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Trichloroethylene degradation and mineralization by pseudomonads and *Methylosinus trichosporium* OB3b

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Abstract To examine the trichloroethylene (C_2HCl_3)-degrading capability of five microorganisms, the maximum rate, extent, and degree of C_2HCl_3 mineralization were evaluated for *Pseudomonas cepacia* G4, *Pseudomonas cepacia* G4 PR1, *Pseudomonas mendocina* KR1, *Pseudomonas putida* F1, and *Methylosinus trichosporium* OB3b using growth conditions commonly reported in the literature for expression of oxygenases responsible for C_2HCl_3 degradation. By varying the C_2HCl_3 concentration from 5 μM to 75 μM, V_{max} and K_m values for C_2HCl_3 degradation were calculated as 9 nmol/(min mg protein) and 4 μM for *P. cepacia* G4, 18 nmol/(min mg protein) and 29 μM for *P. cepacia* G4 PR1, 20 nmol/(min mg protein) and 10 μM for *P. mendocina* KR1, and 8 nmol/(min mg protein) and 5 μM for *P. putida* F1. This is the first report of these Michaelis-Menten parameters for *P. mendocina* KR1, *P. putida* F1, and *P. cepacia* G4 PR1. At 75 μM, the extent of C_2HCl_3 that was degraded after 6 h of incubation with resting cells was 61%–98%; the highest degradation being achieved by toluene-induced *P. mendocina* KR1. The extent of C_2HCl_3 mineralization in 6 h (as indicated by concentration of chloride ion) was also measured and varied from 36% for toluene-induced *P. putida* F1 to 102% for *M. trichosporium* OB3b. Since C_2HCl_3 degradation requires new bio-mass, the specific growth rate (μ_{max}) of each of the C_2HCl_3-degradation microorganisms was determined and varied from 0.080/h (*M. trichosporium* OB3b) to 0.864/h (*P. cepacia* G4 PR1).

Introduction

Chlorinated aliphatic compounds have been used widely as organic solvents and degreasing agents in industry (Janssen et al. 1985) with more than 80×10^6 kg (178×10^6 lb) trichloroethylene (C_2HCl_3) used industrially in the U.S. in 1985 alone (Wackett and Gibson 1988). As a consequence of its extensive usage and improper disposal, C_2HCl_3 is one of the most frequently detected pollutants in hazardous waste sites and in ground water and aquifers (U.S. EPA 1984). To restore these sites, bioremediation is viewed as a cost-effective method (McFarland et al. 1992; Winter et al. 1989). Microorganisms that are capable of oxidizing C_2HCl_3 aerobically include pseudomonads (Ensley and Kurisko 1994; Shields and Reagin 1992; Wackett and Householder 1989; Winter et al. 1989), methanotrophs (Brusseau et al. 1990; Green and Dalton 1989; Oldenhuis et al. 1989; Uchiyama et al. 1992), propane oxidizers (Wackett et al. 1989) and ammonia oxidizers (Rasche et al. 1991; Vannelli et al. 1990).

The methanotroph, *Methylosinus trichosporium* OB3b, may be used for bioremediation since its soluble methane monooxygenase degrades C_2HCl_3 as much as 50 times faster than pseudomonads (Jahng and Wood 1994). *M. trichosporium* OB3b expresses this enzyme in the presence of methane; however, its expression is repressed by low concentrations of copper ion (0.25 μM) (Tsien et al. 1989), and concentrations of copper in excess of 0.25 μM have been found in polluted ground water (Phelps et al. 1992).

With their diverse catabolic activity and ability to adapt to different environments, pseudomonads are well-suited for environmental remediation. With growth on phenol or toluene as a sole carbon source (Ensley and Kurisko 1994; Folsom et al. 1990) or by providing an additional source of carbon and energy (such as lactate) with subsequent induction of enzyme by the addition of toluene or phenol after initial growth (Folsom and Chapman 1991; Nelson et al. 1987; Shields et al. 1989), *P. cepacia* G4 expresses toluene *ortho*-monooxygenase and mineralizes C_2HCl_3 to CO_2 (Nelson et al. 1987). *P. cepacia* G4 PR1, a transposon mutant of *P. cepacia* G4, constitutively expresses

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toluene *ortho*-monooxygenase and grows in Luria-Bertani (LB) medium or medium containing lactate as a sole carbon source (Krumme et al. 1993; Shields and Reagin 1992). *P. putida* F1 degrades C_2HCl_3 using toluene dioxygenase (Wackett and Gibson 1988), which is expressed with growth on toluene supplied in the vapor phase as the sole carbon source (Wackett and Gibson 1988; Zylstra et al. 1988). *P. mendocina* KR1 expresses toluene *para*-monooxygenase with growth on vapor-phase toluene as the sole carbon source, and can also degrade C_2HCl_3 completely (Winter et al. 1989).

To select the best microorganism for C_2HCl_3 remediation, the maximum C_2HCl_3 degradation rate, the extent of both its degradation and mineralization, and the degree of stable expression of the C_2HCl_3-degrading enzyme (Ensley and Kurisko 1994) should be considered. Growth rate is also important because biomass must be replaced, since C_2HCl_3 breakdown products are somewhat toxic to the cells (Oldenhuis et al. 1991; Wackett and Gibson 1988; Wackett and Householder 1989; Zylstra et al. 1989) . *P. cepacia* G4 (Ensley and Kurisko 1994; Folsom and Chapman 1991), *P. cepacia* G4 PR1 (Shields et al. 1994). *P. mendocina* KR1 (Ensley and Kurisko 1994), and methanotrophs (McFarland et al. 1992) have been used in bioreactors to degrade C_2HCl_3; however, comparing degradation rates among these C_2HCl_3-degrading microorganisms is complicated by the lack of uniformity in the experiments reported in the literature as well as a lack of kinetic data. These data are essential for designing efficient bioreactors for *in situ* C_2HCl_3 remediation. In this work, four pseudomonads were studied to obtain V_{max} and K_m for C_2HCl_3 degradation. This is the first report of these values for *P. cepacia* G4 PR1, *P. mendocina* KR1, and *P. putida* F1. Additionally, the extent of C_2HCl_3 degradation and mineralization at 75 μM was determined along with the specific growth rates for the four pseudomonads and *M. trichosporium* OB3b.

Materials and methods

Bacteria and culture conditions

P. cepacia G4 (Folsom et al. 1990) and *P. cepacia* G4 PR1 (Shields and Reagin 1992) were provided by Professor Reagin, University of West Florida, Fla. *P. mendocina KR*1 (Yen et al., 1991) was obtained from Dr. Yen at Amgen Inc., Thousand Oaks, Calif., and *P. putida* F1 (Wackett and Gibson 1988) was provided by Professor Gibson at the University of Iowa, Iowa City, Iowa. *M. trichosporium* OB3b (Little et al. 1988) was provided by Professor Lidstrom at the California Institute of Technology,. Pasadena, Calif. *P. cepacia* G4 PR1 was cultured in LB medium (Maniatis et al. 1982) with 50 μg/ml kanamycin sulfate. Expression of the toluene oxygenases for *P. cepacia* G4, *P. mendocina* KR1, and *P. putida* F1 was done in two stages. *P. cepacia* G4 was grown overnight in M9 medium (Maniatis et al. 1982) with 5 mM phenol as the sole carbon source by inoculating from a −85°C glycerol stock (15% v/v); then 1%–2% of this seed culture was used to inoculate M9 medium with 5 mM phenol for further growth. *P. mendocina* KR1 and *P. putida* F1 were precultured in M9-glucose (0.4% w/v) (Maniatis et al. 1982) by inoculating from a −85°C glycerol stock (15% v/v); then 1%–2% of this seed culture was used to inoculate in M9 medium with toluene (0.4% v/v) supplied in the vapor phase as the sole carbon source. *M. trichosporium* OB3b was cultured by streaking a −85°C glycerol stock (15% v/v) onto a Higgins nitrate minimal salt (Park et al. 1991) plate with $CuSO_4$ deleted and incubating in 1:4 (v/v) methane/air gas mixture. After 4–5 days, a few loops-full of colonies were inoculated into Higgins nitrate minimal salt medium with $CuSO_4$ deleted. A 1:4 (v/v) methane/air gas mixture was bubbled into the inoculated culture medium for 5 min twice per day at a flow rate of 270 ml/min at 238 kPa. The gas mixture was sterilized through a 0.22-μm sterile bacterial air vent (Gelman Sciences, Ann Arbor, Mich.). All pseudomonads were grown in 50 ml cultures at 30°C in 250-ml conical flasks with shaking at 250 rpm (Series 25, New Brunswick Scientific Co. Inc., Edison, N.J.). All pseudomonads were grown for 16–24 h to reach the exponential phase of growth [absorbance at 600 nm (A_{600}) of 0.5–3.0]. *M. trichosporium* OB3b was grown either in 50-ml cultures in 250-ml side-arm flasks or 100-ml cultures in 500-ml conical flasks. Flasks were shaken at 250 rpm at 30 °C for 3–4 days to reach $A_{600} = 0.7–1.5$.

Specific growth rate and total cell protein

For *P. cepacia* G4 and *P. cepacia* G4 PR1, cell growth of 20-ml cultures was monitored by measuring the absorbance at 600 nm with a Milton Roy Spectronic 20 D spectrophotometer (Fischer Scientific, Tustin, Calif.). The initial A_{600} was always less than 0.05, and the specific growth rate was determined from data with the absorbance less than 1.0. Growth of 20-ml cultures of *P. mendocina* KR1, *P. putida* F1, and *M. trichosporium* OB3b was monitored using a 250-ml side-arm flask and a Klett-Summerson photoelectric colorimeter (Klett Mfg. Co. Inc., N.Y., N.Y.). A 100-μl aliquot of a glycerol stock at −85°C was used for the growth rate study of *M. trichosporium* OB3b instead of inoculating from a Higgins nitrate minimal-salt plate. For the determination of total cell protein, cells were lysed using a sonic dismembrator model 300 (Fischer Scientific, Tustin, Calif.) equipped with a micro-tip for 10 min at 60% of maximum output. The total cell protein was assayed using the BioRad Protein Assay (Hercules, Calif.) based on the Bradford method. Bovine serum albumin (0–0.7 mg/ml) was used to establish the protein standard curve.

Preparation of resting cell suspensions

For the determination of V_{max} and K_m of C_2HCl_3 degradation, exponentially growing cells were harvested by centrifugation (5000 *g* for 10 min at 4°C) and washed twice with cold (4°C) 0.1 M potassium phosphate buffer (PPB) at pH 7.0· (Maniatis et al. 1982). Cell suspensions used for determining the extent of C_2HCl_3 degradation and mineralization were centrifuged at 25°C (5000 *g* for 10 min) and washed twice with prewarmed 0.1 M PPB at 30°C. All cell suspensions were diluted to an A_{600} of 1.0 with prewarmed 0.1 M PPB at 30°C. For *M. trichosporium* OB3b, 20 mM sodium formate was added (when specified) to 0.1 M PPB 10 min before C_2HCl_3 addition, and 20 mM lactate or glutamate was added for *P. cepacia* G4 PR1 when indicated. Cell suspensions prepared were used within 1 h from the time of harvesting.

Rates of C_2HCl_3 degradation

5 ml of a resting cell suspension were transferred to prewarmed 60-ml glass serum vials. Vials were sealed with a Teflon-coated silicone septum (Wheaton, Millville, N.J.) and an aluminum crimp seal. A 0.1 M C_2HCl_3 stock standard was prepared daily (in

a vial with no headspace gas) by dissolving C_2HCl_3 (Fisher Scientific, Tustin, Calif.) in *N*, *N*-dimethylformamide (Fisher Scientific, Tustin, Calif.). A 0.1 M C_2HCl_3 stock was added using a 10-µl gas-tight syringe (Hamilton, Reno, Nev.) to triplicate sets of vials for each C_2HCl_3 concentration (5–75 µM assuming all the C_2HCl_3 remains in the liquid). The vials were shaken vigorously for 30 s then inverted and shaken at room temperature using a IKA-Vibrax-VXR shaker at 200 rpm (IKA-Works Inc., Cincinnati, Ohio).

A 5-µl gas sample was removed from the headspace of each sealed vial using a 50-µl gas-tight syringe (Hamilton, Reno, Nev.) at specific time intervals (5, 15, 30 and 60 min), and injected into a Varian GC-3600 gas chromatograph equipped with an electron-capture detector (Varian Associates, Sunnyvale, Calif.). The concentration of C_2HCl_3 was analyzed using a 1.8 m × 3.2 mm × 0.089 mm 0.1% AT-1000 on Graphpac GC, 80/100 mesh stainless-steel packed column (Alltech Associates Inc., Deerfield, Ill.) The injector and detector temperature were 170°C and 190°C respectively. The GC was operated isothermally at 150°C with nitrogen carrier gas (30 ml/min). Under these conditions, the C_2HCl_3 peak retention time was 1.5–1.6 min. The C_2HCl_3 degradation rates were determined by averaging the three peak heights of the three vials for each sample time and comparing results to the average results of negative controls consisting of 0.1 M PPB and C_2HCl_3 (5–75 µM) in crimped vials (zero-time values were determined with the negative control by averaging them over the first 5 min). The initial C_2HCl_3 degradation rate at each concentration was determined on the basis of the disappearance of the C_2HCl_3 peak between zero time and 5 min or between 5 min and 15 min. To determine the K_m values, the actual C_2HCl_3 concentration in the liquid phase was calculated using a dimensionless Henry's constant at 25°C (Folsom et al. 1990). Kinetic parameters for C_2HCl_3 degradation, V_{max} and K_m, were determined from the Lineweaver-Burk plot assuming that C_2HCl_3 degradation followed the Michaelis-Menten kinetic model.

Extent of C_2HCl_3 degradation

The extent of C_2HCl_3 degradation was determined in experiments independent of those used to determine the rate of degradation. C_2HCl_3 (75 µM) was added to three 60-ml vials containing 5 ml resting cell suspension ($A_{600} = 1.0$), and the vials were inverted and shaken at room temperature using an IKA-Vibrax-VXR shaker at 200 rpm. Over 6 h, a 5-µl gas sample was removed hourly from the headspace of each sealed vial using a 50-µl gas-tight syringe and C_2HCl_3 concentrations were determined with a GC as described above. The negative control consisted of triplicate vials crimped with 5 ml 0.1 M PPMB and 75 µM C_2HCl_3, and the average of 5-min samples was used to establish the C_2HCl_3 zero-time baseline value. Other negative controls consisted of heat-killed *P. cepacia* G4 PR1, non-induced *P. cepacia* G4, non-induced *P. mendocina* KR1, and non-induced *P. putida* F1 cell suspensions with C_2HCl_3 in 0.1 M PPB. The percentage C_2HCl_3 degradation was calculated as:

$$\frac{[(\text{average zero-time peak height}) - (\text{average sample peak height})] \times 100}{(\text{average zero-time peak height})}$$

Extent of C_2HCl_3 mineralization

10 ml resting of a cell suspension were used for these experiments to ensure an appropriate volume to submerge the electrodes. C_2HCl_3 (75 µM) was added to the vials which were inverted and shaken at room temperature using an IKA-Vibrax-VXR shaker at 200 rpm. Chloride ion concentrations were determined over a 6-h period by sacrificing duplicate or triplicate vials every hour using a model 13-620-519 chloride-ion-selective electrode and a model 13-620-47 reference silver chloride electrode (Fisher Scientific, Tustin, Calif.) attached to a Corning pH/ion analyzer 350 (Corning Incorporated, Corning, N.Y.) and calibrated with five NaCl standards (0.01–0.5 mM) made in 0.1 M PPB at room temperature. Liquid samples were analyzed for chloride ion generation by removal of the aluminium crimp tops and addition of 2% (v/v) 5 M sodium nitrate (ionic strength adjuster) to a well-stirred 25-ml beaker. Chloride ion readings were obtained within 5 min of the transfer of liquid samples to the beakers. The negative control consisted of a triplicate set of 10 ml 0.1 M PPB with 75 µM C_2HCl_3. The baseline chloride ion level was established using 10 ml appropriate resting cell suspension without C_2HCl_3 by measuring the chloride ion concentration within 1 h. Other negative controls consisted of heat-killed *P. cepacia* G4 PR1, non-induced *P. cepacia* G4, non-induced *P. mendocina* KR1 and non-induced *P. putida* F1 with C_2HCl_3 in 0.1 M PPM, which were monitored hourly for 6 h. For these controls, zero-time chloride ion concentrations were subtracted from samples to obtain the actual amount of chloride ion. The percentage of C_2HCl_3 mineralization was calculated as:

$$\frac{[(\text{average sample } Cl^-, \text{mM}) - (\text{average zero-time } Cl^-, \text{mM})] \times 100}{3(0.075\,\text{mM})},$$

and the mineralization efficiency (%) was calculated as (percentage C_2HCl_3 mineralized) × 100/(percentage C_2HCl_3 degraded) (Jahng and Wood 1994).

Results

Rates of growth and total protein concentration (Table 1)

The specific growth rate (μ_{max}) was obtained from the average of the maximum slope of the growth curve (ln A_{600} versus time) obtained from duplicate or triplicate flasks. Since *P. cepacia* G4 PR1 was grown in complex medium, it had the highest μ_{max} (0.864 ± 0.025/h in LB medium with 50 µg/ml kanamycin sulfate). The μ_{max} for *P. cepacia* G4 in M9 medium with 5 mM phenol as the sole carbon is 0.350 ± 0.024/h. *P. mendocina* KR1 and *P. putida* F1 cultured in M9 with 0.4% (v/v) toluene have μ_{max} values of 0.366 ± 0.011/h and 0.445 ± 0.020/h respectively. These μ_{max} values agrees well with the reported growth rate of toluene-induced *P. putida* F1 of 0.462/h (Wackett and Householder 1989) at 30°C and 0.45/h (Duetz et al. 1994) at 28°C as well as 0.38/h (Duetz et al. 1994) 28°C for *P. mendocina* KR1. The μ_{max} for *M. trichosporium* OB3b in Higgins nitrate minimal-salt medium with $CuSO_4$ deleted was determined to be 0.080 ± 0.005/h with the methane/air gas mixture replenished twice per day. This μ_{max} value agrees well with that of Park et al. ($\mu_{max} = 0.08$/h) (Park et al. 1991).

To determine the total protein content of the cells using a spectrophotometer (used to calculate V_{max} for C_2HCl_3 degradation), the pseudomonads and *M. trichosporium* OB3b were suspended in 0.1 M PPB with A_{600} adjusted to 1.0. The protein concentration of *P. cepacia* G4 PR1 was found to be the highest at 0.276 ± 0.001 mg/ml. The protein concentration ranged from 0.188 mg/ml to 0.259 mg/ml for the other strains (Table 1).

Table 1 Average specific growth rates and average total protein concentrations of microorganisms. All microorganisms were grown at 30 °C with shaking at 250 rpm. Luria-Bertani (*LB*) medium contained 50 μg/ml kanamycin sulfate. Higgins nitrate minimal-salt medium (*HNMS*) was prepared using distilled deionized water with $CuSO_4$ deleted. Values are mean ± S.D. for $n = 3$

Strains	Media	μ_{max} (h^{-1})	Protein content at $A_{600} = 1.0$ (mg/ml)
P. cepacia G4 PR1	LB	0.864 ± 0.025	0.276 ± 0.001
P. cepacia G4	M9 + 5 mM phenol	0.350 ± 0.024	0.259 ± 0.008
P. mendocina KR1	M9 + 0.4% (v/v) toluene	0.366 ± 0.011	0.188 ± 0.003
P. putida F1	M9 + 0.4% (v/v) toluene	0.445 ± 0.020	0.218 ± 0.004
M. trichosporium OB3b	HNMS + 1:4/methane:air	0.080 ± 0.005	0.200 ± 0.001

Table 2 Initial average rates of trichloroethylene (C_2HCl_3) degradation using resting cells as a function of C_2HCl_3 concentration. C_2HCl_3 concentrations in the liquid phase were determined using a Henry's law constant of 0.4 (Folsom et al. 1990). Values are means ± SD for $n = 3$

Microorganisms	C_2HCl_3 degradation rate [nmol/(min mg protein)] for an initial concentration in the liquid phase of:							
	0.93 μM	1.85 μM	3.70 μM	5.56 μM	7.41 μM	9.26 μM	11.11 μM	13.89 μM
P. cepacia G4 PR1	0.56 ± 0.09	1.13 ± 0.17	2.04 ± 0.28	2.46 ± 0.37	3.15 ± 0.38	4.37 ± 0.44	6.18 ± 0.06	7.90 ± 0.88
P. cepacia G4	1.66 ± 0.16	3.25 ± 0.36	4.18 ± 1.16	5.39 ± 0.34	7.01 ± 2.02	6.77 ± 0.19	6.21 ± 0.39	7.00 ± 0.51
P. mendocina KR1	1.63 ± 0.57	3.29 ± 0.76	5.98 ± 1.92	6.79 ± 0.53	7.47 ± 1.19	8.24 ± 0.49	10.38 ± 1.09	11.74 ± 2.69
P. putida F1	1.17 ± 0.33	1.48 ± 0.27	3.49 ± 0.57	2.84 ± 0.29	3.92 ± 1.21	5.41 ± 1.36	6.37 ± 0.75	7.15 ± 1.01

Rates of C_2HCl_3 degradation (Table 2)

The actual C_2HCl_3 concentration in the liquid phase was varied from 0.93 μM to 13.89 μM [well below the concentration that is toxic to these organisms (Folsom et al. 1990; Oldenhuis et al. 1991, 1989; Shields and Reagin 1992)] to measure the initial rate of its degradation by the microorganisms. The value for V_{max} represents the maximum degradation rate of C_2HCl_3, and K_m represents the affinity of the intact resting cells for C_2HCl_3 (including its transport through the cellular membrane). The C_2HCl_3 concentration for these experiments in the liquid phase is significantly less than that predicted by assuming that all the added C_2HCl_3 remains in the liquid phase because of its partitioning between the 55 ml gas and 5 ml liquid (Folsom et al. 1990). The actual C_2HCl_3 concentration in the liquid phase was calculated using the dimensionless Henry's constant (ratio of C_2HCl_3 concentration in the gas and liquid = 0.4) (Folsom et al. 1990).

To show that there were not any limitations for the transport of C_2HCl_3 between the gas and the liquid phases during the course of the degradation rate experiments, two sets of 60-ml glass serum vials were inoculated with C_2HCl_3 to yield liquid concentrations of 20 μM and 50 μM (stock C_2HCl_3 solution injected into 0.1 M PPB) and changes in the C_2HCl_3 gas concentrations were determined at 1, 3, 5, 10, 15, 30, and 60 min after C_2HCl_3 addition. Using the average GC peak heights for C_2HCl_3 obtained after 15–60 min equilibration time for the zero-time values, it was determined that C_2HCl_3 equilibrium was established between the gas and liquid phases within the first 5 minutes of its addition (after 5 min, 108 ± 5% and 110 ± 16% of the average C_2HCl_3 peak heights were obtained). These results agree with those of Folsom et al. (1990) and Wackett and Gibson (1988), who reported that transport limitations for C_2HCl_3 between the gas and liquid phases were negligible for these degradation rate experiments.

The initial C_2HCl_3 degradation rates as a function of its concentration are presented in Table 2. V_{max} and K_m were calculated using a Lineweaver-Burk plot of the initial degradation rates (Table 3). V_{max} and K_m for *P. cepacia* G4 PR1 are 18 nmol/(min mg protein) and 29 μM respectively. These are the first reported V_{max} and K_m values for *P. cepacia* G4 PR1. For comparison, a maximal C_2HCl_3 degradation rate of approx. 1 nmol/(min mg protein) was measured at 20 μM C_2HCl_3 in batch culture by Shields and Reagin (1992).

Additional V_{max} values were obtained in this work with two independent experiments using *P. cepacia* G4 PR1 resting cell suspensions supplemented with 20 mM glutamate or 20 mM lactate. These substrates were chosen since they are tricarboxylic acid cycle intermediates and may supply additional NADH to drive the C_2HCl_3 reaction. Based on four different C_2HCl_3 concentrations (5, 20, 30, and 40 μM), a V_{max} of 15 and 18 nmol/(min mg protein) and K_m value of 13 μM and 17 μM were obtained for glutamate and lactate respectively. These results agree well with results obtained using cells that degraded C_2HCl_3 in buffer alone (V_{max} = 18 nmol/min mg protein). To corroborate further that C_2HCl_3 oxidation was not reductant-limited in *P. cepacia* G4 PR1, independent

Table 3 Comparison of Michaelis-Menten kinetic parameters for C_2HCl_3 degradation. The initial degradation rate (V) was determined at a given C_2HCl_3 concentration

Microorganisms	V_{max} [nmol/(min mg protein)]	K_m (μM)	Initial V [nmol/(min mg protein)]	C_2HCl_3 (μM)	Reference
P. cepacia G4	9	4	—	—	This study
P. cepacia G4	8	3	—	—	Folsom et al. 1990
P. cepacia G4 PR1	18	29	—	—	This study
P. cepacia G4 PR1[c]	15	13	—	—	This study
P. cepacia G4 PR1[d]	18	17	—	—	This study
P. cepacia G4 PR1	—	—	~ 1	20	Shields and Reagin 1992
P. mendocina KR1	20	10	—	—	This study
E. coli FM5/pKY287 (containing *tmoABCDE* of KR1)	1–2	—	—	—	Winter et al. 1989
P. putida F1	8	5	—	—	This study
P. putida F1	—	—	1.8	80	Wackett and Gibson 1988
M. trichosporium OB3b[a]	—	—	37.5	75	This study
M. trichosporium OB3b[b]	—	—	40.4	75	This study
M. trichosporium OB3b	—	—	~ 35[a]	80	Tsien et al. 1989
M. trichosporium OB3b[b]	509[a, b]	145[b]	~ 47[a]	200	Oldenhuis et al. 1991; Oldenhuis et al. 1989

[a] For *M. trichosporium* OB3b at $A_{600} = 1.0$, a conversion factor of 0.57 g protein/(g cell) was used based on the basis of a calibration curve of total cellular protein concentration versus dried cell weight (unpublished results)

[b] C_2HCl_3 degradation rate performed with 20 mM sodium formate in 0.1 M potassium phosphate buffer

[c] Maximum C_2HCl_3 degradation rate obtained with 20 mM glutamate

[d] Maximum C_2HCl_3 degradation rate obtained with 20 mM lactate

experiments in the presence of 20 mM reducing equivalents of succinate, lactate, glutamate, or acetate were performed to measure an initial degradation rate at 20 μM C_2HCl_3. The initial degradation rates were 1.66 ± 0.11, 1.58 ± 1.11, 2.88 ± 0.31, and 2.50 ± 0.54 nmol/(min mg protein) for succinate, lactate, glutamate, and acetate respectively. The initial C_2HCl_3 degradation rate without a source of reducing equivalents at 20 μM C_2HCl_3 was 2.04 ± 0.28 nmol/(min mg protein) (Table 2). Therefore, the degradation rates determined for *P. cepacia* G4 PR1 were not limited by the concentration of reducing equivalents.

V_{max} for *P. cepacia* G4 was determined to be 9 nmol/(min mg protein) with a K_m of 4 μM. These results agree well with that of Folsom et al. (1990) [8 nmol/(min mg protein) and 3 μM], who also showed that *P. cepacia* G4 cultured in a chemostat under continuous induction of 10 mM phenol can degrade C_2HCl_3 at 6.1 nmol/(min mg protein) (Folsom and Chapman 1991). The addition of a co-substrate, such as lactate, with phenol was studied to compare the C_2HCl_3 degradation rate for *P. cepacia* G4 PR1. A 20 mM solution of lactate in M9 with the addition of 5 mM phenol for *P. cepacia* G4 increased biomass production but lowered the C_2HCl_3 degradation rate, and similar results were observed by Folsom and Chapman (1991).

V_{max} and K_m values for *P. putida* F1 were determined to be 8 nmol/(min mg protein) and 5 μM. These are also the first reported V_{max} and K_m values for *P. putida* F1. The most frequently and highest reported initial rate is 1.8 nmol/(min mg protein) for *P. putida* F1 at 80 μM of C_2HCl_3 concentration (Oldenhuis et al. 1989; Wackett and Gibson 1988; Winter et al. 1989). At 75 μM C_2HCl_3, an initial degradation rate of 7.15 nmol/(min mg protein) was measured for *P. putida* F1 in this work; this value is almost four times higher than the reported value of 1.8 nmol/(min mg protein).

For *P. mendocina* KR1, V_{max} and K_m were found to be 20 nmol/(min mg protein) and 10 μM. There are no reported values for V_{max} and K_m for *P. mendocina* KR1 for comparison. The most often reported value for C_2HCl_3 degradation for *P. mendocina* KR1 is 1–2 nmol/(min mg protein) (Winter et al. 1989), which is for recombinant *E. coli* cell suspensions containing the *P. mendocina* KR1 *tmoABCDE* genes (*E. coli* FM5/pKY287).

The initial C_2HCl_3 degradation rate for *M. trichosporium* OB3b was measured as 37.5 nmol/(min mg protein) at 75 μM C_2HCl_3. Upon addition of 20 mM sodium formate to the resting cell suspension, the initial degradation rate increased to 40.4 nmol/(min mg protein). Tsien et al. (1989) previously reported the maximum initial C_2HCl_3 degradation rate to be 20 nmol/(min mg cell) at 80 μM C_2HCl_3. Using a dry cell weight of 0.35 mg/ml at $A_{600} = 1.0$ (unpublished result for *M. trichosporium* OB3b) and 0.2 mg total protein/ml at $A_{600} = 1.0$ (Table 1) for *M. trichosporium* OB3b, 1 mg/ml dried cell weight is equivalent to 0.57 mg/ml total cell protein. Thus, the reported maximum initial C_2HCl_3 degradation rate is equivalent to roughly 35 nmol/(min mg protein), which

agrees well with the initial rate measured in this study. Oldenhuis et al. (1989) reported the initial C_2HCl_3 degradation rate of 27 nmol/(min mg cell) at 200 μM C_2HCl_3 with addition of 20 mM sodium formate in batch-cultured *M. trichosporium* OB3b; this is equivalent to roughly 48 nmol/(min mg protein). A V_{max} of 509 ± 174 nmol (min mg protein) and K_m of 145 ± 61 μM are obtained using *M. trichosporium* OB3b grown in continuous cultures under copper stress with 20 mM formate (Oldenhuis et al. 1991).

Using the independent first-hour results obtained for the extent of C_2HCl_3 degradation at 75 μM shown in Fig. 1, a different set of maximum initial degradation rate can be calculated as compared with those based on the 0–5 min or 5–15 min data at 75 μM (Table 2): 1.31 versus 7.90 nmol/(min mg protein) for *P. cepacia* G4 PR1, 0.90 versus 7.00 nmol/(min mg protein) for *P. cepacia* G4, 4.10 versus 11.74 nmol/(min mg protein) for *P. mendocina* KR1, and 1.38 versus 7.15 nmol/(min mg protein) for *P. putida* F1. Thus, the proper time scale of measurement is critical for the determination of the degradation rate when using resting cells. Besides the time scale, differences in the total protein concentration in cells can also affect the calculation of initial degradation rates. A total protein concentration of 0.259 mg/ml was measured for *P. cepacia* G4 at $A_{600} = 1.0$. This value is more than twofold higher than the 0.114 mg/ml total protein concentration used by Folsom et al. at $A_{600} = 1.0$ (1990). A higher protein concentration will lead to a lower C_2HCl_3 degradation rate; thus, the V_{max} and K_m values can be affected.

To stabilize the activity of the oxygenases, cells were centrifuged and washed twice at 4°C before the C_2HCl_3 degradation rate at various concentrations was determined; however, the resting cells may have been sensitive to the cold environment. Hence, the effect of cold washes (4°C PPB) compared to warm washes (30°C PPB) on the C_2HCl_3 degradation rate was assessed using *P. cepacia* G4 PR1. Although the initial C_2HCl_3 degradation rates with warm washing were slightly higher than those with cold PPB washing at low C_2HCl_3 concentrations, no differences in initial rates were measured for higher concentrations (data not shown, initial rates determined as before at 5, 10, 20, 30, 40, 50, 75 μM). The K_m value decreased from 29 μM (cold washing) to 16 μM (warm washing), and the V_{max} value was decreased slightly from 18 (cold washing) to 15 nmol/(min mg protein) (warm washing).

Extent of C_2HCl_3 degradation (Fig. 1)

Along with V_{max} and K_m, the degree to which the C_2HCl_3 degradation reaction was sustained was quantified by measuring the extent of degradation by resting cells over 6 h. At 75 μM, the initial extent of

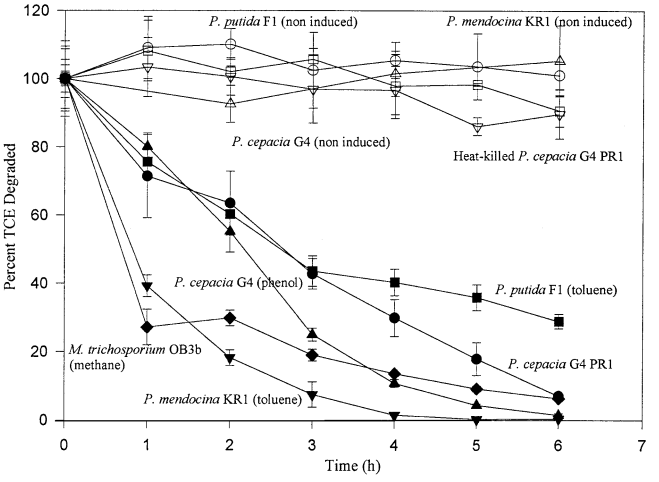

Fig. 1 Extent of trichloroethylene (*TCE*; initial concentration 75 μM) degradation by resting cells as determined from GC measurements. Data are averages of triplicate vials (error bars shown). For the non-induced negative controls, cultures were grown in M9/glucose (0.4% w/v)

degradation after the first hour was similar for *P. cepacia* G4 PR1 (29%), *P. cepacia* G4 (20%) and *P. putida* F1 (24%); however, more extensive degradation of C_2HCl_3 was observed with *P. mendocina* KR1 (61%) and *M. trichosporium* OB3b (73%). After 6 h had elapsed, similar, high levels of C_2HCl_3 degradation (93%–99%) occurred with all the strains (*P. mendocina* KR1 had the most extensive degradation) except *P. putida* F1 (71%). For comparison, Oldenhuis et al. (1989) found that 200 ml *M. trichosporium* OB3b (0.15–0.2 mg cells/ml) degraded approximately 45% of 125 μM C_2HCl_3 after 30 h of C_2HCl_3 addition. With 2 ml cells at $A_{600} = 1.0$, Wackett and Gibson (1988) found that *P. putida* F1 degraded 67% of 15 μM C_2HCl_3 in 6 h. Since there was no significant change in the C_2HCl_3 concentration for all five of the negative controls, its degradation occurred in the induced strains as a result of the enzymatic attack of the oxygenase.

C_2HCl_3 degradation was roughly linear for all the strains except for *M. trichosporium* OB3b (reaching a plateau after 1 h) and *P. putida* F1 (reaching a plateau after 3 h). This decrease in the degradation rate could be due to either the formation of toxic intermediates (Wackett and Gibson 1988) or depletion of reductant (e.g. NADH); however, oxygen does not limit the C_2HCl_3 reaction since it is present in excess, and the resting cells are not actively growing (no substrate present). From Fig. 1, the plateau phenomena were most pronounced for *P. putida* F1 and *M. trichosporium* OB3b, and this suggests that *P. putida* F1 and *M. trichosporium* OB3b were the most sensitive microorganisms to either event. For *M. trichosporium* OB3b, C_2HCl_3 oxidation is limited by the amount of reduc-

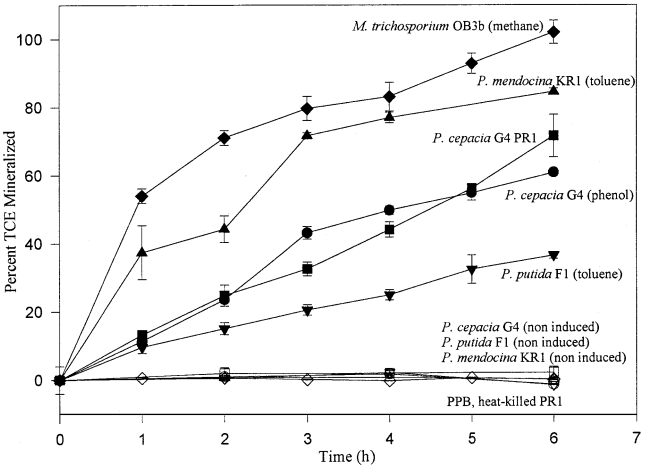

Fig. 2 Extent of trichloroethylene (*TCE*; initial concentration 75 μM) mineralization by resting cells as determined from chloride ion measurements. Data are averages of duplicate or triplicate vials (error bars shown). For the non-induced negative controls, cultures were grown in M9/glucose (0.4% w/v)

tant available in the cells, and the presence of reducing equivalents in the form of formate can enhance the rate and the extent of C_2HCl_3 degradation (Brusseau et al. 1990; Oldenhuis et al. 1989).

Extent of C_2HCl_3 mineralization and efficiency (Fig. 2, Table 4)

After the initial attack by the oxygenases, it is important to determine the degree to which the cells are capable of completely mineralizing the C_2HCl_3 epoxide to CO_2 and chloride ions. As an indicator of complete mineralization (Nelson et al. 1987), the concentration of chloride ions was monitored over 6 h. The trends for the extent of C_2HCl_3 mineralization by the resting cells are similar to those for the extent of its degradation. After 1 h of C_2HCl_3 addition, *P. putida* F1 mineralized C_2HCl_3 the least (10%), and *M. trichosporium* OB3b mineralized it at the fastest rate (54%). After 6 h, *M. trichosporium* OB3b mineralized 102% of the 75 μM C_2HCl_3, and *P. mendocina* KR1 mineralized 84%. The other pseudomonads mineralized C_2HCl_3 to a lower extent (61% for *P. cepacia* G4, 72% *P. cepacia* G4 PR1, and 36% for *P. putida* F1). The C_2HCl_3 mineralization rate was roughly linear for *P. cepacia* G4 PR1, *P. cepacia* G4, and *P. putida* F1. No C_2HCl_3 mineralization was detected for any of the five negative controls; hence, C_2HCl_3 was mineralized in the induced strains as a result of enzymatic attack.

Mineralization efficiency, the percentage C_2HCl_3 mineralized that is degraded (Jahng and Wood 1994), is a good measure of the ability of a microorganism to carry out this process. After 1 h of C_2HCl_3 addition, *M. trichosporium* OB3b had the highest mineralization efficiency (75%) while *P. putida* F1 had the lowest mineralization efficiency (40%). After 6 h, the mineralization efficiency of *M. trichosporium* OB3b was also the highest (109%), and 77%, 62%, and 85% mineralization efficiencies were achieved by *P. cepacia* G4 PR1, *P. cepacia* G4, and *P. mendocina* KR1 respectively. *P. putida* F1 had the lowest mineralization efficiency of all the microorganisms tested with only 51% of the C_2HCl_3 that was degraded mineralized after 6 h. For all the microorganisms tested, the C_2HCl_3 mineralization efficiency value did not change significantly (variation of 6%–18%) during 6 h.

Discussion

To identify a superior microorganism for C_2HCl_3 remediation and to measure important kinetic constants for bioreactor design, the maximum degradation rate as well as the extent of C_2HCl_3 degradation and mineralization were evaluated under consistent experimental conditions. Since the oxidized C_2HCl_3 intermediate is somewhat toxic to the cells (e.g. 0.48 mg *M. trichosporium* OB3b is inactivated/μmol C_2HCl_3 degraded) (Oldenhuis et al. 1991), new biomass must be continuously produced; hence, the specific growth rate was also determined. Since *P. cepacia* G4 PR1 constitutively produces toluene *ortho*-monooxygenase, it could be cultured in complex medium and therefore had the highest specific growth rate. In contrast, *M. trichosporium* OB3b cultured on 25% methane/air mixture had the lowest specific growth rate and grew 11-fold slower than *P. cepacia* G4 PR1.

A comparison of the kinetic parameters shows there is a significant difference in the V_{max} values

Table 4 Average C_2HCl_3 mineralization efficiency by resting cells. The initial C_2HCl_3 concentration was 75 μM. *ND* not determined. Values are means ± SD for $n = 2$ or 3

Microorganisms	C_2HCl_3 mineralization efficiency (%) after:					
	1 h	2 h	3 h	4 h	5 h	6 h
P. cepacia G4 PR1	46 ± 13	66 ± 12	57 ± 7	63 ± 7	68 ± 6	77 ± 6
P. cepacia G4	57 ± 5	52 ± 7	57 ± 4	56 ± 2	57 ± 2	62 ± 1
P. mendocina KR1	61 ± 11	54 ± 6	78 ± 5	78 ± 2	ND	85 ± 1
P. putida F1	40 ± 6	37 ± 3	36 ± 6	41 ± 5	50 ± 8	51 ± 3
M. trichosporium OB3b	74 ± 7	101 ± 5	98 ± 5	96 ± 5	102 ± 4	109 ± 4

[8–20 nmol/(min mg protein)]; however, a greater difference occurs in the kinetic parameter K_m (4–29 μM). The difference in K_m could be due to differences in uptake, transport, or diffusion of C_2HCl_3 and greatly affects reactor performance. For example, if a batch reactor is used to degrade C_2HCl_3 in ground water and Michaelis-Menten enzyme kinetics holds (neglecting the partitioning of C_2HCl_3 between the liquid and the gas phases), then the time required for the degradation is:

$$t = \frac{K_m \ln\left[\frac{c_0}{c}\right] + (c_0 - c)}{X V_{max}}$$

where c_0 and c are initial and final C_2HCl_3 concentrations, respectively, and X is the amount of total cell protein per liter of reactor volume. Therefore, using the V_{max} and K_m constants determined in this work [Table 3, data from Oldenhuis et al. (1991) used for *M. trichosporium* OB3b] and assuming $A_{600} = 1.0$, the time predicted to degrade 99% of 75 μM is 32, 40, 42, 56, and 93 min for *P. mendocina* KR1, *P. cepacia* G4, *P. cepacia* G4 PR1, *P. putida* F1, and *M. trichosporium* OB3b respectively. Except for *M. trichosporium* OB3b, these predicted times match the trend shown after 6 h for thedegradation of 75 μM C_2HCl_3 shown in Fig. 1 (it takes longer to degrade C_2HCl_3 with resting cell suspensions since C_2HCl_3 is somewhat toxic, it partitions between the two phases, and the reducing equivalents are being exhaused because of the lack of nutrients).

C_2HCl_3 mineralization is initiated when it is oxidized by the oxygenase to form the epoxide, and subsequent spontaneous and enzymatic processes convert the trichloroethylene epoxide to other intermediates (primarily carbon monoxide, formate, chloral, and glyoxylate) (Fox et al. 1990; Wackett and Householder 1989; Wackett et al. 1994). Although the V_{max} and K_m values determined by this work are corroborated by the extent of C_2HCl_3 degradation curves shown in Fig. 1, these constants are unable to predict the extent of mineralization shown in Fig. 2. For example, *M. trichosporium* OB3b ranked third for its extent of C_2HCl_3 degradation; however, it has the best extent of mineralization (Figs. 1, 2). Hence, for remediation of C_2HCl_3, emphasis should also be placed on the ability of an organism to mineralize it completely.

The C_2HCl_3 degradation and mineralization experiments (6 h each) were conducted at room temperature, which is not the optimal temperature for the microorganisms studied. Since the optimum temperature for the activity of these oxygenase enzymes is 30 °C, it is likely that the maximum degradation rates could be higher than the values reported in this study. In addition, for C_2HCl_3 mineralization analysis, all liquid samples were measured within 5 min for chloride ion generation to detect the actual chloride ion concentration at the times indicated in Fig. 2. If the samples are stored and chloride ion concentration measurements are delayed, both biotic and abiotic decay of C_2HCl_3 intermediates occurs, which leads to the samples having an incorrect and high chloride ion concentration. Thus, some of the difference between the percentage C_2HCl_3 degradation and percentage mineralization is due to incomplete mineralization of C_2HCl_3 intermediates within cells.

The experimental data indicated some surprising results. Of the four psuedomonads, *P. mendocina* KR1 has the fastest C_2HCl_3 degradation rate and degrades it to the lowest concentration in the shortest time; however, *P. mendocina* KR1 does not have the highest affinity toward C_2HCl_3 (K_m of *P. cepacia* G4 2.5 times lower that of *P. mendocina* KR1). *P. cepacia* G4 has the highest affinity for C_2HCl_3 and degrades it to a greater extent than does *P. cepacia* G4 PR1 and *P. putida* F1; however, it has a low degradation rate (45% of *P. mendocina* KR1). For operating a bioreactor for degrading C_2HCl_3, *P. cepacia* G4 PR1 and *P. cepacia* G4 should perform similarly on the basis of their comparable C_2HCl_3 degradation and mineralization results; however, the greatest advantage of *P. cepacia* G4 PR1 is its ability to express toluene *ortho*-monooxygenase constitutively. Since *P. cepacia* G4 PR1 does not require an inducer, and also has a high specific growth rate, *P. cepacia* G4 PR1 is an excellent microorganism for bioremediation. Furthermore, in a novel bioreactor that has been developed with *P. cepacia* G4 PR1 immobilized on activated carbon, stable C_2HCl_3 degradation and mineralization have been observed with this strain for a period longer than 2 months (unpublished results).

Even though *M. trichosporium* OB3b can degrade and mineralize C_2HCl_3 at the fastest rate, it is probably not a good choice for bioremediation because of its extremely slow growth, high K_m, and extreme sensitivity to copper ion suppression of the expression of soluble methane monooxygenase. On the basis of it having the slowest degradation rate and poorest extent of both C_2HCl_3 degradation and mineralization, *P. putida* F1 might also be a poor choice for C_2HCl_3 degradation. In contrast, *P. mendocina* KR1 is a superior strain for C_2HCl_3 degradation. Thus, it appears to be advantageous to develop *P. mendocina* KR1 strains for constitutive expression of toluene *para*-monooxygenase for the degradation of C_2HCl_3 (as has been pursued by R. J. Steffan et al. at Envirogen Inc., Princeton Research Center, Lawrenceville, N.J. 08648).

The information obtained using resting cells in batch experiments has been shown to correlate well with the kinetic constants obtained using continuous reactors (Folsom and Chapman 1991). Therefore, the kinetic parameters determined in this study can be used as starting values for designing efficient bioreactors for C_2HCl_3 remediation.

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