Biodegradation **15:** 19–28, 2004.

Trichloroethylene degradation by toluene-oxidizing bacteria grown on non-aromatic substrates

Chris M. Yeager[1]*, Kirstin M. Arthur[1], Peter J. Bottomley[1,2] & Daniel J. Arp[1,3]*
[1]*Molecular and Cellular Biology Program,* [2]*Department of Microbiology and Crop and Soil Sciences, and* [3]*Department of Botany and Plant Pathology, Corvallis, OR 97331-2902, USA (*author for correspondence: e-mail: arpd@science.oregonstate.edu)*

Accepted 26 September 2003

Key words: bioremediation, catabolite repression, fructose, *Pseudomonas mendocina*, toluene, trichloroethylene

Abstract

The potential of trichloroethylene (TCE) to induce and non-aromatic growth substrates to support TCE degradation in five strains (*Pseudomonas mendocina* KR1, *Ralstonia pickettii* PKO1, *Pseudomonas putida* F1, *Burkholderia cepacia* G4, *B. cepacia* PR1) of toluene-oxidizing bacteria was examined. LB broth and acetate did not support TCE degradation in any of the wild-type strains. In contrast, fructose supported the highest specific levels of TCE oxidation observed in each of the strains tested, except *B. cepacia* G4. We discuss the potential mechanisms and implications of this observation. In particular, cells of *P. mendocina* KR1 degraded significant amounts of TCE during cell growth on non-aromatic substrates. Apparently, TCE degradation was not completely constrained by any given factor in this microorganism, as was observed with *P. putida* F1 (TCE was an extremely poor substrate) or *B. cepacia* G4 (lack of oxygenase induction by TCE). Our results indicate that multiple physiological traits are required to enable useful TCE degradation by toluene-oxidizing bacteria in the absence of aromatic cosubstrates. These traits include oxygenase induction, effective TCE turnover, and some level of resistance to TCE mediated toxicity.

Abbreviations: DMF – *N,N*-dimethylformamide; TCE – trichloroethylene

Introduction

Aerobic biodegradation of the common groundwater pollutant trichloroethylene (TCE) occurs via cometabolism, whereby microorganisms utilize non-specific oxygenases to catalyze TCE oxidation (Ensley 1991; Arp 1995). Until recently, it was a commonly accepted belief that synthesis of monooxygenases or dioxygenases capable of TCE oxidation was dependent on induction by a natural substrate of the transforming oxygenase. To achieve maximal rates of induction, the inducing substrate is often provided as the sole source of carbon and/or energy for the TCE-degrading microorganism(s). However, reliance upon these natural substrates to support TCE cometabolism can be problematic since they often act as inhibitors of co-substrate oxidation (Folsom et al. 1990; Strand et al. 1990; Hyman et al. 1995). If the physiological growth substrate is in excess, rates of TCE oxidation will be repressed. On the other hand, if too much TCE is oxidized relative to the natural growth substrate, the microorganism will face a net drain of reductant and eventually lose the capacity for growth and cometabolic activity. Since it is difficult to precisely regulate the concentrations of substrates during many bioremediation applications, it is of practical importance to develop or discover systems in which the induction of TCE-degrading oxygenases is not dependent upon the presence of substrates harvested by the same enzymes (e.g., simple aromatics such as phenol or toluene).

Reports that TCE itself, or an oxidation product of this compound, can induce toluene oxygenase activity

* Present address: Los Alamos National Laboratory, Biosciences Division, M888, Los Alamos, NM 87545, USA

in some toluene-oxidizing bacteria including *Pseudomonas putida* F1, *Burkholderia cepacia* G4, *Ralstonia pickettii* PKO1, and *Pseudomonas mendocina* KR1 (Heald & Jenkins 1994; McClay et al. 1995; Leahy et al. 1996; Shingleton et al. 1998) raise the possibility that TCE degradation could be supported by alternative, non-aromatic growth substrates. In each of these microorganisms, TCE oxidation has been shown to be catalyzed by a toluene mono- or dioxygenase (*P. putida* F1, toluene dioxygenase (Wackett & Gibson 1988); *P. mendocina* KR1, toluene 4-monooxygenase (Winter et al. 1989; McClay et al. 1995); *R. pickettii*, toluene 3-monooxygenase (Olsen et al. 1994); *B. cepacia* G4 and PR1, toluene 2-monooxygenase (Shields et al. 1991; Newman & Wackett 1997)). Furthermore, TCE degradation has been demonstrated in toluene-oxidizing strains grown on lactate and glutamate (McClay et al. 1995; Leahy et al. 1996). However, there is some ambiguity among these reports. For example, TCE-mediated induction of toluene dioxygenase in *P. putida* F1 has been observed (Leahy et al. 1996; Shingleton et al. 1998), yet it has also been reported that TCE does not induce toluene-oxidizing activity in this organism (McClay et al. 1995). There have also been conflicting reports on the ability of TCE to induce toluene-oxidizing activity in *B. cepacia* G4 (McClay et al. 1995; Leahy et al. 1996). In each of these studies different growth substrates, culture conditions, and concentrations of TCE were utilized. Taken together, these observations imply that the choice of growth substrate could greatly affect the extent to which TCE can stimulate its own degradation among wild type strains of toluene-oxidizing bacteria.

The objectives of the current study were to examine the TCE-degrading capacity of five toluene-oxidizing strains, *B. cepacia* G4, *B. cepacia* PR1, *P. putida* F1, *P. mendocina* KR1, and *R. pickettii* PKO1, while grown on select sugars, organic acids, and rich media, and to characterize the physiological limitations of TCE transformation under these conditions.

Materials and methods

Bacterial strain and culture conditions

B. cepacia G4 and *B. cepacia* PR1 were provided by Malcolm Shields (University of West Florida, Pensacola), *P. putida* F1 was obtained from the American Type Culture Collection (#700007), *R. pickettii* PKO1 was provided by Ronald Olsen (University of Michigan, Ann Arbor), and *P. mendocina* KR1 was provided by Amgen (Thousand Oaks, CA). All strains were maintained on minimal medium agar plates containing 20 mM lactate (Yeager et al. 1999). Liquid cultures were grown overnight at 30 °C with shaking in sealed serum vials (160 ml) containing minimal media (60 ml) with the indicated carbon source (20 mM). Growth with toluene (1.0 mM initial aqueous phase concentration) was achieved by two sequential additions of 9 μl toluene, as previously described (Yeager et al. 1999). The aqueous concentrations of toluene and TCE in liquid-gas systems at 30 °C were calculated with dimensionless Henry's constants (H_c; (mol/L gas concentration)/(mol/L aqueous concentration)) of 0.343 (Yeager et al. 1999) and 0.494 (Gossett 1987), respectively. The initial aqueous phase concentration of volatile substances in sealed serum vials was calculated by back calculating from: gas phase concentration = total moles added/((aqueous volume/H_c) + gas volume). Toluene (99.8%) and TCE (99+%) were obtained from Aldrich (Milwaukee, WI). To collect cells for experimental assays, cultures were centrifuged, rinsed twice with 50 mM KH_2PO_4—K_2HPO_4 buffer, pH 7.0 (phosphate buffer) and resuspended in fresh phosphate buffer as a concentrated cell solution. The cell suspension was stored at room temperature for $\leq$1 h before use.

TCE degradation by strains grown with various substrates

Cells of each strain were grown overnight in minimal medium containing a select growth substrate (20 mM) or in LB broth and harvested as described above. A portion of the harvested cells was used to inoculate fresh medium (60 ml in 160 ml serum vials sealed with Teflon-lined butyl rubber stoppers) containing the same growth substrate (20 mM) and 1.1, 5.5, or 21.8 μmol of TCE. The initial aqueous phase concentration of TCE was 10, 50, and 200 μM for these additions, respectively. Cells were added to give each culture an initial OD_{600} of 0.05. The amount of TCE in each vial was measured by GC analysis of headspace samples immediately following addition of cells and after the cultures had incubated at 30 °C with shaking for 16–24 h (Table 1). All cultures had ceased growing after 16–24 hand there was no evidence of further TCE consumption with increased incubation periods. In an additional set of experiments (Figure 1), TCE consumption by growing cultures was monitored more

Table 1. TCE degradation by toluene-oxidizing bacteria grown on various substrates

Growth substrate	TCE added (μmol)	TCE Degraded (μmol)[a] F1	KR1	PKO1	G4	PR1
Lactate	1.1	–[b]	1.1, 1.1	–	–	1.1, 1.1
	5.5	–	1.3, 1.5	–	–	5.5, 5.5
Acetate	1.1	ND[c]	ND	ND	ND	ND
	5.5	–	–	–	–	2.9, 2.8
Glucose	1.1	–	0.8, 0.7	–	–	1.1, 1.1
	5.5	–	0.8, 0.8	0.5, 0.2	–	5.3, 5.1
Fructose	1.1	0.4, 0.5	1.1, 1.1	–	–	1.1, 1.1
	5.5	0.9, 1.1	5.5, 5.5	0.6, 1.0	–	5.1, 5.5
Glutamate	1.1	–	0.7, 0.6	–	–	1.1, 1.1
	5.5	–	0.4, 0.6	–	–	5.5, 5.5
LB broth	1.1	–	–	–	–	1.1, 1.0
	5.5	–	–	–	–	4.3, 5.3

[a] TCE consumption was measured in sealed serum vials (160 ml) containing minimal media (60 ml) amended with the indicated substrate and inoculated with the designated strains. The cultures were incubated at 30 °C with shaking for 16–24 h, beyond which time further TCE consumption was not observed. Values from duplicate experiments are presented.
[b] (–): TCE degradation was below the level of detection ($\leq$0.1 μmol).
[c] (ND): experiment not performed.

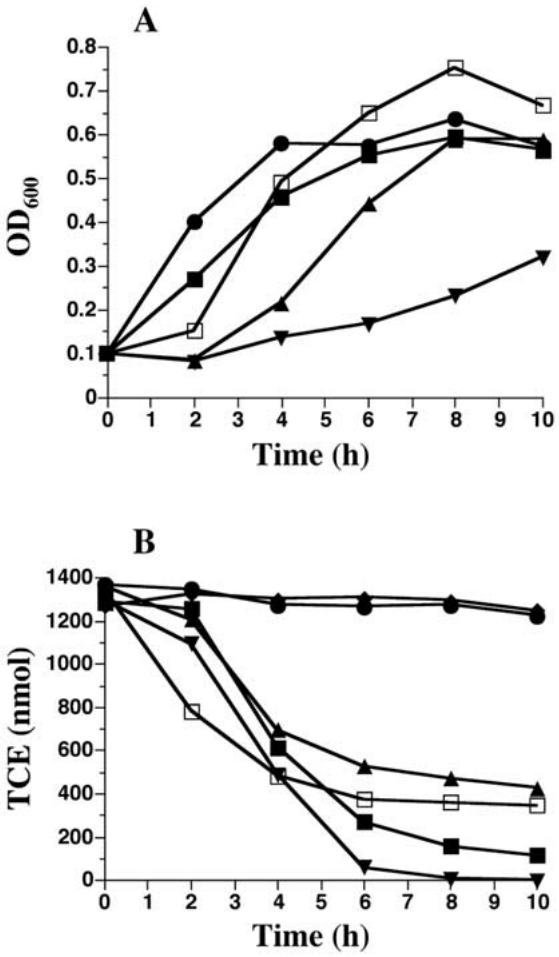

Figure 1. (A) Cell growth of *P. mendocina* KR1 cultures incubated with TCE (initial concentration, 10 μM) in growth media with lactate (■), glucose (▲), glutamate (□), fructose (▼), or LB (●), was monitored via OD_{600} measurements over time. (B) TCE consumption by each of the cultures depicted in (A), along with a control culture with no growth substrate (♦), was monitored periodically by GC analysis.

frequently (as was the OD_{600}) over a 10.5 h incubation period to ensure that comparisons of TCE consumption were made before oxygen limitation became an issue.

Induction of toluene- and TCE-degradative activity

Cells of each strain were grown at 30 °C in serum vials (160 ml) containing minimal medium (60 ml) with fructose (20 mM). When the OD_{600} of the culture reached 0.3–0.4, 5.5 μmol of toluene (58 μM, initial concentration) or TCE (50 μM, initial concentration) diluted in *N,N*-dimethylformamide (DMF) was added to the vials, which were then sealed with Teflon-lined butyl stoppers and incubated at 30 °C for 4 h. In all assays $\leq$12.5 μl of DMF was added to the cultures. These levels of DMF did not alter the growth rates of any of the strains examined, and our initial observations indicated that DMF does not inhibit TCE degradation under these conditions. It was empirically determined that a 4 h induction period yielded the highest levels of oxygenase activity under the conditions examined. Induced cells were harvested, as described above, and assayed for toluene-degrading activity. Alternatively, cells grown overnight on toluene were assayed for toluene-degrading activity or TCE-degrading activity. To measure the toluene or TCE degradation rates, cells (1 mg total protein) were added to sealed serum vials (10 ml) containing phosphate buffer (1 ml final reaction volume) and toluene (817 nmol; 200 μM) or TCE (550 nmol; 100 μM) from a DMF stock solution. The reaction vessels were incubated at 30 °C with shaking and hydrocarbon consumption was monitored by GC analysis of headspace samples over a 30–60 min time period.

Determination of oxygenase activity upon TCE exposure

To examine toluene monooxygenase or dioxygenase activity following TCE exposure, the toluene-dependent O_2 uptake rates of treated cells were determined with a Clark-style O_2 electrode (Yellow Springs Instruments Co., Yellow Springs, OH) mounted in a glass water-jacketed reaction vessel (1.6 ml) maintained at 30 °C. The reaction vessel was filled with phosphate buffer, and cells were added to determine a basal rate of cellular respiration. The basal respiratory rate was subtracted from the toluene-stimulated (250 μM) O_2 uptake rate to obtain the toluene-dependent O_2 uptake rate. It was empirically determined that the toluene-dependent O_2 uptake activity was proportionately related to the toluene-degrading activity as measured by GC analysis in each of the strains examined.

Analytical methods

Toluene and TCE consumption was monitored with a Shimadzu (Kyoto, Japan) GC-8A chromatograph equipped with a flame ionization detector and a capillary column (Alltech, 5 μm RSL 160 polydimethylsiloxane stationary phase, 15 m $\times$ 0.53 mm) with nitrogen as the carrier gas. The column temperature was 120 °C and the injector and detector temperatures were set at 220 °C for all GC analyses. Hydrocarbons were quantified by comparison of peak heights to standard curves constructed from known amounts of authentic compounds. Protein concentrations were determined with the Biuret assay (Gornall et al. 1949) following cell solubilization in 3 M NaOH for 30 min at 65 °C. Bovine serum albumin was used as the standard.

Results

TCE degradation by toluene-oxidizing bacteria grown on non-aromatic substrates

B. cepacia PR1 is a Tn5-derived mutant of *B. cepacia* G4 that constitutively expresses toluene 2-monooxygenase and has been shown to effectively degrade TCE while grown on alternative substrates, such as lactate (Shields & Reagin 1992). In the current study, cells of *B. cepacia* PR1 degraded TCE while grown on each of the substrates tested, albeit somewhat inefficiently with acetate (Table 1). This strain was included in our analyses to serve as a comparative benchmark with which to evaluate TCE degradation by wild type strains of toluene-oxidizing bacteria grown on non-aromatic substrates. TCE degradation by cultures of *P. putida* F1, *P. mendocina* KR1, *R. pickettii* PKO1, *B. cepacia* G4, and *B. cepacia* PR1 was measured following growth on 6 different substrates (Table 1). Several important observations were revealed in this experiment. First, none of the wild type strains consumed TCE when grown on LB broth, or with acetate as the sole C source. Second, among the wild type strains, *P. mendocina* KR1 degraded the most TCE when grown on glutamate, lactate, glucose, and fructose. In contrast, *B. cepacia* G4 did not degrade TCE while growing on any of the non-aromatic growth substrates tested. Third, TCE degradation (when observed) was most pronounced in wild type strains when fructose was supplied as the C source. In fact, fructose-grown *P. mendocina* KR1 degraded TCE to the same extent as *B. cepacia* PR1 under these conditions.

Because O_2 exhaustion probably occurred at different times in the sealed vial treatments of the experiment described in Table 1, and may have influenced the final amounts of TCE degraded, we examined TCE oxidation and concurrent growth of *P. mendocina* KR1 on different C sources over a shorter time (10 h) course (Figure 1A and 1B). LB-grown cells of this strain did not degrade TCE, while cultures growing on glutamate, lactate, glucose, and fructose degraded TCE at similar rates for approximately 4 h (beyond which time cell growth slowed and eventually ceased either due to cytotoxic effects of TCE degradation, or because of oxygen limitation in the sealed serum vials). When the OD_{600} of the *P. mendocina* KR1 cells grown on different substrates is compared with the amount of TCE consumed at a given time (up to 4 h), it is apparent that fructose supported more TCE degradation for a given amount of accumulated biomass than did the other substrates (Figure 1). The results in Table 1 are consistent with the idea that fructose also supports greater specific rates of aerobic TCE degradation in *P. putida* F1 and *R. pickettii* PKO1 than do the other non-aromatic substrates tested.

Physiological limitations to TCE degradation

Even though we tested just a limited number of toluene-oxidizing strains, it is obvious from the data presented in Table 1 that there are considerable differences in the TCE-degrading capacity of these strains

when grown on non-aromatic substrates. Therefore, we performed a series of experiments to assess the possible physiological underpinnings that prevent or constrain TCE oxidation in toluene-oxidizing bacteria when grown on non-aromatic substrates. Because fructose supported the highest levels of TCE degradation in each of the strains tested (except *B. cepacia* G4) (Table 1), we utilized it as the growth substrate (when required) in the following comparative physiological assays. Unfortunately, *R. pickettii* PKO1 was not included in these analyses because fructose-grown cells of this organism were extremely sensitive to toluene (extensive cell clumping) at the concentrations utilized in the assays (50–200 μM).

TCE induction of toluene-oxidizing activity

First, we compared the ability of toluene and TCE to induce toluene-oxidizing activity in fructose-grown cells (Table 2). Toluene-oxidizing activity was used as a proxy for TCE-degrading activity because it is known that TCE is degraded by a toluene mono- or dioxygenase in each of the strains included in this study (see Introduction). Also, with toluene as a substrate, toluene-oxidizing activity does not suffer from inactivation or energy/reductant limitations in these strains, thus providing a robust, steady activity measurement following the induction treatments. We made no attempt to maximize toluene-oxidizing activity in these assays other than to optimize the toluene concentration used and to ensure that toluene consumption was linear over the time course of the experiment.

With the exception of *B. cepacia* PR1, none of the fructose-grown strains exhibited appreciable toluene-degrading activity until induced with TCE or toluene (Table 2, DMF control). Toluene-oxidizing activities were essentially the same among toluene-grown cells of *B. cepacia* G4, *B. cepacia* PR1, *P. putida* F1, and *P. mendocina* KR1, although these rates may have been artificially limited in our assays (e.g., diffusion limited). Additionally, the toluene inducible toluene-oxidizing activities in fructose-grown cells of each strain were comparable (approximately 20–25% of the activity observed in toluene-grown cells). There were striking differences, however, between the toluene-oxidizing activities of the four fructose-grown strains when they were induced with TCE (50 μM). Activity was not detected in fructose-grown cells of *B. cepacia* G4 induced with TCE, while fructose-grown *B. cepacia* PR1 and *P. mendocina* KR1 exhibited 22 and 53% of the activity measured in toluene-grown cells under these conditions. Even though fructose-grown cells of *P. putida* F1 degraded less TCE than *P. mendocina* KR1 and *B. cepacia* PR1 (Table 1), these *P. putida* F1 cells expressed approximately 5 times the toluene-oxidizing activity expressed by fructose-grown, TCE-induced cells of the other two strains.

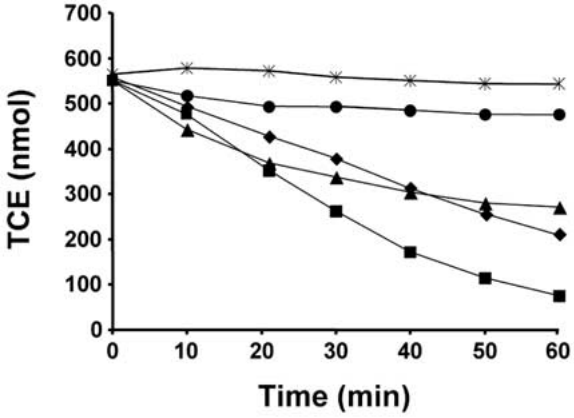

Figure 2. Time course of TCE consumption by *B. cepacia* G4 (■), *P. mendocina* KR1 (▲), *B. cepacia* PR1 (♦), *P. putida* F1 (●), and a no cell control (*). Toluene-grown cells (1.0 mg total protein) were incubated in phosphate buffer with TCE (100 μM initial aqueous phase concentration; 1 ml final reaction volume) at 30 °C with shaking.

TCE consumption profiles

Next, we determined the TCE-degrading activity of *B. cepacia* G4, *B. cepacia* PR1, *P. mendocina* KR1, and *P. putida* F1 without the confounding influence of an additional carbon or energy source. TCE consumption by toluene-grown, washed cells of each of these strains was monitored over a short time period (Figure 2). We utilized toluene-grown cells in this assay because each strain exhibited robust, initial rates of TCE consumption that could be easily monitored, whereas TCE consumption was at or below the detection limit in fructose-grown cells of several strains (i.e., *P. putida* F1 and *B. cepacia* G4). The results show that washed cells of toluene-grown *B. cepacia* G4 and *B. cepacia* PR1 consumed TCE rather steadily up to 40 min, beyond which time a slight loss of activity was observed. In contrast, TCE consumption by *P. mendocina* KR1 declined continuously from 10 min onward and had essentially ceased by 40–50 min. The decline in TCE consumption was even more dramatic in *P. putida* F1, which ceased after 10–20 min.

TCE-dependent oxygenase inactivation

One of the fundamental criticisms of aerobic TCE cometabolism is that it is often constrained by cyto-

Table 2. Specific toluene-degradative activity of *B. cepacia* G4, *P. putida* F1 and *P. mendocina* KR1, and *B. cepacia* PR1 grown with toluene or grown with fructose and induced with toluene, TCE, or DMF

Strain	Toluene degradation rate (nmol min^{-1} mg of $protein^{-1}$)[a]			
	Toluene-grown cells[b]	Fructose-grown cells[c]		
		Induced w/toluene	Induced w/TCE	Induced w/DMF
B. cepacia G4	60.3 ± 4.8[d]	12.0 ± 4.6	0.3 ± 0.3	0.1 ± 0.1
B. cepacia PR1	62.1 ± 10.7	16.3 ± 1.3	13.6 ± 1.2	9.1 ± 1.3
P. mendocina KR1	51.8 ± 8.2	12.0 ± 1.2	6.6 ± 2.4	0.4 ± 0.1
P. putida F1	60.2 ± 7.3	16.1 ± 7.4	31.9 ± 8.9	0.1 ± 0.1

Toluene degradation (initial aqueous phase concentration, 200 μM) was monitored in sealed serum vials (10 ml) containing cells (1 mg total protein) and phosphate buffer (1 ml final reaction volume) over a 30 min time course at 30 °C with shaking.
[b] Cells were grown overnight on toluene (as described in materials and methods).
[c] Fructose-grown cells were induced with toluene (58 μM) or TCE (50 μM) from DMF diluted stock solutions ($\leq$12.5 μl added) or with DMF alone (12.5 μl added).
[d] Values are the mean of 3 experiments $\pm$ SD.

toxic effects. During TCE degradation, it is thought that reactive intermediates can injure cells via specific inactivation of the transforming oxygenase (Fox et al. 1990; Li & Wackett 1992; Newman & Wackett 1997) or by causing general cellular damage that ultimately leads to cell death and cessation of enzymatic activity (Heald & Jenkins 1994; van Hylckama Vlieg et al. 1997; Yeager et al. 2001). Therefore, we examined the effects of TCE degradation on toluene-dependent oxygenase activity and cell growth to determine if toxicity could have influenced the TCE degradation patterns observed in the current study.

To determine if the strain-dependent differences in TCE degradation observed in Figure 2 were due to oxygenase inactivation, toluene-dependent O_2 uptake rates were determined in cells of each strain following exposure to TCE for different time intervals (Figure 3). The irreversible inhibition of toluene-oxidizing activity following TCE degradation varied considerably among the strains, and with the exception of the results for *P. putida* F1, the loss of activity observed in each strain was consistent with the corresponding TCE degradation curves presented in Figure 2. *B. cepacia* G4 and *B. cepacia* PR1 maintained between 70–80% of their toluene-oxidizing activities over the 1 h incubation, whereas the toluene-oxidizing activity of *P. mendocina* KR1 dropped considerably during the first 10 min of TCE exposure (70% loss) and continued to decline to $\sim$12% of the initial value after 1 h. The toluene oxidizing activity of *P. mendocina* KR1 showed more TCE-independent sensitivity to the assay conditions than the other strains. Loss of monooxygenase activity during aeration has been previously reported, and it has been speculated that reactive oxygen species generated in the active site of the enzyme can eventually react with nearby amino acids, thus inactivating the enzyme, unless a substrate is available that can be readily oxidized (Alvarez-Cohen & McCarty 1991; Studts et al. 2000). KR1 lost 30% of its activity over an 1 h incubation in the absence of any substrate, while the other strains lost only 5% of their activities (data not shown). Cells of *P. putida* F1 retained $\sim$75% of toluene-oxidizing activity over the 1 h incubation (Figure 3), despite TCE oxidizing activity being undetectable after 10–20 min in the TCE consumption experiment (Figure 2). These same TCE-treated cells of *P. putida* F1 were incapable of further TCE degradation after incubation with toluene (in the O_2 electrode chamber) indicating that reductant deficit had not caused the inhibition of TCE oxidation (data not shown). These results were highly reproducible with different batches of *P. putida* F1 cells.

TCE-dependent general cellular toxicity

To determine the effects of TCE degradation on the overall cellular health of the toluene-oxidizing strains during growth on non-aromatic substrates, we monitored growth rates of *B. cepacia* PR1, *B. cepacia* G4, *P. putida* F1, and *P. mendocina* KR1 with lactate or fructose in the presence or absence of TCE. TCE (50 μM initial concentration) diminished the growth rate of *B. cepacia* PR1, *P. putida* F1, and *P. mendocina* KR1 with fructose (Figure 4), but had no effect on the growth characteristics of *B. cepacia* G4 (data

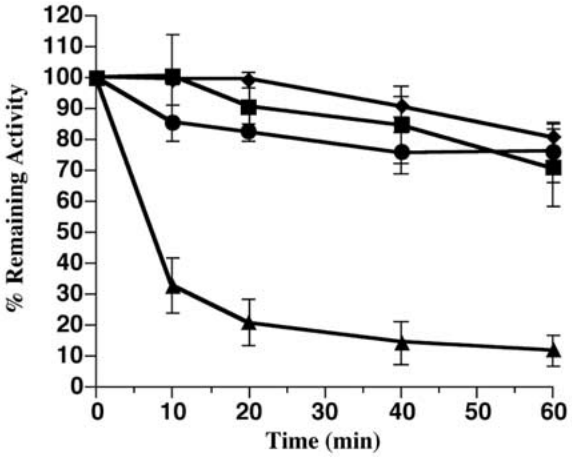

Figure 3. Loss of toluene oxidizing activity in *B. cepacia* G4 (■), *P. putida* F1 (●), *P. mendocina* KR1 (▲) and *B. cepacia* PR1 (♦) cells exposed to TCE for select times. Toluene-grown cells (1.0 mg total protein) were incubated in phosphate buffer with TCE (100 μM initial aqueous phase concentration; 1 ml final reaction volume) at 30 °C with shaking for the indicated times, washed twice in phosphate buffer, and assayed for toluene-dependent O_2 uptake activity. Values are the mean of 3 experiments ± SD.

not shown). In cells of *B. cepacia* PR1 and *P. mendocina* KR1 that had been pretreated with propyne to inactivate toluene monooxygenase activity (Yeager et al. 1999), no reduction in fructose-dependent growth was observed in the presence of TCE (data not shown). We were unaware of a specific inactivator for toluene dioxygenase, so a similar control experiment was not performed with *P. putida* F1. These experiments indicate that the effect of TCE on the fructose-supported growth rates of *B. cepacia* PR1 and *P. mendocina* KR1 (and probably *P. putida* F1) was turnover-dependent. The growth rate of cells of each of these strains utilizing lactate was not affected by the addition of TCE (Figure 4).

Discussion

The results of this study demonstrate that *P. putida* F1, *R. pickettii* PKO1, and *P. mendocina* KR1, each harboring distinct toluene-oxidizing systems, were capable of degrading TCE when grown on non-aromatic substrates. However, there were significant strain-dependent and substrate-dependent differences in the amount of TCE degraded. Our results indicate that multiple physiological traits were responsible for the strain-dependent differences including: oxygenase induction, kinetics of TCE oxidation, and resistance or

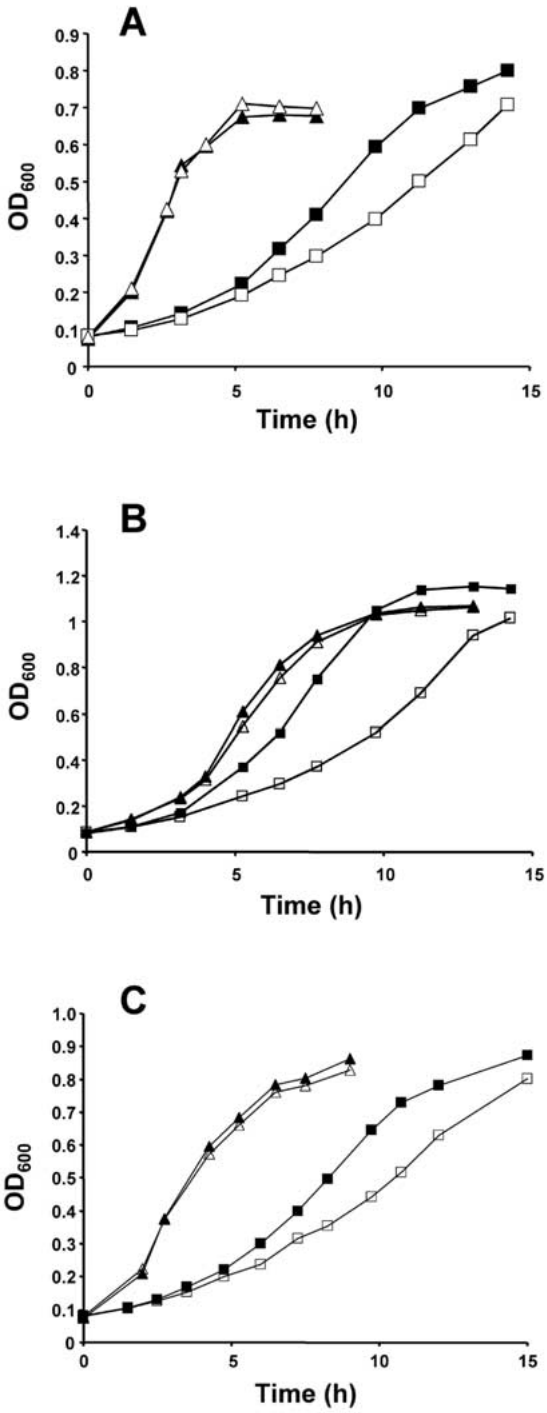

Figure 4. Growth of (A) *P. mendocina* KR1, (B) *B. cepacia* PR1 or (C) *P. putida* F1 cells in minimal media containing fructose (squares) or lactate (triangles) with (open symbols) or without (closed symbols) 50 μM TCE. Cultures were incubated in sealed serum vials at 30 °C with shaking and periodically samples were removed to measure the OD_{600} of the culture.

susceptibility to TCE-dependent oxygenase inactivation and general cellular toxicity. Additionally, the specific rate of TCE degradation by *P. mendocina* KR1 (and likely other toluene-oxidizing strains) during growth with non-aromatic compounds was highly dependent on the choice of substrate. In particular, it was found that fructose was remarkable in its ability to support TCE degradation in *P. mendocina* KR1.

The failure of TCE to induce its own degradation in toluene-oxidizing strains grown on LB or acetate was most likely due to catabolite repression. It has been demonstrated that carbon catabolite repression is often involved in transcriptional regulation of bacterial oxygenases in strains that grow on phenol, toluene and other simple aromatic compounds (Holtel et al. 1994; Ampe et al. 1998; Cases et al. 1999; Sze & Shingler 1999). Indeed, complete repression of oxygenase activity during rapid growth in rich media, or exponential silencing, is apparently quite common (Yuste et al. 1998; Sze & Shingler 1999; Carmona et al. 2000; Tover et al. 2001). Acetate has also been shown to inhibit synthesis of several bacterial oxygenases, including phenol hydroxylase in *Ralstonia eutropha* (Ampe et al. 1998) and styrene monooxygenase in *Pseudomonas fluorescens* ST (Santos et al. 2000).

Among the strains tested in this study, *B. cepacia* G4 was most susceptible to catabolite repression, as it exhibited virtually no TCE-degrading or toluene-oxidizing activity in the presence of the non-aromatic substrates. In a previous study, cells of *B. cepacia* G4 exhibited toluene-dependent oxygenase activity following growth on lactate in the presence of 2.35 mM TCE (Leahy et al. 1996), which suggests that high concentrations of TCE (mM range) could induce some level of toluene 2-monooxygenase activity in *B. cepacia* G4. However, requiring high levels of contaminant for induction would be disadvantageous in many bioremediation scenarios, thus the use of *B. cepacia* G4 as a bioremediation agent is expected to be hampered by catabolite repression when non-aromatic growth substrates are present.

Unlike *B. cepacia* G4, cells of *P. putida* F1 were capable of high levels of oxygenase expression in the presence of the alternative substrate, fructose, yet overall levels of TCE degradation by this bacterium were poor because the activity was extremely short-lived. Several previous studies have also demonstrated a precipitous loss of TCE-oxidizing activity in whole cells of *P. putida* F1 and the authors suggested that toluene dioxygenase is rapidly inactivated during the transformation (Wackett & Gibson 1988; Wackett & Householder 1989). However, we found that the toluene-oxidizing activity of *P. putida* F1 cells was not inactivated upon TCE degradation. Indeed, our results imply that TCE may be a better enzyme inhibitor than a substrate of the *P. putida* F1 toluene oxidizing system, or that the enzyme contains different active sites for TCE and toluene oxidation. This would explain the observation that a recombinant strain of *Deinococcus radiodurans*, expressing toluene dioxygenase, was able to effectively sustain toluene degradation, but not TCE degradation (Lange et al. 1998).

Among the wild type toluene-oxidizing strains tested, *P. mendocina* KR1 was the most successful TCE degrader when grown with non-aromatic substrates. No single factor repressed TCE oxidation in this organism as in *B. cepacia* G4 (catabolite repression) or *P. putida* F1 (kinetics of TCE turnover). Nonetheless, *P. mendocina* KR1 did not exhibit any exceptional TCE-degrading qualities. In comparison to the other toluene-oxidizing strains examined, KR1 (1) exhibited moderate levels of oxygenase activity when grown on non-aromatic substrates in the presence of TCE (Table 2); (2) degraded intermediate levels of TCE in resting cell assays (Figure 2); and 3) was relatively sensitive to the toxic effects associated with TCE oxidation (Figures 3 and 4). Even so, because TCE degradation was sustained for many hours in this bacterium under a variety of growing conditions, *P. mendocina* KR1 was obviously capable of synthesizing toluene 4-monooxygenase and diverting sufficient reductant/energy to repair or resynthesize enzymes or other cellular constituents damaged upon TCE oxidation during growth on non-aromatic substrates.

As mentioned earlier, we found that fructose was remarkable in its ability to support high specific rates of TCE degradation in toluene-oxidizing bacteria growing on non-aromatic substrates. A link between growth on fructose and relatively high levels of oxygenase activity in some *Pseudomonas* strains was previously observed and attributed to a novel form of carbon catabolite regulation (Cases et al. 1999; Cases & de Lorenzo 2000). On the other hand, high specific rates of oxygenation reactions have also been correlated with slow cell growth (as was observed for TCE oxidation in fructose-grown cultures of *P. mendocina* KR1 in the current study). For instance, the TCE transformation efficiency of *B. cepacia* G4 was reported to be up to 10 times greater under non-growing or slow growth conditions than during periods of rapid growth (Landa et al. 1994; Mars et al. 1996). To explain this

phenomenon, it has been theorized that during periods of rapid bacterial growth electrons are preferentially shuttled towards anabolic processes, depriving oxygenases of their reductant needs; whereas, reductant (usually in the form of NADH) availability may be greater during slow growth due to a decrease in the demand for this commodity in anabolic processes relative to its generation via the TCA cycle (Leahy et al. 1996; Dahlen & Rittmann 2002b). Indeed, respiration uncoupled from growth has been observed previously in various strains of *P. mendocina*, including KR1, suggesting that this species may be capable of diverting NADH for TCE degradation during growth on alternative substrates (Verdoni et al. 1992; Leahy et al. 1996).

In contrast to the scenario described above, Dahlen & Rittmann (2002a) calculated that only 1% of the available cellular NADH was required for oxygenation reactions in a mixed-culture bioreactor designed to degrade phenol and 2,4-dichlorophenol. The authors suggested that by increasing the specific growth rate of the cultures, the cellular content (thus activity) of phenol monooxygenase increased, and that this factor had a greater effect on monooxygenase activity in the bioreactor than did NADH availability. Obviously, a better understanding of the intricate relationship between the oxygenase activity of xenobiotic-degrading microorganisms and specific growth rates, NADH availability, and carbon catabolite regulation is necessary to fully interpret the high specific rates of TCE degradation observed in bacteria growing on fructose and to provide information that will allow further optimization of oxygenase-catalyzed reactions.

Acknowledgements

We thank Suzanne Miller for preliminary experiments and conversations that served to initiate these investigations, and Norman Hommes and Alisa Vangnai for technical support. Funding for this study was provided by the office of Research and Development, U.S. Environmental Protection Agency, under Agreement PR-0345 through the Western Region Hazardous Substance Research Center.

References

Alvarez-Cohen L & McCarty PL (1991) Effects of toxicity, aeration, and reductant supply on trichloroethylene transformation by a mixed methanotrophic culture. Appl. Environ. Microbiol. 57: 228–235

Ampe F, Leonard D & Lindley ND (1998) Repression of phenol catabolism by organic acids in *Ralstonia eutropha*. Appl. Environ. Microbiol. 64: 1–6

Arp DJ (1995) Understanding the diversity of trichloroethylene cooxidations. Curr. Opin. Biotechnol. 6: 352–358

Carmona M, Rodriguez MJ, Martinez-Costa O & de Lorenzo V (2000) *In vivo* and *in vitro* effects of (p)ppGpp on the σ^{54} promoter *Pu* of the TOL plasmid of *Pseudomonas putida*. J. Bacteriol. 182: 4711–4718

Cases I & de Lorenzo V (2000) Genetic evidence of distinct physiological regulation mechanisms in the σ^{54} *Pu* promoter of *Pseudomonas putida*. J. Bacteriol. 182: 956–960

Cases I, Perez-Martin J & de Lorenzo V (1999) The IIANtr (PtsN) protein of *Pseudomonas putida* mediates the C source inhibition of the σ^{54}-dependent *Pu* promoter of the TOL plasmid. J. Biol. Chem. 274: 15562–15568

Dahlen EP & Rittmann BE (2002a) A detailed analysis of the mechanisms controlling the acceleration of 2,4-DCP monooxygenation in the two-tank suspended growth process. Biodegradation 13: 117–130

Dahlen EP & Rittmann BE (2002b) Two-tank suspended growth process for accelerating the detoxification kinetics of hydrocarbons requiring initial monooxygenation reactions. Biodegradation 13: 101–116

Ensley BD (1991) Biochemical diversity of trichloroethylene metabolism. Annu. Rev. Microbiol. 45: 283–299

Folsom BR, Chapman PJ & Pritchard PH (1990) Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: kinetics and interactions between substrates. Appl. Environ. Microbiol. 56: 1279–1285

Fox BG, Borneman JG, Wackett LP & Lipscomb JD (1990) Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. Biochemistry 29: 6419–6427

Gornall AG, Bardawill CJ & David MM (1949) Determination of serum proteins by means of the Biuret reaction. J. Biol. Chem. 177: 751–766

Gossett JM (1987) Measurement of Henry's law constants for C_1 and C_2 chlorinated hydrocarbons. Environ. Sci. Technol. 21: 202–208

Heald S & Jenkins RO (1994) Trichloroethylene removal and oxidation toxicity mediated by toluene dioxygenase of *Pseudomonas putida*. Appl. Environ. Microbiol. 60: 4634–4637

Holtel A, Marques S, Mohler I, Jakubzik U & Timmis KN (1994) Carbon source-dependent inhibition of *xyl* operon during expression of the *Pseudomonas putida* TOL plasmid. J. Bacteriol. 176: 1773–1776

Hyman MR, Russell SA, Ely RL, Williamson KJ & Arp DJ (1995) Inhibition, inactivation, and recovery of ammonia-oxidizing activity in cometabolism of trichloroethylene by *Nitrosomonas europaea*. Appl. Environ. Microbiol. 61: 1480–1487

Landa AS, Sipkema EM, Weijma J, Beenackers AA, Dolfing J & Janssen DB (1994) Cometabolic degradation of trichloroethylene by *Pseudomonas cepacia* G4 in a chemostat with toluene as the primary substrate. Appl. Environ. Microbiol. 60: 3368–3374

Lange CC, Wackett LP, Minton KW & Daly MJ (1998) Engineering a recombinant *Deinococcus radiodurans* for organopollutant degradation in radioactive mixed waste environments. Nat. Biotechnol. 16: 929–933

Leahy JG, Byrne AM & Olsen RH (1996) Comparison of factors influencing trichloroethylene degradation by toluene-oxidizing bacteria. Appl. Environ. Microbiol. 62: 825–833

Li S & Wackett LP (1992) Trichloroethylene oxidation by toluene dioxygenase. Biochem. Biophys. Res. Commun. 185: 443–451

Mars AE, Houwing J, Dolfing J & Janssen DB (1996) Degradation of toluene and trichloroethylene by *Burkholderia cepacia* G4 in growth-limited fed-batch culture. Appl. Environ. Microbiol. 1996: 886–891

McClay K, Streger SH & Steffan RJ (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

Newman LM & Wackett LP (1997) Trichloroethylene oxidation by purified toluene 2-monooxygenase: products, kinetics, and turnover-dependent inactivation. J. Bacteriol. 179: 90–96

Olsen RH, Kukor JJ & Kaphammer B (1994) A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PKO1. J. Bacteriol. 176: 3749–3756

Santos PM, Blatny JM, DiBartolo I, Valla S & Zennaro E (2000) Physiological analysis of the expression of the styrene degradation gene cluster in *Pseudomonas fluorescens*. Appl. Environ. Microbiol. 66: 1305–1310

Shields MS, Montgomery SO, Cuskey SM, Chapman PJ & Pritchard PH (1991) Mutants of *Pseudomonas cepacia* G4 defective in catabolism of aromatic compounds and trichloroethylene. Appl. Environ. Microbiol. 57: 1935–1941

Shields MS & Reagin MJ (1992) Selection of a *Pseudomonas cepacia* strain constitutive for the degradation of trichloroethylene. Appl. Environ. Microbiol. 58: 3977–3983

Shingleton JT, Applegate BM, Nagel AC, Bienkowski PR & Sayler GS (1998) Induction of the tod operon by *trichloroethylene* in *Pseudomonas putida* TVA8. Appl. Environ. Microbiol. 64: 5049–5052

Strand SE, Bjelland MD & Stensel HD (1990) Kinetics of chlorinated hydrocarbon degradation by suspended cultures of methane-oxidizing bacteria. Res. J. Wat. Poll. Control Fed. 62: 124–129

Studts JM, Mitchell KH, Pikus JD, McClay K, Steffan RJ & Fox BG (2000) Optimized expression and purification of toluene 4-monooxygenase hydroxylase. Protein Expr. Purif. 20: 58–65

Sze CC & Shingler V (1999) The alarmone (p)ppGpp mediates physiological-responsive control at the σ^{54}-dependent Po promoter. Mol. Microbiol. 31: 1217–1228

Tover A, Ojangu EL & Kivisaar M (2001) Growth medium composition-determined regulatory mechanisms are superimposed on CatR-mediated transcription from the *pheBA* and *catBCA* promoters in *Pseudomonas putida*. Microbiology-(UK) 147: 2149–2156

van Hylckama Vlieg JET, De Koning W & Janssen DB (1997) Effect of chlorinated ethene conversion on viability and activity of *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. 63: 4961–4964

Verdoni N, Aon M & Lebeault JM (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

Wackett LP & Gibson DT (1988) Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. 54: 1703–1708

Wackett LP & Householder SR (1989) Toxicity of trichloroethylene to *Pseudomonas putida* F1 is mediated by toluene dioxygenase. Appl. Environ. Microbiol. 55: 2727–2725

Winter RB, Yen KM & Ensley BD (1989) Efficient degradation of trichloroethylene by a recombinant *Escherichia coli*. Bio/Technology 7: 282–285

Yeager CM, Bottomley PJ & Arp DJ (2001) Cytotoxicity associated with trichloroethylene oxidation in *Burkholderia cepacia* G4. Appl. Environ. Microbiol. 67: 2107–2115

Yeager CM, Bottomley PJ, Arp DJ & Hyman MR (1999) Inactivation of toluene 2-monooxygenase in *Burkholderia cepacia* G4 by alkynes. Appl. Environ. Microbiol. 65: 632–639

Yuste L, Canosa I & Rojo F (1998) Carbon-source-dependent expression of the PalkB promoter from the *Pseudomonas oleovorans* alkane degradation pathway. J. Bacteriol. 180: 5218–5226