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ORIGINAL PAPER

Toluene dioxygenase expression correlates with trichloroethylene degradation capacity in *Pseudomonas putida* F1 cultures

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Abstract Trichloroethylene (TCE) is extensively used in commercial applications, despite its risk to human health via soil and groundwater contamination. The stability of TCE, which is a useful characteristic for commercial application, makes it difficult to remove it from the environment. Numerous studies have demonstrated that TCE can be effectively removed from the environment using bioremediation. *Pseudomonas putida* F1 is capable of degrading TCE into less hazardous byproducts via the toluene dioxygenase pathway (TOD). Unfortunately, these bioremediation systems are not self-sustaining, as the degradation capacity declines over time. Fortunately, the replacement of metabolic co-factors is sufficient in many cases to maintain effective TCE degradation. Thus, monitoring systems must be developed to predict when TCE degradation rates are likely to decline. Herein, we show evidence that *tod* expression levels correlate with the ability of *P. putida* F1 to metabolize TCE in the presence of toluene. Furthermore, the presence of toluene improves the replication of *P. putida* F1, even when TCE is present at high concentration. These findings may be applied to real world applications to decide when the bioremediation system requires supplementation with aromatic substrates, in order to maintain maximum TCE removal capacity.

Keywords Toluene dioxygenase · *Pseudomonas putida* · Trichloroethylene · Bioremediation

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Introduction

Chlorinated solvents such as trichloroethylene (TCE) are extensively used in commercial applications due to their high stability and low probability of combusting. TCE is commonly used for a wide variety of applications, which includes industrial cleaning products, dry cleaning solutions, and fumigants. Despite their commercial utility, chlorinated solvents are dangerous for human health (Council 1994); therefore care must be taken to avoid contaminating the environment with these substances. Unfortunately, leaking storage tanks

and improper disposal practices lead to significant release of chlorinated solvents into the soil and groundwater (Ensley 1991). Therefore, recent efforts have focused on removing spilled chlorinated solvents from the environment (Council 1994).

TCE is a suspected human carcinogen; therefore, it is essential to remove it from the environment. Unfortunately, it is fairly resistant to environmental removal and specific detoxification processes targeting this chlorinated solvent must be developed. Although physical and chemical methods may be used to decontaminate the environment, they are labor intensive and costly. Fortunately, there is a more cost effective solution; bioremediation approaches are a well accepted alternative to chemical methods of hazardous chemical removal Revah and Morgan-Sagastume (2005)These methods utilize specialized microbes, which are added to the contaminated area, to degrade TCE into less toxic or nontoxic byproducts (Ensley 1991). Either anaerobic or aerobic bacteria can be used, but anaerobic techniques are preferred, since aerobic degradation pathways produce carcinogenic vinyl chlorides Maltoni and Lefemine (1975)In anaerobic detoxification schemes, bacteria that express monoxygenase or dioxygenase enzymes oxidize TCE, rendering it inactive (Ensley 1991; Wackett and Gibson 1988. A common strain used for this process is *Pseudomonas putida* F1, which expresses the toluene dioxygenase (TOD) C1C2BA (Zylstra et al. 1989).

Aerobic degradation of TCE via co-metabolism occurs via the TOD pathway in *P. putida* F1 (Zylstra et al. 1989; Heald and Jenkins 1994. The pathway requires a nonspecific oxygenase, TOD, to catalyze the conversion of TCE to formate, water, chloride, and hydrogen ions (Ensley 1991). Compared to TCE, these degradation products are less hazardous and are more easily removed from the environment by natural processes. In order for the TOD pathway to function, co-substrates such as aliphatics and aromatics must be present; thus, toluene or phenol is frequently added to the contaminated area to catalyze the breakdown of chlorinated solvents (Chen et al. 2008). This approach of linking TCE degradation to the metabolism of aromatic hydrocarbons has been effectively employed in both soil Fan and Scow (1993); Mu and Scow 1994and groundwater (Hopkins 1995; Hopkins et al. 1993a; Hopkins et al. 1993b). Due to this success, significant research efforts have been made to improve the efficiency and effectiveness of TCE bioremediation. One of the major limitations of this process is that degradation products of TCE transformation reduce critical cellular functions such as respiration and growth (Van Hylckama Vlieg et al. 1997; Wackett and Householder 1989). Thus, the capacity of bacteria to degrade TCE is thought to be inherently limited, unless the inhibitory components are removed, a process which is extremely difficult and impractical. Fortunately, recent studies discovered that supplanting the cultures with aromatic substrates such as toluene can restore TCE degradation rates in *P. putida* (Morono et al. 2004).

In order to optimize the co-metabolic degradation of TCE, it is essential to develop efficient monitoring systems for the detoxification capacity of microbes in the bioremediation system. Much attention has been focused on modeling co-metabolic kinetics since improved understanding of TCE degradation reactions allows more efficient and effective in situ applications of co-metabolism in bioremediation. However, rapid and cost effective techniques that monitor the capacity of the system to oxidize TCE are still needed.

Materials and methods

Bacterial strains and culture conditions

The *P. putida* F1 (DSM6899) strain was obtained from the German Collection of Microorganisms and Cell Cultures. For the preparation of TDO-induced cell suspensions, cells were grown on toluene (142 μmol) as the sole carbon and energy source in mineral salts medium at 28°C for 8 h in 50 mL serum bottles (capacity, 68 mL) that were sealed with butyl rubber stoppers and crimp caps. For experiments, each bottle contained 12 mL of mineral medium, to which the TDO-induced bacterial inoculum was added. Where indicated, a small amount of cotton embedded with toluene (142 μmol) was added to a tube, which was then placed in the serum bottles. Three replicate serum bottles were incubated at 28°C on a rotary shaker at 110 rpm.

Cells were harvested at different time intervals from 1 mL cell cultures by centrifugation at 6,000 rpm (10 min, 4°C). The supernatants were used for measuring toluene and TCE concentration, and the bacterial pellets were washed twice with 1 mL 50 mM KH_2PO_4–

K_2HPO_4 buffer, pH = 7.0 (phosphate buffer) and stored at −84°C until use. Bacterial growth was monitored by determining the optical density at 600 nm (OD_{600}). A growth curve was generated using average OD_{600} values at different time intervals.

For establishing the number of viable bacteria, samples were serially diluted in physiological saline, and 100 µL of each dilution was spread onto 220 agar plates in replicates. Plates were incubated at 30°C for 24 h. For calculating the colony forming units (CFUs) per milliliter, dilutions yielding between 30 and 300 colonies were used.

Total RNA extraction and confirmation of RNA integrity

Pseudomonas putida F1 were lysed and total RNA from the cell pellets was isolated using an RNAspin MiNi RNA isolation kit (GE Healthcare UK Limited, Buckinghamshire, UK), according manufacturer's recommendations. RNase-free DNase I treatment was performed during the isolation procedure to eliminate the remaining DNA.

The integrity of RNA is a critical first step in obtaining meaningful gene expression data Fleige and Pfaffl (2006); Lekanne Deprez et al. 2002; Kabir et al. 2003). RNA quality was checked by electrophoresis on a 2% native agars gel, stained with ethidium bromide, and viewed with a UV transilluminator. Total RNA concentration and purity was assessed by spectrometry using a V-550UV/VIS spectrophotometer (JASCO, Tokyo). RNA was quantified at 260 nm and purity was confirmed by obtaining an $OD_{260}/OD_{280} = 1.80 \sim 2.00$. Following isolation, the precipitated RNA was then resuspended in 60 µL of RNase-free water and stored −84°C for further use.

Complementary DNA (cDNA) synthesis

Reverse transcription (RT) reactions were performed using an Exscript® RT reagent kit (Takara Bio Inc., Otsu, Japan) in accordance with the manufacturer's instructions with random hexamer primers. Reactions were performed in a total volume of 20 µL under the following conditions: reverse transcription at 42°C for 15 min, enzyme denaturation by heating at 95°C for 2 min, and rapid cooling at 4°C. To check for residual DNA, reverse transcriptase control samples (RT-negative control samples) were prepared for each RNA sample using identical cDNA synthesis procedure except for the omission of reserve transcriptase. All cDNA samples and RT-control samples were diluted 1:5 and stored at −20°C for use as templates in real-time PCR analysis. cDNA concentrations were determined by optical density at 260 nm.

Primer design and generation of the standard curve

The sequences of the *rpo*D gene in *P. putida* F1 were obtained from GeneBank. *rpoD* primers were designed using Primers3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primers.cgi) and were synthesized by Invitrogen (Tokyo, Japan). The sequences of the primers are shown in Table 1. For relative quantification of gene expression, a standard curve was generated using RNA extracted from cells cultured with toluene (300 µmol) for 10 h at 28°C. This RNA was used to synthesize cDNA, and serial dilution of this cDNA was used to generate a real-time PCR standard curve.

Real-time quantitative PCR

Real-time PCR was carried out in capillary glass tubes in a LightCycler system (Roche Diagnostics, Mannherm, Germany) running software version 3.5 (Roche). Reactions were set up using SYBR® Premix Ex Taq™ (Takara Bio Inc.), according to the manufacturer's instructions. Briefly, the final 20 µL PCR mixture included 0.2 µM of each primer, 2 µL diluted cDNA, and 10 µL of SYBR® 2× premix Ex Taq™.

Table 1 Primers designed in this study

Gene	Nucleotide sequence (5′–3′)	GC%	Product size
tod	ATCCTGCGAGGCCACAAG (R)	60	119
	TTCCTCGCTGTAGACGTTGTTG (F)	50	
rpo	*D* CAGTTGCTCAAGCACCTCAG (R)	55	109
	GTTCGGCGATTTCCAGATAG (F)	50	

Cycle conditions consisted of heating to 95°C for 10 s, followed by 45 cycles at 95°C for 5 s, and 60°C for 20 s. Finally, melting curve analysis was performed to eliminate reactions in which primer dimers and other artifacts appeared.

Biomass quantification

Biomass was quantified as the mass of viable cell number. The viability of the culture was determined by diluting a culture sample and counting the number of colonies (CFU). The culture density was estimated by measuring the optical density at 600 nm (OD_{600}) on an Ubest-55 UV/VIS spectrophotometer (JASCO, Tokyo, Japan).

Gas chromatography

Toluene and TCE were analyzed by gas chromatography conducted with a series gas chromatograph equipped with a 30 m column and electron capture or flame ionization detection systems (Hewlett Packard). Calculations were made using dimensionless techniques. Samples were applied with an autosampler, and peak integrations were obtained with an integrator. The following operating conditions were used: injector temperature, 40°C and detector temperature, 250°C.

TCE and toluene degradation

The amount of TCE and toluene in each vial was measured by GC analysis, as described above. TCE and toluene degradation were assayed in 50 mL serum bottles that were sealed with Teflon-lined butyl rubber stoppers. The 1 mL samples used for organic substance analysis were removed with a 1 mL syringe and extracted with 1 mL of hexane. The percentage of TCE and toluene degradation were calculated from the mean concentrations in 3 serum bottles by comparison with the mean concentrations in an equal number of uninoculated controls in which the amount of organic substance lost never exceeded 5%. The statistical significance of TCE and toluene degradation in experimental solutions was assessed relative to the differences between inoculated and uninoculated serum bottles.

Results

Growth curves of *P. putida* F1 cultures

We prepared toluene-induced cultures of *P. putida* F1 by growing cultures in salts medium plus 142 µmol toluene for 8 h. To investigate the effects of toluene and TCE on culture growth, we subcultured toluene-induced cultures into new vials. Each vial contained salts medium plus a cotton ball spiked with either 142 µmol toluene, 111 µmol TCE, or both. As expected, toluene enhanced the growth of *P. putida* F1, while TCE inhibited growth of the cultures (Fig. 1). Even when TCE was present, toluene was sufficient to partially rescue the growth curve (Fig. 1).

Degradation of TCE and Toluene by *P. putida* F1

Next, we analyzed the capacity of various culture conditions to degrade TCE over a 10 h time course, measuring TCE concentrations every 2 h. To quantify the levels of TCE present at each time point, we harvested supernatants from the salts medium and extracted TCE with hexane for subsequent measurement by gas chromatography. Bacterial cultures grown in the presence of TCE alone had less TCE (140 mg/L) compared to the no bacteria control cultures (180 mg/L) (Fig. 2). However, the addition of toluene to the TCE cultures increased the capacity

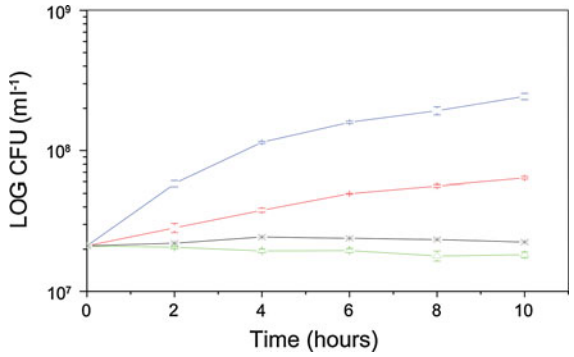

Fig. 1 Growth curves of *P. putida* F1 which had been pregrown on salts medium supplemented with 142 µmol toluene for 8 h, then subcultured to salts medium plus 142 µmol toluene (*squares*), 142 µmol toluene and 111 µmol TCE (*circles*), or 111 µmol TCE (*triangles*). As a control, the growth curve of *P. putida F1* cells grown on only salts medium are displayed as asterisks. All cultures were performed in triplicate, and the standard error bars for each group are displayed

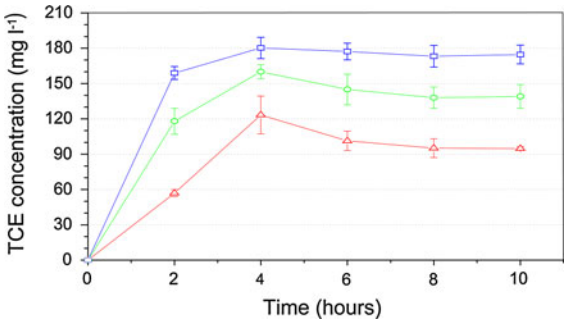

Fig. 2 Time course of TCE degradation in *P. putida* F1. As indicated, either 111 µmol TCE or 142 µmol toluene was added to each vial of toluene induced cells. The amount of degradation of TCE was determined at 2 h intervals by gas chromatography. *Squares* no bacterial TCE control; *diamonds* salts medium plus TCE, and *triangle* salts mediums plus toluene and TCE. All cultures were performed in triplicate, and the standard error bars for each group are displayed

of *P. putida* F1 to degrade TCE substantially (to 95 mg/L) (Fig. 2). These results suggest that providing both TCE and its co-metabolite toluene increases the capacity of *P. putida* F1 to degrade TCE.

As a co-metabolite in the TOD pathway, toluene is also subject to degradation by *P. putida* F1. To assess how toluene levels were changing over time in our cultures, we quantified toluene concentrations in each culture at 2 h intervals, using gas chromatography. When no bacteria were present in the salts medium, toluene levels remained steady at 250 mg/L (Fig. 3). When bacteria were seeded into the culture, toluene levels were reduced to 140 mg/L by 6 h. We observed further decreases in toluene concentration at 6 h (to 100 mg/L) when both TCE and toluene were present (Fig. 3). Interestingly, after 4 h of culture, the capacity of toluene plus TCE cultures to remove toluene plateaued, while cultures containing only toluene continued to metabolize toluene (Fig. 3). This was not due a reduction in *P. putida* F1 viability at this time point since CFUs/mL continued to increase past 4 h (Fig. 5). However, the rapid decrease in toluene between 6 and 10 h is likely due to exponential increases in *P. putida* F1 growth, which provided enough metabolic capacity to efficiently degrade toluene (Fig. 4).

In order to examine the relationship between TCE degradation capacity and bacterial growth, we repeated the experiment from Fig. 2 and counted CFUs/mL present at each time point (Fig. 5). When no cells were added to the culture, TCE remained at 180 mg/L for the duration of the time course. However, when *P.putida* F1 cells were present, the level of TCE was reduced from 180 mg/mL to 140 mg/L over the course of the experiment. The addition of toluene to the TCE cultures further reduced the TCE concentration to 40 mg/L. The results of this independent experiment were similar to those observed in Fig. 2. Interestingly, when toluene and TCE were both included in the cultures, *P. putida* F1 multiplied from 2×10^7 to 6.5×10^7 CFU/mL, while the number of

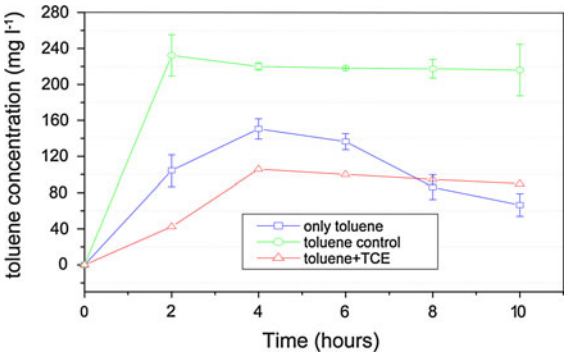

Fig. 3 Time course for toluene concentration variation. Toluene (initially amount 142 µmol) was added to each vial, and the amount of degradation was determined at 2 h intervals. *Circles* no bacterial, toluene control; *squares* salts medium plus toluene; and *triangle* salts mediums plus toluene and TCE. All cultures were performed in triplicate, and the standard error bars for each group are displayed

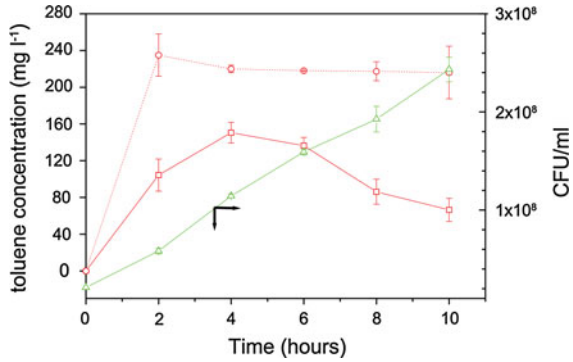

Fig. 4 Toluene degradation in *P. putida* F1 and bacterial multiplication. Cultures were seeded with142 µmol toluene at 28°C, pH = 7.1. *Circles* TCE concentration in no bacterial toluene control; *squares* TCE concentration in bacteria plus toluene cultures; and *triangles* growth curve of *P. putida* F1 incubated with salts medium and toluene. All cultures were performed in triplicate, and the standard error bars for each group are displayed

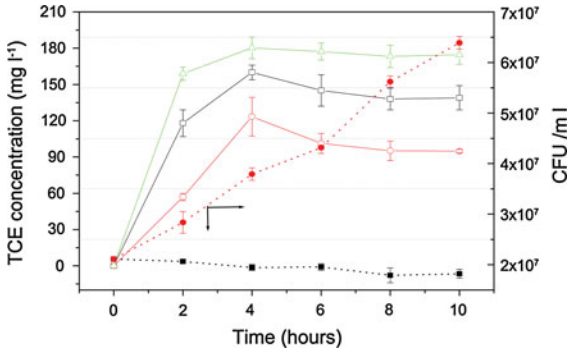

Fig. 5 Time course of TCE degradation (*open symbols*) in *P. putida* F1 and bacterial multiplication (*closed symbols*). *Triangles* 111 μmol TCE no bacteria control; *squares* bacteria plus 111 μmol TCE; and *circles* bacteria plus 142 μmol toluene and 111 μmol TCE. All cultures were performed in triplicate, and the standard error bars for each group are displayed

cells in TCE only cultures actually decreased over the same time course. Thus, it is likely that the increased multiplication of *P. putida* F1 cells partially accounts for the increased capacity of these cultures to degrade TCE.

Analysis of toluene dioxygenase expression

TOD, the enzyme required for toluene and TCE co-metabolism, may be regulated at the transcriptional level. We investigated whether the presence of TCE and toluene in *P. putida* F1 cultures influenced the expression of the *tod* gene by real-time quantitative PCR (Table 2). We harvested bacterial pellets at each timepoint, isolated RNA, and generated cDNA for subsequent use in *tod* and reference gene (*rpoD*) quantitation. Following 2 h of culture, we did not observe a significant effect of TCE, toluene, or TCE + toluene on *tod* expression in *P. putida* F1 cells (Table 2). By 4 h, *tod* expression was reduced approximately tenfold from the 2 h time point. Conversely, when toluene was present, the levels of *tod* mRNA levels continued to increase nearly tenfold from 2 to 4 h, then remained stable for the remainder of the time course (Table 2). When the cultures were supplemented with both toluene and TCE, *tod* levels were reduced, but not as strongly as when TCE alone was present (Table 2).

The expression of a gene of interest is most accurately quantified through comparison to a reference gene, whose expression is not expected to change as a result of the treatment Fan and Scow (1993, 2003); Vandecasteele et al. 2001). For these studies, we selected the *rpoD* gene, which is known to maintain consistent expression throughout the growth curve (Savli et al. 2003); thus, *rpoD* expression levels allow us to normalize the expression of *tod* between samples. Although TCE lead to a reduction in *tod* levels, it did not significantly influence *rpoD* levels until 10 h of culture (table 2). On the other hand, toluene increased *rpoD* expression, producing a similar trend as the *tod* expression (Table 2). The combination of TCE and toluene produced *rpoD* levels that were intermediated to either chemical alone (Table 2). The expression changes in *rpoD* levels are likely related to differences in bacterial multiplication and viability between the different culture conditions.

Finally, using the delta delta Ct method of normalization, we were able to separate effects of bacterial growth and viability changes, which may influence reference gene expression from treatment specific

Table 2 The copies of *tod* and *rpod* genes by different cluture conditions in *P. putida* F1. Mean (SD)

Culture conditions	Time (h)					
		2	4	6	8	10
TCE	*tod*	1.07 (1.10)	0.11 (0.93)	0.10 (0.48)	0.14 (0.86)	0.14 (0.23)
	rpoD	0.22 (5.02)	0.15 (5.72)	0.21 (5.01)	0.28 (0.86)	0.02 (0.75)
Toluene	*tod*	1.21 (10.30)	11.97 (6.80)	10.31 (6.51)	10.21 (13.30)	10.15 (16.50)
	rpoD	0.32 (2.71)	1.01 (5.51)	0.95 (7.95)	0.69 (8.23)	0.65 (5.61)
TCE +	*tod*	1.11 (5.71)	0.66 (3.23)	1.04 (0.50)	0.51 (4.01)	1.03 (4.52)
Toluene	*rpoD*	0.09 (0.23)	0.22 (0.10)	0.32 (0.91)	0.17 (0.58)	0.16 (0.86)

Mean 10^8 cpoies/mL

SD 10^6

Table 3 The relative expression of *tod* by different culture conditions in *P. putida* F1. Mean (SD)

Culture conditions	Time (h)				
	2	4	6	8	10
TCE	1.00 (0.08)	0.14 (0.01)	0.85 (0.04)	0.89 (0.03)	0.98 (0.02)
Toluene	1.30 (0.07)	1.52 (0.06)	1.78 (0.06)	1.24 (0.05)	1.18 (0.04)
TCE + Toluene	1.12 (0.07)	0.52 (0.03)	0.51 (0.02)	0.44 (0.01)	0.38 (0.01)

effects (Lekanne Deprez et al. 2002; Ullmannova and Haskovec 2003; Vandecasteele et al. 2001; Fey et al. 2004). When normalized to *rpoD* expression, *tod* levels mirrored the trend observed in Table 2. Toluene cultures exhibited enhanced *tod* mRNA levels, while TCE cultures exhibited severely repressed *tod* levels (Table 3). When both toluene and TCE were added to the cultures, the level of *tod* expression was intermediate (Table 3).

Discussion

Pseudomonas putida F1 is a favored bioremediation organism at sites contaminated with chlorinated solvents. For peak effectiveness, TOD, the critical enzyme in the pathway, must be stably expressed. In addition, cells must remain viable and competent to transport co-metabolic substrates across the cell membrane. Furthermore, modulation of enzymatic activity or co-factor availability is likely to play an important role in bioremediation success. Therefore, it is essential to understand the kinetics of substrate degradation and their influence on bacterial growth and TOD regulation.

Our results demonstrate that toluene enhanced *tod* expression, while TCE represses *tod* expression. Furthermore, the addition of toluene to TCE cultures is sufficient to partially rescue *tod* expression. Therefore, regulation of *tod* mRNA levels, which likely correlates with increased protein and enzymatic activity, is a potential mechanism of toluene enhancement of TCE degradation. Several studies concluded that toluene enhances the ability of *P. putida* F1 to degrade TCE (Morono et al. 2004; Bordel et al. 2007; Hori et al. 2005; Leahy et al. 1996). Our results provide an additional mechanism of toluene's enhancement of TCE degradation. Furthermore, we observed a decrease in *tod* levels when TCE was present, which suggests that the negative effects of TCE on *P. putida* growth may be related to reduction in *tod* levels. Indeed, several studies have identified TCE as an inhibitor of bacterial growth (Mu and Scow 1994; Chu and Alvarez-Cohen 1999; Nakano et al. 1999; Alpaslan Kocamemi and Çeçen 2007. Importantly, real-time PCR analysis of catabolic gene expression has successfully been applied in situ in groundwater (Fey et al. 2004; Labrenz et al. 2004; Wilson et al. 1999) and soil (Fey et al. 2004; Labrenz et al. 2004; Wilson et al. 1999), suggesting this approach is feasible for real-world application.

Ideally, bioremediation schemes would include techniques to monitor the capacity of the biomass to remove the noxious substance. Previous studies have suggested that the addition of aromatic substrates, such as phenol and toluene, may restore the capacity of *P. putida* F1 to degrade TCE. Our data suggest that monitoring *tod* levels can provide vital information about the health and TCE degradation capacity of the biomass. Furthermore, our results suggest that high levels of TCE and low levels of toluene may inhibit the growth of *P. putida* F1 in the biomass, thereby reducing bioremediation efficacy. This observation may explain why other groups (Morono et al. 2004; Bordel et al. 2007; Hori et al. 2005; Leahy et al. 1996) have observed that the addition of aromatic substrates to *P. putida* F1 cultures restores TCE degradation capacity.

In conclusion, similar to previously published reports, we observed that TCE inhibits *P. putida* F1 metabolism, while toluene enhances co-metabolism. Furthermore, toluene is beneficial for *P. putida* F1 growth, while TCE stunts bacterial growth. Real-time PCR analysis of *tod* expression suggests that toluene may influence co-metabolism of TCE by enhancing expression of the key enzyme in the pathway, *tod*. Likewise, TCE inhibits *tod* expression, indicating that this may be a contributing factor to the decrease in TCE degradation capacity observed over time.

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