APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1990, p. 1279–1285
0099-2240/90/051279-07$02.00/0

Vol. 56, No. 5

Phenol and Trichloroethylene Degradation by *Pseudomonas cepacia* G4: Kinetics and Interactions between Substrates†

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Received 8 February 1990/Accepted 15 February 1990

Intact cells of *Pseudomonas cepacia* G4 completely degraded trichloroethylene (TCE) following growth with phenol. Degradation kinetics were determined for both phenol, used to induce requisite enzymes, and TCE, the target substrate. Apparent K_s and V_{max} values for degradation of phenol by cells were 8.5 μM and 466 nmol/min per mg of protein, respectively. At phenol concentrations greater than 50 μM, phenol degradation was inhibited, yielding an apparent second-order inhibitory value, K_{SI}, of 0.45 mM as modeled by the Haldane expression. A partition coefficient for TCE was determined to be 0.40 ± 0.02, [TCE_{air}]/[TCE_{water}], consistent with Henry's law. To eliminate experimental problems associated with TCE volatility and partitioning, a no-headspace bottle assay was developed, allowing for direct and accurate determinations of aqueous TCE concentration. By this assay procedure, apparent K_s and V_{max} values determined for TCE degradation by intact cells were 3 μM and 8 nmol/min per mg of protein, respectively. Following a transient lag period, *P. cepacia* G4 degraded TCE at concentrations of at least 300 μM with no apparent retardation in rate. Consistent with K_s values determined for degradation, TCE significantly inhibited phenol degradation.

Trichloroethylene (TCE) is a volatile chlorinated organic compound which has been used extensively as a solvent and degreasing agent. TCE and other chemical wastes have been shown to migrate through soils from disposal sites and threaten groundwater aquifers across the nation. TCE, a suspected carcinogen in rats (9), has been shown to be converted under anaerobic conditions to vinyl chloride, another highly toxic chemical (6). Due to its widespread contamination of soil and water and its potential health threat, TCE has received much attention recently.

Several organisms have been isolated and characterized which possess the ability to act on TCE. One organism, strain G4 (12), requires toluene, *o*-cresol, *m*-cresol, or phenol for TCE degradation activity (11, 12). This organism, identified as a strain of *Pseudomonas cepacia* (S. O. Montgomery, M. S. Shields, P. J. Chapman, and P. H. Pritchard, unpublished data), has been shown to employ a novel toluene-degradative pathway involving sequential hydroxylation of toluene at *ortho* and *meta* positions to form 3-methylcatechol (13). TCE is completely degraded by this organism to CO_2, Cl^-, and unidentified, nonvolatile products (12).

Biodegradation of TCE and other environmental contaminants has the potential for being a cost-effective remediation technology. Though TCE is readily degraded in the laboratory (11, 12, 15–17), its persistence in the environment indicates that naturally occurring organisms are limited for this activity. These limitations need to be identified and characterized so that TCE degradation can be stimulated either by supplementing the contaminated site (14) or by constructing bioreactors. Characterization of cellular degradation kinetics is relevant to both scenarios since intact organisms would probably be used in most bioremediation processes. The physiological basis for TCE degradation by *P. cepacia* G4 has been shown to reside in its ability to degrade toluene (11). We report here basic kinetics for degradation of phenol and TCE by intact cells, including inhibitory interactions between these two substrates.

MATERIALS AND METHODS

Bacterial strain, culture conditions, and chemicals. *P. cepacia* G4 used in this study was isolated and characterized previously (12; Montgomery et al., unpublished data). Cells were grown in a stirred chemostat (150-ml liquid volume) with 5 mM phenol as the sole carbon source in a defined basal salts medium (BSM) (3), pH 7.5, at a dilution rate of 0.10 to 0.050/h. Phenol was redistilled prior to use. Cells used in the various assays were harvested from the chemostat, centrifuged for 10 min at 26,000 × *g*, and suspended in BSM to 23 μg of protein per ml unless otherwise stated. Phenol and TCE degradation rate assays were performed at room temperature and initiated within 1 h of harvesting unless otherwise indicated. Cell protein (in micrograms per milliliter) was determined by the BCA method (Pierce Chemical Co., Rockford, Ill.), using bovine serum albumin as a protein standard. The correlation between cell protein concentrations and A_{600} was linear up to an A_{600} of 1, which allowed for direct conversion of turbidity measurements to cell protein concentrations (114 μg/ml at an A_{600} of 1).

Phenol disappearance assay. Rates at which cells degraded phenol were determined by monitoring changes in phenol concentration, using a modified colorimetric assay (7). In this assay, 0.1 ml of 10 mM phenol in water (final concentration, 100 μM) was added to a 10-ml suspension of cells. At defined intervals, 1-ml samples were transferred to 1-ml snap-cap Microfuge tubes containing 50 μl of 2 N NH_4OH and 25 μl of 2% 4-aminoantipyrene (Aldrich Chemical Co., Inc., Milwaukee, Wis.). The tubes were closed and the contents were mixed; 25 μl of 8% $K_3Fe(CN)_6$ (Sigma Chemical Co., St. Louis, Mo.) was added, and the contents were mixed again and centrifuged (14,000 × *g*) for 2 min. The A_{500}

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of the supernatant was measured. Phenol concentrations were calculated by reference to a standard curve. Generally, phenol disappearance rates were calculated from six determinations over a 10-min time period. Rates of phenol disappearance were calculated and reported as nanomoles per minute per milligram of protein.

TCE disappearance assays. Three assays for TCE degradation were used: a standard bottle assay (Montgomery et al., unpublished results), a no-headspace bottle assay, and a syringe assay. A 100- to 300-ml suspension of cells was mixed by magnetic stirring and aerated with compressed air. Samples of this solution were placed either in a bottle or a syringe (depending on the assay employed). A stock solution of TCE in methanol, 2 to 20 mM, was prepared, and samples were added to give the desired final TCE concentration. No more than 10 μl of methanol per ml of reaction solution was added.

(i) Standard assay. A 2-ml cell suspension was placed in a 20-mm crimp-seal bottle (17-ml total capacity), and 10 μl of 4 mM TCE was added. The bottle was capped with a Teflon-lined rubber septum (Supelco, Inc., Bellefonte, Pa.) and crimped before incubation at 26°C (inverted and shaken at 200 rpm). *n*-Pentane (2 ml) was injected with a syringe through the septum to terminate the reaction at selected times. For each time point, triplicate determinations were made.

(ii) No-headspace bottle assay. A 2-ml cell suspension and a glass bead (2- to 3-mm diameter) were placed in a 2-ml crimp-seal vial, and 10 μl of 4 mM TCE was added; the bottle was capped with no trapped air and shaken vigorously for 15 s before incubation at 26°C. The glass bead facilitated thorough mixing of the contents. To obtain samples, a syringe needle was inserted to vent air into the bottle and a gastight syringe was used to withdraw and transfer 1.5 ml of solution to a 20-mm crimp-seal bottle containing 1.5 ml of *n*-pentane, which was then capped with a Teflon-lined septum. For each time point, samples were prepared and analyzed in triplicate.

(iii) Syringe assay. A 50-ml cell suspension was drawn into a 50-ml gastight syringe containing a small magnetic stir bar. All air bubbles were removed before the addition of 10 to 50 μl of TCE (10 to 40 mM), and the solution was mixed for 2 min on a magnetic stir plate. The syringe containing cells and TCE was mounted alongside a second 50-ml syringe containing *n*-pentane on a syringe pump, and the two were connected with Teflon tubing and a T connector. At designated time intervals, from 10 to 30 min, the pump was turned on for 20 s to deliver 2 ml each of the reaction solution and pentane to a 20-mm crimp-seal bottle. The bottle was immediately capped with a Teflon-lined septum.

Bottles containing the water-pentane mixtures from all of the procedures described above were mixed for at least 30 min at 200 rpm. The pentane phase was transferred to a 2-ml crimp-seal vial, and the contents were analyzed for TCE by using a gas chromatograph equipped with a capillary column and an electron capture detector. The detection limit was less than 10 nM. Rates of TCE disappearance were reported as nanomoles of TCE per minute per milligram of protein. Cell-free controls were run in parallel to assess the abiotic loss of TCE from the system. Less than 1% loss of TCE per h was usually observed.

Calculations and equations. Inhibition of phenol degradation at high phenol concentrations was modeled by a Haldane expression, which incorporates a second-order inhibitory term, K_{SI}, into the Michaelis-Menten expression (equation 1). K_s is the Michaelis constant for cellular kinetics and is analogous to K_m for enzymatic reactions. [*S*] is the substrate concentration, and v_0 is the initial reaction rate.

$$v_0 = \frac{V_{max}[S]}{[S] + \frac{1}{K_{SI}}[S]^2 + K_s} \text{ (Haldane expression)} \quad (1)$$

The air-water partition behavior of TCE was expressed by using Henry's law with a dimensionless Henry's law constant, H″ (equation 2).

$$H'' = \frac{[TCE_{air}]}{[TCE_{water}]} \text{ (Henry's law)} \quad (2)$$

Retardation of phenol degradation by TCE was modeled by a competitive-inhibition relationship. Data for TCE inhibition of phenol degradation were plotted as the inverse rate ($1/v_0$) versus inhibitor concentration ([I]) in the form of a Dixon plot according to equation 3 (2). K_I represents the inhibitor-binding affinity, similar to K_s (K_m).

$$\frac{1}{v_0} = \frac{[S] + K_s}{V_{max}[S]} + \frac{1}{V_{max}} \cdot \frac{K_s[I]}{K_I[S]} \text{ (Dixon plot)} \quad (3)$$

RESULTS

Characterization of phenol degradation rates. Changes in phenol degradation rates were monitored initially because phenol was selected as the primary growth substrate and was required for catabolic enzyme induction. No significant changes in phenol disappearance rates (100 μM initial concentration) were observed over a pH range of 6 to 8, and all subsequent assays were performed at a pH of 7.5.

Kinetic parameters for phenol degradation by cells were determined by measuring phenol disappearance rates. Phenol disappearance exhibited first-order rate increases with phenol concentrations up to 50 μM and decreases in rate at higher concentrations (Fig. 1). The term K_s was employed instead of K_m because rates were measured by using intact organisms and not purified enzymes. Values are described as apparent because no attempt was made to vary the concentration of oxygen or other cosubstrates. The hyperbolic curve was calculated from K_s and V_{max} values estimated from the Lineweaver-Burk plot. The best fit to the observed results was calculated by using the Haldane expression (equation 1), which incorporates an inhibitory term, K_{SI}. The apparent values for K_s, V_{max}, and K_{SI} were 8.5 μM, 466 nmol/min per mg of protein, and 454 μM, respectively.

To determine whether cellular degradation activity varied following removal of the carbon source used for growth, cells grown on phenol were harvested, washed, suspended in BSM, and then incubated while being mixed and aerated at 30°C. Phenol degradation rates were determined for samples of this suspension at intervals over an 8-h time period. Rates of phenol disappearance remained essentially constant, though a short lag period before the onset of phenol disappearance was noted after 8 h of incubation (data not shown).

Development of an assay to monitor TCE disappearance. Though TCE is highly volatile, for convenience it is often reported as an aqueous concentration in closed systems containing both air and water phases. To determine the effect of TCE volatility on its aqueous concentrations, time-dependent changes in TCE concentration were determined for two sets of uninoculated controls. In one set, total TCE was extracted by addition of pentane to the bottle. The total amount of TCE remained essentially constant for the

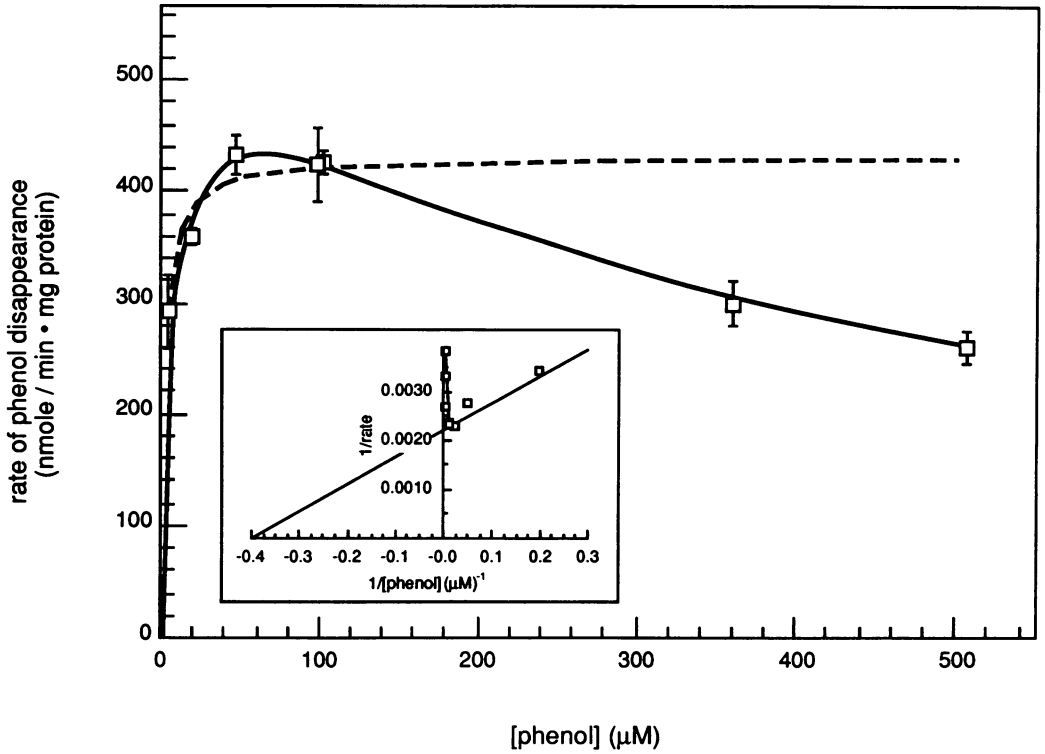

FIG. 1. Phenol biodegradation kinetics. Phenol was added to give initial concentrations of 10 to 500 μM, and rates of phenol disappearance, determined in duplicate, were then plotted against initial phenol concentrations. The hyperbolic curve (– – –) was calculated from K_s and V_{max} values estimated from the Lineweaver-Burk plot (inset). The best fit to the observed results (—) was calculated by using the Haldane expression (equation 1).

duration of the experiment, at 31 nmol per bottle (15.5 μM, assuming that all of the TCE was dissolved in the 2-ml aqueous phase) (Fig. 2). For the second set, 1.5 ml of the aqueous solution was withdrawn from the bottle and extracted with 1.5 ml of pentane. The concentration of TCE in the water phase decreased rapidly by the first sampling time (15 s after the addition of TCE with vigorous shaking) and thereafter remained essentially constant at 3.8 μM (Fig. 2). The experiment was repeated with different amounts of total TCE, and the actual concentrations in both the air and water phases were determined. From these data, a dimensionless Henry's law constant was calculated to be 0.40 ± 0.02 at 25°C (equation 2).

Cell-mediated TCE disappearance in the standard bottle assay was also determined (Fig. 2). Reported concentrations are for total extracted TCE, as though all the TCE is dissolved in the water phase. When this experiment was continued for longer times, TCE eventually degraded to undetectable levels. Under these conditions, the initial aqueous TCE concentration was about 15 μM but the actual aqueous concentration rapidly dropped to about 4 μM, as established for uninoculated controls. Following initial redistribution of TCE from water to air, the air-entrained TCE redissolves in the water to replace the water-entrained TCE degraded by the organisms, leading to complete removal of the TCE from the bottle.

The rate of TCE transfer from air to water may slow TCE degradation by waterborne organisms. Aqueous TCE concentrations will also affect degradation rates at low enough levels. To determine whether degradation rates were affected by these factors, a no-headspace bottle assay was run in addition to the three experiments whose results are depicted in Fig. 2. Though the total amounts of TCE in each bottle were the same, the steady-state aqueous concentrations were different. Even so, the rate of TCE disappearance was only slightly faster for the no-headspace bottle assay than for the standard bottle assay (Table 1). These results suggest that the rate of TCE redistribution from air to water was much faster than degradation and was not a limiting factor in these well-mixed systems.

To accurately determine the kinetics of TCE disappearance it is important that the TCE measured in the system be accurately related to its aqueous concentration. Measurement of TCE concentrations in a no-headspace system was simplified by development of the syringe assay. This method gave highly reproducible results. In two separate experiments (eight sets of duplicate time points collected over 4 h), TCE disappearance rates (20 μM initial concentration) were calculated to be 2.54 and 2.51 nmol/min per mg of protein, with r^2 values of 0.97 and 0.98. This method was used to determine TCE disappearance rates over a range of initial TCE concentrations (Fig. 3). Data were plotted as a Lineweaver-Burk plot to estimate K_s and V_{max}. These values were then used to generate the hyperbolic curve shown in Fig. 3. The apparent K_s for TCE was estimated to be 3 μM. A more accurate estimate was difficult to obtain because of limitations in assaying low TCE concentrations. A representative value of V_{max} for TCE disappearance was 7.9 nmol/min per mg of protein, though the absolute value varied somewhat with different batches of cells, ranging from 4 to 8 nmol/min per mg of protein.

Like many organic solvents, TCE is hydrophobic and can

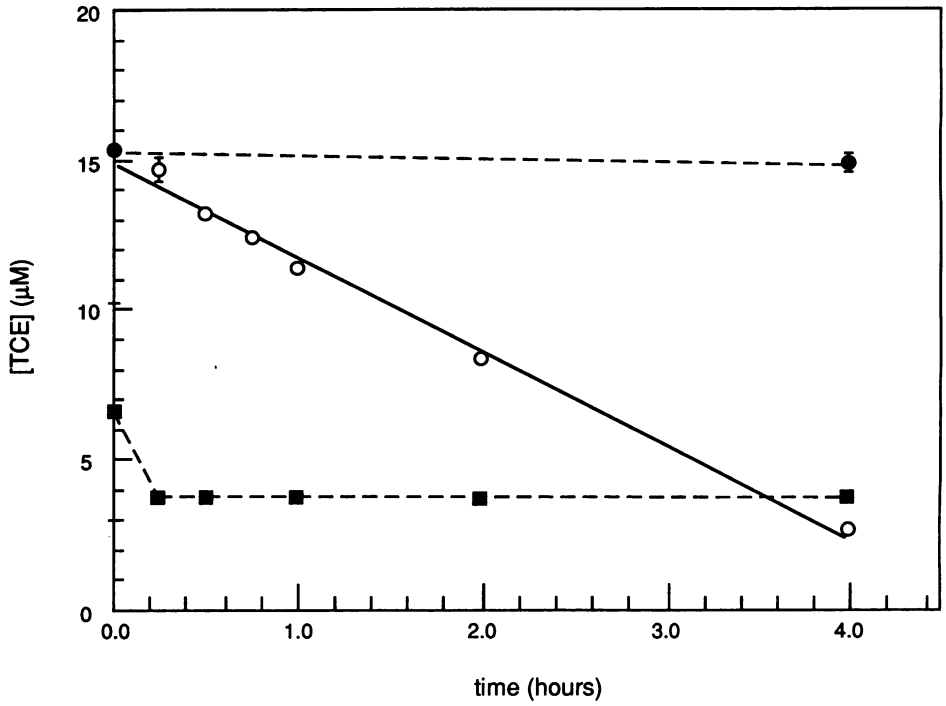

FIG. 2. Partitioning and degradation of TCE in the standard bottle assay. The standard bottle assay was used to measure time-dependent changes in TCE concentration for two sets of uninoculated controls, total TCE (●) and TCE found only in the aqueous phase (■), and for a suspension of cells (20 μg of protein per ml) (○). Triplicate samples were obtained for each point.

potentially express toxic or inhibitory effects on cells by partitioning into cellular components, such as lipids and polysaccharides. To determine the effect of higher concentrations on degradation activity, the rates of TCE disappearance were determined for concentrations of 20, 80, 140, and 180 μM (Fig. 4). At TCE concentrations above 50 μM, an apparent lag of 40 to 60 min was observed before the onset of TCE disappearance. Uninoculated controls demonstrated no significant loss of TCE from the system. Though there was an initial inhibition of degradation, cells could apparently overcome those effects and resume TCE degradation at near-maximum rates. This experiment was repeated several times with TCE concentrations up to 310 μM, with similar results. Though the lag time was variable, the rates of TCE disappearance remained essentially constant for all concentrations between 20 and 310 μM TCE.

Interactions between TCE and phenol which could affect cometabolic degradation. Since TCE degradation requires catabolic enzymes involved in toluene, cresol, and phenol utilization (11), it appeared likely that the fortuitous degradation of TCE would be inhibited by those compounds.

TABLE 1. Comparison of the standard bottle assay with the no-headspace bottle assay

Assay	Total TCE (nmol/bottle)	Aqueous TCE concn (μM)	Cellular activity[a] (nmol/min per mg of protein)
Standard	31	3.8	3.5
No-headspace bottle	28	14.0	4.7

[a] Cellular activities for TCE disappearance rate were determined for a suspension of cells, 15 μg of protein per ml, by both of the methods indicated.

Earlier work indicated that phenol inhibited TCE degradation (Montgomery et al., unpublished data). To study this inhibition in more detail, the reverse relationship was studied because the degradation rates for phenol are significantly faster than those for TCE. Rates of phenol degradation, concentration fixed at 25 μM, were determined in the presence of different concentrations of TCE, concentrations ranging from 0 to 35 μM. TCE concentrations were determined at the end of the 15-min assay. Under these conditions, less than 1 μM change in TCE concentration was observed in 15 min in the absence of phenol. TCE was observed to inhibit phenol degradation in a concentration-dependent manner (Fig. 5). The inset to Fig. 5 depicts a Dixon plot of inhibitor concentration versus substrate degradation rate. Though more than one set of data are required to determine the mechanism of inhibition, the data shown would estimate K_I to be less than 25 μM and the K_s/K_I ratio to be about 1 for either competitive or noncompetitive inhibition mechanisms. TCE inhibition of phenol was consistent with the hypothesis that TCE and phenol serve as substrates for the same enzyme(s).

DISCUSSION

P. cepacia G4 grown on phenol as the sole carbon source produces catabolic enzymes required for both phenol and TCE degradation. The apparent K_s for phenol degradation was 8.5 μM (for cells harvested from continuous culture). A more accurate value could not be obtained because of limitations in the sensitivity of the method at low phenol concentrations. Less precise K_s values determined from carbon-limited continuous cultures of several *Pseudomonas* species and *Trichosporon cutaneum* ranged from less than 10 to about 30 μM (4, 10, 18). Inhibition of phenol degradation

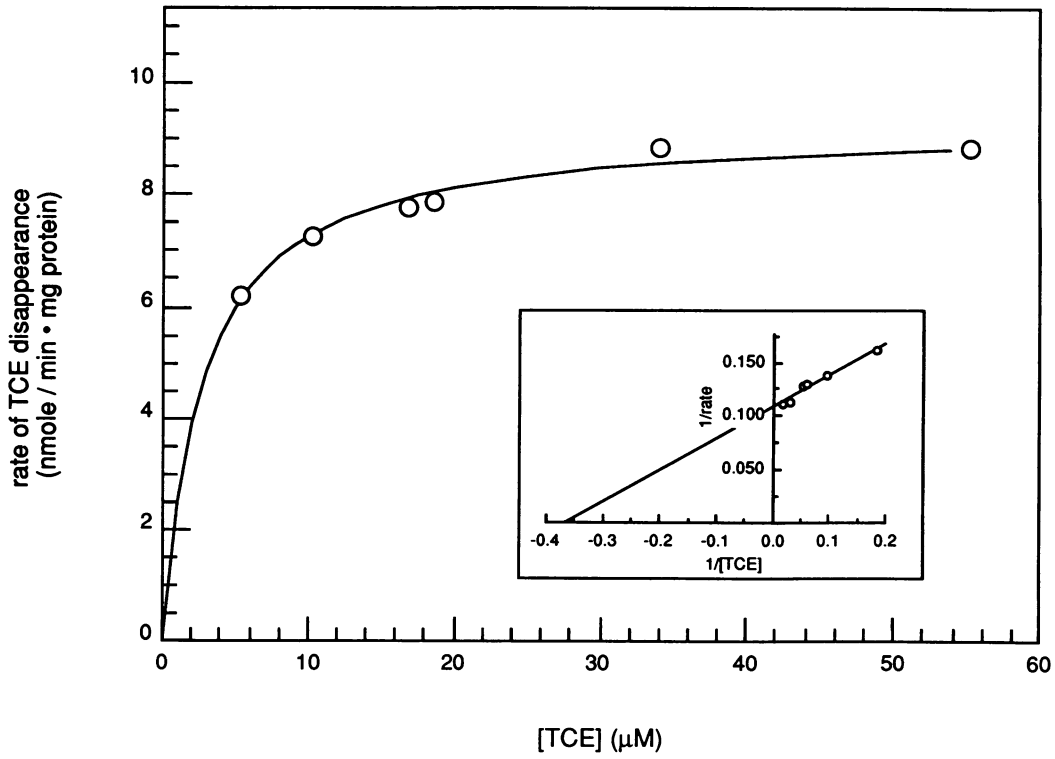

FIG. 3. TCE degradation kinetics. Rates of disappearance were determined from eight sets of duplicate samples over 4 h for initial TCE concentrations (5 to 50 μM). Inset depicts Lineweaver-Burk plot of data.

at higher phenol concentrations has been shown previously to be modeled best by use of a Haldane function (equation 1) (4). Previously reported apparent K_{SI} values ranged between 1 and 5 mM (4, 18); these values are higher than our estimate for *P. cepacia* G4 of 0.45 mM. The apparent V_{max} for phenol degradation was about 466 nmol/min per mg of protein. Because of these kinetic characteristics, phenol was rapidly degraded to low concentrations when phenol was the sole carbon source in continuous culture.

Following induction of requisite catabolic enzymes by phenol, *P. cepacia* G4 was capable of degrading TCE to below detection limits. To determine the kinetics of TCE degradation, accurate measurements of aqueous TCE concentrations were required. Wackett and Gibson (15) proposed that calculations using Henry's law may not accurately predict aqueous TCE concentrations because of additional factors such as sorption by cells or vessel surfaces. We found a good correlation between published values for the (dimensionless) Henry's law constants for TCE, 0.38 and 0.41 at 25°C and 1 atm (ca. 100 kPa) (5, 8), and our value for cell-free controls, 0.40 ± 0.02. Evidently, adsorption to vessel surfaces does not significantly affect partitioning. In contrast, sorption by cell components may be part of the TCE uptake process. TCE degradation rates were not retarded by air-water partitioning, presumably because rates of redissolving air-entrained TCE in water were rapid enough to impose no limitation in these well-mixed systems. Consequently, air-entrained TCE was completely available for degradation. In a two-phase system, the best assessment of degradative activity is to monitor the loss of total TCE from the bottle.

To simplify the determination of kinetic constants, a no-headspace assay was developed. Using this one-phase

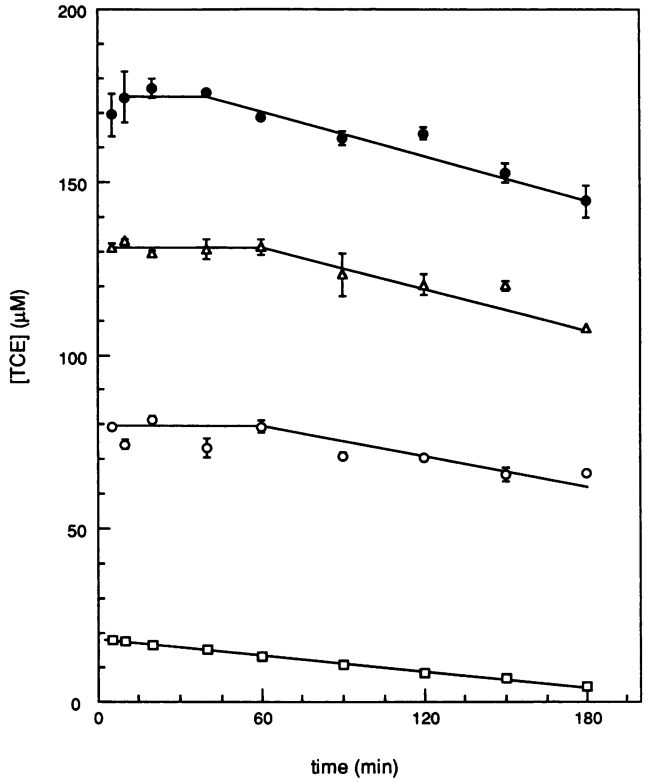

FIG. 4. TCE degradation at higher initial TCE concentrations. TCE disappearance was determined for 20 (□), 80 (○), 140 (△), and 180 (●) μM TCE (29 μg of protein per ml). Duplicate samples were obtained for each point.

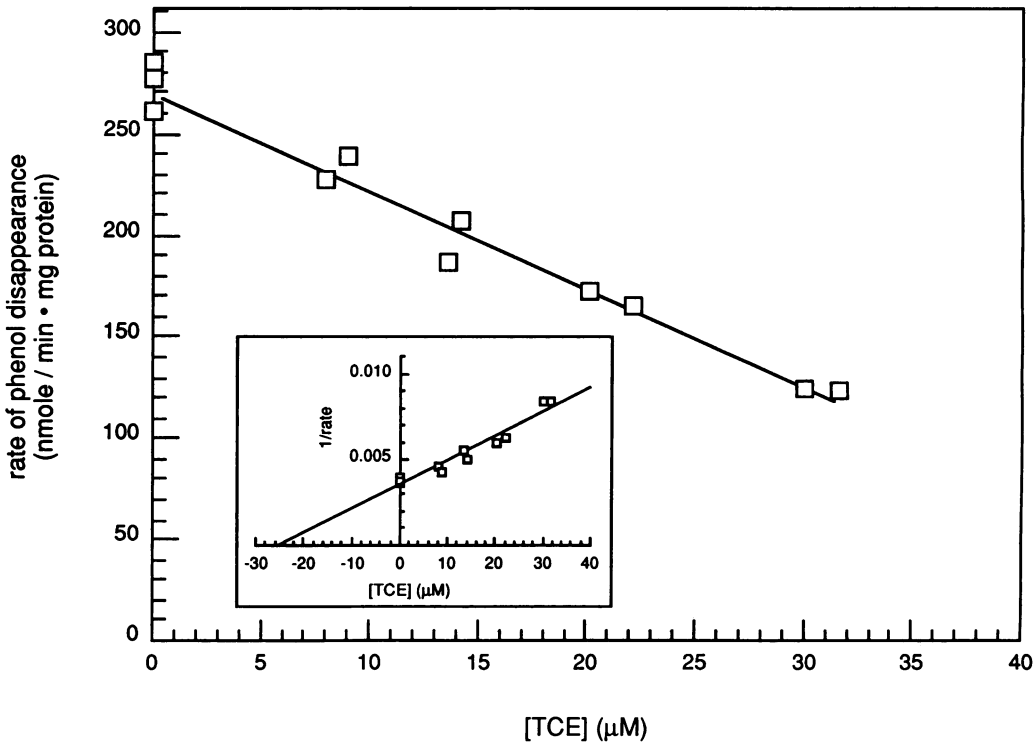

FIG. 5. TCE inhibition of phenol degradation. Phenol disappearance rates were plotted against the TCE concentration (9 μg of protein per ml) by using the syringe assay. TCE concentrations were determined by extracting triplicate samples at the end of each assay. The inset depicts the results plotted as a Dixon plot, inverse rate versus inhibitor concentration (equation 3).

system, the apparent K_s and V_{max} values for TCE degradation were determined to be 3 μM and 8 nmol/min per mg of protein, respectively. The apparent K_s for intact cells represents a combination of several activities, including uptake, sorption, and degradation, so the degradative enzymes may not actually be saturated and operating at maximal rates if TCE uptake is slow. At concentrations higher than 50 μM, TCE transiently inhibited its own degradation, followed by complete recovery of degradative activity. Kinetic constants determined for *P. cepacia* G4 indicated that TCE would be degraded to low concentrations at rates near the V_{max} for aqueous TCE concentrations up to at least 300 μM.

Due to significant differences between methods reported in the literature, direct comparison of TCE degradation rates between different organisms is difficult. Even though Henry's law predicts that aqueous concentrations are two to three times greater than air-entrained concentrations, aqueous concentrations would actually be 5- to 10-fold less than air-entrained concentrations because of the large differences in air and water volumes in the bottles used. With these limitations noted, TCE degradation rates reported here for *P. cepacia* G4 are similar to previously published rates for *Pseudomonas mendocina* (17), *Pseudomonas putida* F1 (15), and *Nitrosomonas europaea* (1) and apparently greater than rates calculated from data reported for methanotrophs (15). Winter et al. (17) reported retardation of TCE degradation by *P. mendocina* at concentrations higher than 125 μM TCE. By using the Henry's law constant and the volumes of air and water in their assay system, the inhibitory TCE concentration is calculated to be closer to 20 μM. Wackett and Gibson (15) reported first-order kinetics for initial TCE degradation by *P. putida* F1 for concentrations less than 80 μM (30 μM corrected value) and complete inhibition at 320 μM (130 μM corrected value). Although reporting TCE concentrations as if all TCE is found in the water of two-phase systems is more convenient, it can introduce misleading conclusions concerning concentration-dependent toxic or inhibitory effects.

Though the volatility of TCE requires special care in determining kinetic parameters, it offers advantages impacting the design of TCE remediation processes. TCE can be air stripped from solution, thereby simplifying the chemical composition of the waste to be treated. Air-entrained TCE can also be redissolved in the water of a bioreactor designed for TCE remediation. Thus, K_s, partitioning, and phase transfer rates for TCE favor the removal of air-entrained TCE and allow degradation to undetectable levels by waterborne organisms.

The bioconversion of TCE by *P. cepacia* involves the action of an enzyme(s) whose natural substrates appear to be toluene and phenol (11, 13). A degree of inhibitory interaction between the natural substrate and other chemicals serving as substrates is to be expected. In the case of TCE degradation, in which phenol is employed as both the inducer and the substrate, inhibition is likely to be severe since the K_s values for both TCE and phenol are similar (5 to 10 μM). Experimental evidence bears this out. At equal concentrations of phenol and TCE, a decrease of about 50% in the rate of phenol degradation was observed. From equation 3, which describes simple competitive inhibition, decreases in phenol degradation rates caused by TCE indicated that the K_s/K_I ratio for phenol and TCE was approximately 1, consistent with their similar K_s values. Theoretically, phenol will inhibit TCE degradation, the interaction of primary concern in TCE bioremediation, according to the same principle. Though the exact mechanism of inhibition

was not determined, a competitive mechanism is suspected between the natural substrate, phenol, and the fortuitous substrate, TCE. To overcome inhibitory limitations on TCE degradation efficiencies, phenol could be replaced by a gratuitous inducer or the requisite catabolic enzymes could be placed under alternative genetic regulation. Another approach would be to investigate bioreactor designs which minimize inhibition and maximize TCE degradation. Model bench scale reactors will be employed to test the validity of using cellular kinetic parameters in the assessment of bioreactor performance characteristics and to aid in the identification and characterization of additional limiting factors.

ACKNOWLEDGMENTS

This work was supported by the U. S. Environmental Protection Agency. B.R.F. was supported by a fellowship from the National Research Council.

We thank S. Cuskey and M. Shields for their comments during the preparation of the manuscript.

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