grown on lactate, KR1 was the only one of the four strains which exhibited significant levels of TCE degradation. With all of the strains examined, cells grown on lactate or lactate plus TCE degraded significantly less TCE over an 18-h period than cells grown on lactate plus toluene (Fig. 1A).

In order to determine whether TCE was acting as an inducer of toluene metabolism, the toluene-degradative activities of F1, G4, PKO1, and KR1 were compared by using cells grown on lactate and on lactate plus TCE. The results of these experiments (Table 4) showed that the activity of cells grown in the presence of TCE was 4.6 to 11.8 times higher than the activity of cells grown in the presence of lactate alone and was 36 to 105% of the activity observed in toluene-induced cells, suggesting that TCE or an oxidation product induced the degradation of toluene in these strains.

Promoter probe analysis of transcriptional activation of the PKO1 toluene 3-monooxygenase operon promoter by TCE. To confirm that the apparent induction of toluene degradation by TCE results specifically from the transcriptional activation of genes encoding the PKO1 toluene monooxygenase and not from the induction of other enzymes, transcription from the toluene 3-monooxygenase (*tbuA1UBVA2C* [5]) operon promoter from PKO1 was monitored by using the pKRZ1 promoter probe vector. Workers in our laboratory have shown previously that the *tbuA1UBVA2C* promoter, which is located in a 352-bp *Xho*I-*Sma*I fragment which includes the region immediately upstream of the structural genes, is positively regulated in *trans* by the *tbuT* gene product in the presence of aromatic effectors, such as toluene (6). The results of our experiments (Table 5) conducted with PKO1 harboring the *tbuA1UBVA2C* promoter probe pKRZ1::352-bp *Xho*I/*Sma*I showed that cells grown in the presence of TCE exhibited more than 10 times more expression from the *tbuA1UBVA2C* operon promoter, as measured by β-galactosidase activity, than cells grown in the absence of TCE or an aromatic effector. Moreover, the expression by TCE-induced cells was not significantly different from the expression observed with cells grown in the presence of toluene, indicating that TCE or an oxidation product is a relatively strong effector for transcriptional activation of the *tbuA1UBVA2C* operon in PKO1.

DISCUSSION

TCE degradation by strains of toluene-oxidizing bacteria which degrade toluene via dioxygenases or 2-, 3-, or 4-monooxygenases was studied under various conditions relevant to

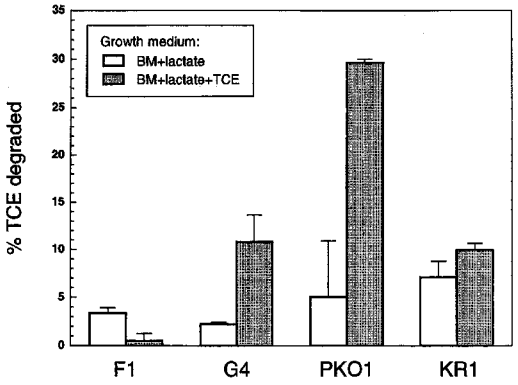

FIG. 4. TCE degradation (after 18 h) by toluene-oxidizing bacteria under initially aerobic conditions in the absence of an exogenous electron donor. Cells were grown on BM containing lactate in the presence and in the absence of TCE, washed, and resuspended as described in the text. TCE, added at an initial nominal concentration of 40 μM, was extracted with pentane, and the amount of TCE was determined by gas chromatography. The error bars represent standard deviations for two samples.

TABLE 4. Specific toluene-degradative activities of F1, G4, PKO1, and KR1 grown in BM containing sodium lactate (17.8 mM) in the presence and in the absence of TCE (2.35 mM)

Strain	Growth medium	Specific toluene-degradative activity (nmol/mg/min)[a,b]	% of activity observed with cells grown in BM + lactate + toluene[a]
F1	BM + lactate	7.1 ± 1.0	10.9 ± 1.6
	BM + lactate + TCE	41.1 ± 2.5	63.0 ± 3.9
G4	BM + lactate	0.7 ± 0.6	8.9 ± 8.1
	BM + lactate + TCE	8.0 ± 0.3	105.0 ± 3.4
PKO1	BM + lactate	1.4 ± 0.7	12.5 ± 6.5
	BM + lactate + TCE	6.5 ± 0.3	57.1 ± 2.6
KR1	BM + lactate	3.6 ± 2.2	5.0 ± 3.2
	BM + lactate + TCE	25.6 ± 0.5	35.9 ± 0.7

[a] Mean ± standard deviation.
[b] Rates were determined by using procedures described in Materials and Methods.

subsurface aquifer environments. Accordingly, no attempt was made to optimize either the conditions for growth or TCE degradation for each individual strain. Moreover, degradation was monitored by using cell suspensions having relatively low cell densities compared with some previous studies (21, 35, 63, 68). Our data show that under the conditions described above, quantitative aspects of TCE degradation by toluene-oxidizing bacteria are associated with the mechanism for toluene oxidation, the availability of electron donors and acceptors, the growth conditions, and the ability to denitrify.

The apparent differences in the abilities of mono- and dioxygenase strains to degrade TCE which were observed in this study cannot be attributed to a greater susceptibility of the dioxygenase strains to cytotoxic effects of TCE or its oxidation products, since the monooxygenase strains exhibited higher degrees of growth inhibition by TCE (Table 1). Nor can our results be readily ascribed to a failure to induce or maintain expression of toluene-oxidizing activity in dioxygenase strains or to the irreversible inactivation of toluene dioxygenases, since the toluene-degrading activity of F1 at both the beginning and the end of the 18-h incubation period, as measured in (re)aerated cell suspensions, was several times higher than the activity of either G4 or PKO1. Previous studies with F1 (63, 68) and another dioxygenase strain of *P. putida* (21) have shown that the rates of degradation of TCE by these strains diminish rapidly with time, a phenomenon which has been tentatively attributed to the toxic effects of products of TCE oxidation by dioxygenases (63). These products may include TCE-dioxetane, 1,2-dihydroxy-TCE, phosgene, formyl chloride, glyoxylate, formate, and carbon monoxide (33).

TCE degradation by toluene-oxidizing bacteria was also correlated with the ability to maintain toluene oxygenase activity over time. For example, toluene-induced cells of G4 and PKO1, which did not exhibit a decrease in toluene-degradative activity over time, degraded more than twice as much TCE between 4 and 18 h of incubation as KR1, which lost more than 50% of its toluene-degradative activity by the end of the experiment. Moreover, the activity was decreased to an even greater extent in the absence of TCE. These results are consistent with the previous findings of Winter et al. (66), who showed that TCE degradation by KR1 was maintained for a longer period when toluene, an inducer and carbon and energy source, was present.

The observation that the toluene-degradative activity of KR1 was partially maintained by the presence of TCE suggested that TCE was acting as an inducer. The results of experiments conducted to determine the toluene-degradative activity of cells grown on lactate and on lactate plus TCE strongly suggest that TCE or an oxidation product at least partially induces toluene-degradative activity not only in KR1, as previously reported (35), but also in F1, G4, and PKO1. Moreover, we found that G4, PKO1, and KR1 are able to degrade TCE when they are grown in the presence of TCE but in the absence of an aromatic inducer. In support of these findings, promoter probe analyses conducted with PKO1 showed that TCE or an oxidation product activates transcription from the toluene 3-monooxygenase promoter at levels similar to the level observed when toluene was an inducer. TCE was also found to induce transcription when the same promoter probes were placed in *Pseudomonas aeruginosa* PAO1, which does not cooxidize TCE, indicating that TCE itself can act as an inducer of toluene degradation in PKO1 (6a). Some of our results are in contrast to the results of McClay et al. (35), who reported that TCE did not induce toluene-degradative activity in F1 or G4. The apparent discrepancy in these findings can probably be explained by differences in the methods used to assess induction. In our study, the induction of toluene-degradative activity was measured by using resting cells which had been previously grown in the presence of a high concentration of TCE (2.35 mM), while in the study of McClay et al., activity was measured by using cells grown in the absence of TCE and then exposed to TCE as resting cells (i.e., in the absence of an energy substrate to support induction).

In experiments performed under hypoxic conditions or with lactate added as an electron donor, the effects of oxygen, nitrate, and lactate on TCE degradation were found to be complex, interrelated, and sometimes strain dependent. In the absence of an exogenous electron donor, TCE degradation by almost all strains decreased significantly under hypoxic conditions (approximately 1.7 mg of dissolved oxygen per liter). The addition of the electron donor lactate stimulated growth but decreased TCE degradation by G4 and PKO1 under both aerobic and hypoxic conditions. However, in the case of denitrifying strain PKO1, TCE degradation was enhanced by nitrate under conditions in which dissimilatory nitrate reduction was observed, which strongly suggests (i) that the lower level of TCE degradation in the presence of lactate may have been a consequence of electron acceptor limitation imposed by the introduction of a growth substrate and (ii) that PKO1 was able to overcome this limitation by utilizing nitrate as an alternative electron acceptor, which is consistent with our previous finding that nitrate enhances toluene degradation by PKO1 under

TABLE 5. Induction of β-galactosidase activity from the *tbuA1UBVA2C* promoter in PKO1(pKRZ1::352-bp *Xho*I/*Sma*I) cells grown in BM amended with kanamycin and lactate in the presence of inducer

Inducer	Corrected β-galactosidase activity (Miller units)[a]	% of activity
None	0.04 ± 0.05	7.9
TCE	0.39 ± 0.01	81.1
Toluene	0.48 ± 0.04	100.0

[a] Mean ± standard deviation. Corrected values were calculated by subtracting the β-galactosidase activity of PKO1(pKRZ1) cells grown under the same conditions. β-Galactosidase assays were done by using procedures described in Materials and Methods.

hypoxic conditions (49). Taken together, the results of our study suggest that the oxidation of TCE is influenced by growth and respiration, possibly as mediated through the availability of NADH. Specifically, NADH may be preferentially partitioned to anabolic processes during growth, thus decreasing its availability as a cosubstrate for the toluene oxygenase and thereby depressing the rate of TCE oxidation. The enhancement of TCE degradation which was observed with PKO1 in the presence of nitrate as an alternative electron acceptor could therefore result from concomitant increases in the rate of respiration and, correspondingly, the rate of NADH generation via the tricarboxylic acid cycle. The results obtained with KR1, which exhibited respiration uncoupled from growth, as previously documented for another strain of *P. mendocina* (61, 62), may be explained by the same hypothesis; i.e., TCE oxidation was not depressed by lactate to the extent observed with PKO1 and G4 because this organism did not grow under these conditions. Clearly, additional work will be required to confirm these findings and to clarify the relationship of TCE degradation to respiration and growth in toluene-oxidizing bacteria.

In view of evidence showing that TCE can act as an inducer for its own degradation, it may be possible to suggest bioremediation strategies which do not require addition of aromatic inducers, such as toluene, which could competitively inhibit TCE degradation (7). If aromatic substrates are not used, some other electron donor must be available to maintain TCE degradation, and noncompetitive substrates, such as lactate, have been shown to stimulate TCE oxidation (23). In situ, sufficient dissolved carbon may be available to serve this purpose. As we have shown, however, the capacity for TCE degradation may be decreased as a consequence of electron acceptor limitation when electron donors are present. In this paper we report that nitrate can serve as an alternative electron acceptor for the degradation of TCE by denitrifying strains, such as PKO1. With appropriate microbial populations, it is conceivable that the addition of nitrate to stimulate in situ TCE degradation may represent a feasible method for the enhancement of TCE removal in subsurface environments.

ACKNOWLEDGMENTS

This research was supported by National Institute of Environmental Health Sciences Superfund research and educational grant ES-04911.

We thank David Gibson and Parmely Pritchard for providing bacterial strains and Ananda Chakrabarty for providing plasmid pKRZ1. The assistance of Mark Mikesell in the development of protocols for TCE degradation analysis is gratefully acknowledged.

REFERENCES

1. **Anid, P. J., P. J. J. Alvarez, and T. M. Vogel.** 1993. Biodegradation of monoaromatic hydrocarbons in aquifer columns amended with hydrogen peroxide and nitrate. Water Res. **27:**685–691.
2. **Baker, K. H., and D. S. Herson.** 1990. In situ bioremediation of contaminated aquifers and subsurface soils. Geomicrobiol. J. **8:**133–146.
3. **Barbaro, J. R., J. F. Barker, L. A. Lemon, and C. I. Mayfield.** 1992. Biotransformation of BTEX under anaerobic, denitrifying conditions: field and laboratory observations. J. Contam. Hydrol. **11:**245–272.
4. **Bradley, P. M., C. M. Aelion, and D. A. Vroblesky.** 1992. Influence of environmental factors on denitrification in sediment contaminated with JP-4 jet fuel. Ground Water **30:**843–848.
5. **Byrne, A. M., J. J. Kukor, and R. H. Olsen.** 1995. Sequence analysis of the gene cluster encoding toluene-3-monooxygenase from *Pseudomonas pickettii* PKO1. Gene **154:**65–70.
6. **Byrne, A. M., and R. H. Olsen.** 1995. Cascade regulation of the *Pseudomonas pickettii* PKO1 toluene-3-monooxygenase operon, abstr. Q-221, p. 438. *In* Abstracts of the 95th General Meeting of the American Society for Microbiology 1995. American Society for Microbiology, Washington, D.C.

6a. **Byrne, A. M., and R. H. Olsen.** Unpublished data.

7. **Chang, H.-L., and L. Alvarez-Cohen.** 1995. Transformation capacities of chlorinated organics by mixed cultures enriched on methane, propane, toluene, or phenol. Biotechnol. Bioeng. **45:**440–449.
8. **Cohen-Bazaire, G., W. R. Sistrom, and R. Y. Stanier.** 1957. Kinetic studies of pigment synthesis by non-sulphur purple bacteria. J. Cell. Comp. Physiol. **49:**25–68.
9. **Dabrock, B., M. Klebeler, B. Averhoff, and G. Gottschalk.** 1994. Identification and characterization of a transmissible linear plasmid from *Rhodococcus erythropolis* BD2 that encodes isopropylbenzene and trichloroethene catabolism. Appl. Environ. Microbiol. **60:**853–860.
10. **Ensley, B. D.** 1991. Biochemical diversity of trichloroethylene metabolism. Annu. Rev. Microbiol. **45:**283–299.
11. **Fan, S., and K. M. Scow.** 1993. Biodegradation of trichloroethylene and toluene by indigenous microbial populations in soil. Appl. Environ. Microbiol. **59:**1911–1918.
12. **Folsom, B. R., P. J. Chapman, and P. H. Pritchard.** 1990. Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: kinetics and interactions between substrates. Appl. Environ. Microbiol. **56:**1279–1285.
13. **Fuller, M. E., D. Y. Mu, and K. M. Scow.** 1995. Biodegradation of trichloroethylene and toluene by indigenous populations in vadose sediments. Microb. Ecol. **29:**311–325.
14. **Gersberg, R. M., W. J. Dawsey, and M. D. Bradley.** 1991. Biodegradation of monoaromatic hydrocarbons in groundwater under denitrifying conditions. Bull. Environ. Contam. Toxicol. **47:**230–237.
15. **Ghiorse, W. C., and J. T. Wilson.** 1988. Microbial ecology of the terrestrial subsurface. Adv. Appl. Microbiol. **33:**107–172.
16. **Gibson, D. T., M. Hensley, H. Yoshioka, and T. J. Mabry.** 1970. Formation of (+)-cis-2,3-dihydroxy-1-methylcyclohexa-4,6-diene from toluene by *Pseudomonas putida*. Biochemistry **9:**1626–1630.
17. **Gibson, T. L., A. S. Abdul, and R. H. Olsen.** 1988. Microbial degradation of aromatic hydrocarbons in hydrogeologic material: microcosm studies, p. 53–69. *In* Proceedings of the Second National Outdoor Action Conference on Aquifer Restoration: Groundwater and Geophysical Methods, vol. 1. National Water Well Association, Dublin, Ohio.
18. **Haigler, B. E., C. A. Pettigrew, and J. C. Spain.** 1992. Biodegradation of mixtures of substituted benzenes by *Pseudomonas* sp. strain JS150. Appl. Environ. Microbiol. **58:**2237–2244.
19. **Haigler, B. E., and J. C. Spain.** 1991. Biotransformation of nitrobenzene by bacteria containing toluene degradative pathways. Appl. Environ. Microbiol. **57:**3156–3162.
20. **Hanson, R. S., and J. A. Phillips.** 1981. Chemical composition, p. 328–364. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
21. **Heald, S., and R. O. Jenkins.** 1994. Trichloroethylene removal and oxidation toxicity mediated by toluene dioxygenase of *Pseudomonas putida*. Appl. Environ. Microbiol. **60:**4634–4637.
22. **Hopkins, G. D., and P. L. McCarty.** 1995. Field evaluation of *in situ* aerobic cometabolism of trichloroethylene and three dichloroethylene isomers using phenol and toluene as primary substrates. Environ. Sci. Technol. **29:**1628–1637.
23. **Hopkins, G. D., J. Munakata, L. Semprini, and P. L. McCarty.** 1993. Trichloroethylene concentration effects on pilot field-scale in-situ groundwater bioremediation by phenol-oxidizing microorganisms. Environ. Sci. Technol. **27:**2542–2547.
24. **Hopkins, G. D., L. Semprini, and P. L. McCarty.** 1993. Microcosm and in situ field studies of enhanced biotransformation of trichloroethylene by phenol-utilizing microorganisms. Appl. Environ. Microbiol. **59:**2277–2285.
25. **Hutchins, S. R.** 1991. Biodegradation of monoaromatic hydrocarbons by aquifer microorganisms using oxygen, nitrate, or nitrous oxide as the terminal electron acceptor. Appl. Environ. Microbiol. **57:**2403–2407.
26. **Hutchins, S. R., W. C. Downs, J. T. Wilson, G. B. Smith, D. A. Kovacs, D. D. Fine, R. H. Douglass, and D. J. Hendrix.** 1991. Effect of nitrate addition on biorestoration of a fuel-contaminated aquifer: field demonstration. Ground Water **29:**571–580.
27. **Johnson, G. R., and R. H. Olsen.** 1995. Nucleotide sequence analysis of genes encoding a toluene/benzene-2-monooxygenase from *Pseudomonas* sp. strain JS150. Appl. Environ. Microbiol. **61:**3336–3346.
28. **Johnson, G. R., and R. H. Olsen.** Unpublished data.
29. **Kaphammer, B., J. J. Kukor, and R. H. Olsen.** 1990. Cloning and characterization of a novel toluene degradative pathway from *Pseudomonas pickettii* PKO1, abstr. K-145, p. 243. *In* Abstracts of the 90th Annual Meeting of the American Society for Microbiology 1990. American Society for Microbiology, Washington, D.C.
30. **Krumme, M. L., K. N. Timmis, and D. F. Dwyer.** 1993. Degradation of trichloroethylene by *Pseudomonas cepacia* G4 and the constitutive mutant strain G4 5223 PR1 in aquifer microcosms. Appl. Environ. Microbiol. **59:**2746–2749.
31. **Kukor, J. J., M. D. Mikesell, and R. H. Olsen.** 1993. Catechol dioxygenase kinetic parameters for BTEX degradation in oxygen-limited (hypoxic) environments, abstr. K-85, p. 275. *In* Abstracts of the 93rd General Meeting of the American Society for Microbiology 1993. American Society for Microbiology, Washington, D.C.
32. **Lee, M. D., J. M. Thomas, R. C. Borden, P. B. Bedient, C. H. Ward, and J. T.**

Wilson. 1986. Biorestoration of aquifers contaminated with organic compounds. Crit. Rev. Environ. Control **18:**29–89.
33. **Li, S., and L. P. Wackett.** 1992. Trichloroethylene oxidation by toluene dioxygenase. Biochem. Biophys. Res. Commun. **185:**443–451.
34. **Major, D. W., C. I. Mayfield, and J. F. Barker.** 1988. Biotransformation of benzene by denitrification in aquifer sand. Ground Water **26:**8–14.
35. **McClay, K., S. Streger, and R. J. Steffan.** 1995. Induction of toluene oxidation activity in *Pseudomonas mendocina* KR1 and *Pseudomonas* sp. strain ENVPC5 by chlorinated solvents and alkanes. Appl. Environ. Microbiol. **61:**3479–3481.
36. **Mikesell, M. D., J. J. Kukor, and R. H. Olsen.** 1994. Metabolic diversity of aromatic hydrocarbon-degrading bacteria from a petroleum-contaminated aquifer. Biodegradation **4:**249–259.
37. **Mikesell, M. D., and R. H. Olsen.** 1992. Degradation of aromatic hydrocarbons under anoxic conditions by *Pseudomonas* sp. W31, abstr. Q-109, p. 353. *In* Abstracts of the 92nd General Meeting of the American Society for Microbiology 1992. American Society for Microbiology, Washington, D.C.
38. **Mikesell, M. D., and R. H. Olsen.** Unpublished data.
39. **Mikesell, M. D., R. H. Olsen, and J. J. Kukor.** 1991. Stratification of anoxic BTEX-degrading bacteria at three petroleum-contaminated sites, p. 351–362. *In* R. Hinchee and R. F. Olfenbuttel (ed.), *In situ* bioreclamation. Butterworth-Heinemann, Stoneham, Mass.
40. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. **Morgan, P., and R. J. Watkinson.** 1992. Factors limiting the supply and efficiency of nutrient and oxygen supplements for the *in situ* biotreatment of contaminated soil and groundwater. Water Res. **26:**73–78.
42. **Mu, D. Y., and K. M. Scow.** 1994. Effect of trichloroethylene (TCE) and toluene concentrations on TCE and toluene biodegradation and the population density of TCE and toluene degraders in soil. Appl. Environ. Microbiol. **60:**2661–2665.
43. **National Cancer Institute.** 1976. Carcinogenesis bioassay of trichloroethylene. CAS no. 79-01-6. U.S. Department of Health, Education, and Welfare publication (NIH) 76-802. U.S. Department of Health, Education, and Welfare, Washington, D.C.
44. **Nelson, M. J., J. V. Kinsella, and T. Montoya.** 1990. *In situ* biodegradation of TCE contaminated groundwater. Environ. Prog. **9:**190–196.
45. **Nelson, M. J. K., S. O. Montgomery, W. R. Mahaffey, and P. H. Pritchard.** 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol. **53:**949–954.
46. **Nelson, M. J. K., S. O. Montgomery, and P. H. Pritchard.** 1988. Trichloroethylene metabolism by microorganisms that degrade aromatic compounds. Appl. Environ. Microbiol. **54:**604–606.
47. **Olsen, R. H., and J. Hansen.** 1976. Evolution and utility of a *Pseudomonas aeruginosa* drug resistance factor. J. Bacteriol. **125:**837–844.
48. **Olsen, R. H., J. J. Kukor, and B. Kaphammer.** 1994. A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PKO1. J. Bacteriol. **176:**3749–3756.
49. **Olsen, R. H., M. D. Mikesell, and J. J. Kukor.** 1994. Enumeration and characterization of BTEX-degrading bacteria from hypoxic environments functional with mixed electron acceptors. Res. Microbiol. **145:**47–49.
50. **Page, W. J., and H. L. Sadoff.** 1976. Physiological factors affecting transformation of *Azotobacter vinelandii*. J. Bacteriol. **125:**1080–1087.
51. **Rajagopal, R.** 1986. Conceptual design for a groundwater quality monitoring strategy. Environ. Prof. **8:**244–264.
52. **Rothmel, R. K., D. L. Shinabarger, M. R. Parsek, T. L. Aldrich, and A. M. Chakrabarty.** 1991. Functional analysis of the *Pseudomonas putida* regulatory protein CatR: transcriptional studies and determination of the CatR DNA-binding site by hydroxyl-radical footprinting. J. Bacteriol. **173:**4717–4724.
53. **SAS Institute, Inc.** 1990. The SAS system for personal computers. SAS Institute, Inc., Cary, N.C.
54. **Shields, M. S., S. O. Montgomery, P. J. Chapman, S. M. Cuskey, and P. H. Pritchard.** 1989. Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium G4. Appl. Environ. Microbiol. **55:**1624–1629.
55. **Shields, M. S., S. O. Montgomery, S. M. Cuskey, P. J. Chapman, and P. H. Pritchard.** 1991. Mutants of *Pseudomonas cepacia* G4 defective in catabolism of aromatic compounds and trichloroethylene. Appl. Environ. Microbiol. **57:**1935–1941.
56. **Shields, M. S., and M. J. Reagin.** 1992. Selection of a *Pseudomonas cepacia* strain constitutive for the degradation of trichloroethylene. Appl. Environ. Microbiol. **58:**3977–3983.
57. **Smibert, R. M., and N. R. Krieg.** 1981. Systematics. General characterization, p. 409–443. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
58. **Sokal, R. R., and F. J. Rohlf.** 1980. Biometry, 2nd ed. W. H. Freeman and Co., New York.
59. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:**159–271.
60. **U.S. Environmental Protection Agency.** 1980. Ambient water quality criteria for trichloroethylenes. Publication 440/5-80-073. National Technical Information Service, Springfield, Va.
61. **Verdoni, N., M. A. Aon, and J. M. Lebeault.** 1992. Metabolic and energetic control of *Pseudomonas mendocina* growth during transitions from aerobic to oxygen-limited conditions in chemostat cultures. Appl. Environ. Microbiol. **58:**3150–3156.
62. **Verdoni, N., M. A. Aon, J. M. Lebeault, and D. Thomas.** 1990. Proton motive force, energy recycling by end product excretion, and metabolic uncoupling during anaerobic growth of *Pseudomonas mendocina*. J. Bacteriol. **172:**6673–6681.
63. **Wackett, L. P., and D. T. Gibson.** 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. **54:**1703–1708.
64. **Wackett, L. P., and S. R. Householder.** 1989. Toxicity of trichloroethylene to *Pseudomonas putida* F1 is mediated by toluene dioxygenase. Appl. Environ. Microbiol. **55:**2723–2725.
65. **Whited, G. M., and D. T. Gibson.** 1991. Toluene-4-monooxygenase, a three-component enzyme system that catalyzes the oxidation of toluene to *p*-cresol in *Pseudomonas mendocina* KR1. J. Bacteriol. **173:**3010–3016.
66. **Winter, R. B., K.-M. Yen, and B. D. Ensley.** 1989. Efficient degradation of trichloroethylene by a recombinant *Escherichia coli*. Bio/Technology **7:**282–285.
67. **Yeh, W.-K., D. T. Gibson, and T.-N. Liu.** 1977. Toluene dioxygenase: a multicomponent enzyme system. Biochem. Biophys. Res. Commun. **78:**401–410.
68. **Zylstra, G. J., L. P. Wackett, and D. T. Gibson.** 1989. Trichloroethylene degradation by *Escherichia coli* containing the cloned *Pseudomonas putida* F1 toluene dioxygenase genes. Appl. Environ. Microbiol. **55:**3162–3166.