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Trichloroethylene degradation by butane-oxidizing bacteria causes a spectrum of toxic effects

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Abstract The physiological consequences of trichloroethylene (TCE) transformation by three butane oxidizers were examined. *Pseudomonas butanovora*, *Mycobacterium vaccae*, and *Nocardioides* sp. CF8 utilize distinctly different butane monooxygenases (BMOs) to initiate degradation of the recalcitrant TCE molecule. Although the primary toxic event resulting from TCE cometabolism by these three strains was loss of BMO activity, species differences were observed. *P. butanovora* and *Nocardioides* sp. CF8 maintained only 4% residual BMO activity following exposure to 165 μM TCE for 90 min and 180 min, respectively. In contrast, *M. vaccae* maintained 34% residual activity even after exposure to 165 μM TCE for 300 min. Culture viability was reduced 83% in *P. butanovora*, but was unaffected in the other two species. Transformation of 530 nmol of TCE by *P. butanovora* (1.0 mg total protein) did not affect the viability of BMO-deficient *P. butanovora* cells, whereas transformation of 482 nmol of TCE by toluene-grown *Burkholderia cepacia* G4 caused 87% of BMO-deficient *P. butanovora* cells to lose viability. Together, these results contrast with those previously reported for other bacteria carrying out TCE cometabolism and demonstrate the range of cellular toxicities associated with TCE cometabolism.

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Introduction

After years of industrial use accompanied by accidental spills and deliberate dumping, trichloroethylene (TCE) is now a common contaminant of soils and groundwater. Aerobic cometabolism is an effective means of transforming TCE, thus providing a promising approach to the bioremediation of contaminated sites. Bacterial species and mixed cultures that can cometabolize TCE have been isolated from contaminated aquifers (Hamamura and Arp 2000; Coleman et al. 2002; Brzostowicz et al. 2003). One of the factors limiting the effectiveness of cometabolism is the cytotoxicity of the TCE transformation product(s). Previously, TCE cometabolism was shown to cause inactivation of the transforming oxygenase in several classes of bacteria, including ammonia-oxidizing (*Nitrosomonas europaea*), methane-oxidizing (*Methylosinus trichosporium* OB3b), and toluene-oxidizing (*Pseudomonas putida* F1; Rasche et al. 1991; van Hylckama Vlieg et al. 1996; Newman and Wackett 1997). Additionally, research with *M. trichosporium* OB3b, toluene-oxidizing *Burkholderia cepacia* G4, and a mixed culture of soluble methane monooxygenase (sMMO)-containing methane oxidizers, showed significant losses in cellular viability during TCE transformation (Alvarez-Cohen and McCarty 1991; van Hylckama Vlieg et al. 1997; Yeager et al. 2001). Exposure of *B. cepacia* G4 to 250 μM TCE for 90 min resulted in only partial (52%) decrease in toluene monooxygenase (T2MO) activity, while acetate-coupled respiration decreased 98% and the number of viable cells was reduced by three orders of magnitude (Yeager et al. 2001).

Butane-oxidizing bacteria are an attractive choice for the augmentation of indigenous microbial communities at sites requiring bioremediation (Kim et al. 1997, 2000; Pardi et al. 2001). While several butane-oxidizing bacteria have been shown capable of TCE cometabolism (Wackett et al.

1989; Hamamura et al. 1999), the kinetic properties of TCE transformation by these bacteria have not been examined. Further, the nature and extent of transformation-dependent TCE toxicity on butane-oxidizing bacteria has not been studied. The enzyme butane monooxygenase (BMO) initiates butane oxidation. Like other catabolic monooxygenases, BMO has a relatively wide substrate range, including certain gaseous and liquid alkanes and a number of chlorinated hydrocarbons. However, BMOs in different organisms have been shown to be biochemically distinct. For example, soluble BMO from *P. butanovora* has been biochemically and genetically characterized and is similar to sMMO from methanotrophs (Arp 1999; Hamamura et al. 1999; Sluis et al. 2002). In contrast, *Nocardioides* sp. CF8 appears to maintain a copper-containing BMO that is associated with the cell membrane (Hamamura et al. 2001). The BMO of *Mycobacterium vaccae* is distinguished from the BMOs of *P. butanovora* and *Nocardioides* sp. CF8 by its distinct [^{14}C]acetylene labeling pattern and its partial inhibition by the copper chelator, allylthiourea (ATU; Hamamura et al. 1997). Since the BMOs from these three organisms were shown to be biochemically diverse, it was intriguing to study the physiological responses of their hosts to TCE exposure and to compare them with other well studied systems.

The type and severity of TCE transformation-dependent damage occurring in bacterial systems has various implications. For example, the regioselectivity of different transforming monooxygenases can have very different impacts on the bacterial host and its community, or the host may utilize epoxidases or other repair mechanisms allowing for greater tolerance to TCE transformation. The objective of this study was to examine the physiological consequences of TCE transformation by three butane oxidizers, *P. butanovora*, *M. vaccae*, and *Nocardioides* sp. CF8, that have distinctly different BMOs and to compare the results with published work on other bacteria.

Materials and methods

Bacterial strains and growth conditions

P. butanovora (ATCC 43655) was cultured in sealed bottles (720 ml) containing 300 ml of liquid medium and 420 ml of air, with 30 ml of *n*-butane gas (99.0%; Airgas, Randor, Pa.) added as an overpressure. The growth medium consisted of 2 mM $MgSO_4 \cdot 7H_2O$, 400 μM $CaCl_2 \cdot 2H_2O$, phosphate buffer (pH 7.2 presented as 60 mM $(NH_4)_2HPO_4$, 7 mM $Na_2HPO_4 \cdot 7H_2O$, 15 mM KH_2PO_4), and the trace elements described by Wiegant and de Bont (1980). The *P. butanovora* mutant strain *bmoX::lacZ::kan* has a DNA cassette disrupting *bmoX* and rendering the host BMO-deficient and kanamycin-resistant (unpublished data). *P. butanovora bmoX::lacZ::kan* was grown in 50 ml of the medium described above with 5 mM sodium citrate and 25 μg ml^{-1} kanamycin. *M. vaccae* JOB5 (ATCC 29678) was grown in medium described by Hamamura et al. (1997). Cultures (300 ml) were grown in 720-ml sealed bottles with 180 ml of butane gas and 150 ml of oxygen added as overpressure. *Nocardioides* sp. CF8 was grown in 300 ml of the same medium used for *M. vaccae*, with 180 ml of butane gas added as overpressure. *B. cepacia* G4 was grown as described by Yeager et al. (2001) in sealed 160-ml vials containing 60 ml of minimal medium and 94 μmol of toluene. At 4 h prior to harvest, an additional 94 μmol of toluene was added. All cultures were incubated at 30°C in an orbital shaker at 150 rpm and harvested during the log or late log growth phase for experimental use. Prior to experiments, cells were washed three times with the same phosphate buffer used for growing the individual cultures and resuspended in the same buffer as concentrated cell suspensions.

TCE exposure

TCE degradation was monitored by gas chromatography. Teflon-faced butyl septa (Supelco) were used to seal serum vials (7.7 ml) which contained 5 mM sodium butyrate or sodium lactate, TCE (2.2, 3.3, 5.5, 10.9, 21.9, 54.8, 110, or 165 μM initial aqueous concentration after addition of cells), and sufficient phosphate buffer to bring the volume to 800 μl and were equilibrated for at least 30 min in a reciprocating shaker with constant shaking at 30°C. Concentrated cell suspensions (200 μl containing 1.0 mg total protein *P. butanovora*, *Nocardioides* sp. CF8, or 0.75 mg *M. vaccae*) were added to initiate the experiments. To monitor TCE consumption, samples of the gas phase (20–100 μl) were removed using a gas-tight syringe (Hamilton) for analysis by gas chromatography (see below). TCE concentration and exposure time were selected to maximize TCE transformation. Cometabolism (e.g. ethylene oxidation; Hamamura et al. 1999) can be slow if the cellular reductant supply is limiting. Therefore, an exogenous source of reductant was provided in the form of sodium lactate (10 mM) for *P. butanovora* and sodium butyrate (10 mM) for *M. vaccae* and *Nocardioides* sp. CF8. These reductants were the most effective among those studied (lactate, citrate, butyrate, acetate). Acetylene is a potent inactivator of BMO (Hamamura et al. 1999). Cells that had been exposed to acetylene were included to confirm the function of BMO in TCE transformation. After the desired incubation period, the reaction mixture was transferred to a microcentrifuge tube and the cells were sedimented. Cells were resuspended, washed three times, and then resuspended in fresh phosphate buffer for post-exposure assays for residual BMO activity, oxygen uptake, and cellular viability (see below for assay descriptions).

For determination of TCE degradation kinetics, vials of butane-grown cells containing different initial TCE concentrations were monitored for TCE degradation. The rate of degradation by *P. butanovora* was linear over the first 20 min of exposure and determined with 0-, 12-, and 20-min time-points. Likewise, the rate of degradation by *M. vaccae* was linear over the first 60 min and determined with 0-, 20-, 40-, and 60-min time points; and the rate of degradation by *Nocardioides* sp. CF8 was linear over the first 30 min and

determined with 0-, 1-, 5-, and 30-min time points (data not shown).

Assays for BMO activity, oxygen uptake, and cellular viability

BMO activity was measured using the ethylene oxidation assay (Hamamura et al. 1999). This method exploits ethylene as an alternative substrate for BMO in the presence of an exogenous source of reductant. Cell suspensions (1 ml) were incubated for up to 30 min at 30°C with shaking in 7.7-ml sealed serum vials containing phosphate buffer and supplemented with 5 mM sodium lactate (*P. butanovora*) or 5 mM sodium butyrate (*Nocardioides* sp. CF8, *M. vaccae*). Ethylene gas (20% v/v) was added to initiate the assay. Samples of the gas phase (100 μl) were removed for analysis by gas chromatography for ethylene oxide accumulation (see below).

Aeration in the absence of enzyme substrate is suggested to cause the inactivation of monooxygenases (Alvarez-Cohen and McCarty 1991; Chu and Alvarez-Cohen 1999). Therefore, treatments were included with no TCE added (0 μM) to establish whether any loss of BMO activity occurred from aeration alone. To help determine if the presence of oxygen was responsible for the loss of BMO activity, reaction vials containing cells were purged of air and exposed to nitrogen during the incubation period (N_2-exposed).

Oxygen uptake measurements were made using a Clark-style oxygen electrode (Yellow Springs, Ohio) mounted in a glass water-jacketed reaction vessel (1.6 ml) at 30°C. The reaction chamber was filled with phosphate buffer. Following TCE exposure, cells were washed and resuspended in phosphate buffer to 200 μl. Cells (50 μl) were added to the reaction vessel and the endogenous oxygen uptake rate was determined. Sodium butyrate (5 mM; *P. butanovora*) or 1-butanol (5 mM; *Nocardioides* sp. CF8 and *M. vaccae*) was added to the vessel to determine a substrate-dependent oxygen uptake rate. Butyrate and 1-butanol are metabolites of butane oxidation (Arp 1999) and do not require functional BMO for their further metabolism (Vangnai et al. 2002). Several other substrates were tested to determine their ability to promote rates of oxygen uptake. Sodium lactate, sodium citrate, sodium acetate, sodium formate, and ethanol were also tested, but generally did not support rates of oxygen uptake above endogenous levels.

To account for any toxicity associated with exposure to TCE in the absence of TCE transformation, acetylene-treated cells were exposed to 22 μM and 165 μM initial TCE concentrations for the predetermined exposure times prior to washing and plating. To determine residual cellular viability following TCE exposure, cells were washed and resuspended in phosphate buffer to 1.0 ml. Serial dilutions of the resuspended cells were made and aliquots (100 μl) were plated onto R2A agar plates (Difco). R2A agar contains pyruvate to degrade hydrogen peroxide, a primary causative agent of oxidative stress. Colonies were counted after 2–3 days of incubation at 30°C.

Effect of TCE degradation on viability of surrounding cells

Butane-grown *P. butanovora* or toluene-grown *B. cepacia* G4 cells were mixed with *P. butanovora bmoX::lacZ::kan* (9:1 ratio for *P. butanovora* wild type to mutant, 9:1 or 6:1 ratio for *B. cepacia* G4 to *P. butanovora bmoX::lacZ::kan*) and exposed to TCE for 90 min as described above. Following TCE exposure, cells were washed and resuspended in phosphate buffer to 1 ml. The number of viable *P. butanovora bmoX::lacZ::kan* cells remaining was determined by preparing serial dilutions to 1×10^{-6} in sterile phosphate buffer and plating 100-μl aliquots onto R2A agar plates with kanamycin (25 μg ml^{-1}). Colonies (*P. butanovora bmoX::lacZ::kan*) were counted after 2–3 days of incubation at 30°C.

Analytical and other methods

Ethylene oxide accumulation was analyzed with a GC-8A chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) and a stainless steel column (0.3×61.0 cm) packed with Porapak Q 80–100 mesh (Alltech, Deerfield, Ill.). TCE transformation was monitored using the same GC-8A FID chromatograph with a capillary column (15.0 m×0.53 mm; Alltech). Ethylene oxide and TCE calibration curves were obtained by performing a headspace gas analysis with vials containing known amounts of each compound. A dimensionless Henry's constant, 0.494 at 30°C (Gossett 1987), was used to account for aqueous and gaseous partitioning of the total TCE mass in the vials. Protein concentrations were determined by the Biuret assay (Gornall et al. 1949), following cell solubilization in 3 M NaOH for 30 min at 65°C.

Results

TCE exposure results in severe loss of BMO activity

Time-courses of TCE transformation are shown for each of the three butane-grown species (Fig. 1). An initial concentration of 165 μM TCE (initial aqueous concentration) was used for determining the time-course of TCE transformation for *P. butanovora*. Since the initial rate of TCE transformation by *M. vaccae* and *Nocardioides* sp. CF8 was approximately five times less than that of *P. butanovora*, an initial TCE concentration of 22 μM was used to accurately measure TCE disappearance by GC. The rate of TCE transformation decreased with time for all three strains. Even after 4 h of exposure, TCE transformation by *M. vaccae* remained measurable. In contrast, TCE transformation by *P. butanovora* and *Nocardioides* sp. CF8 ceased after 90 min and 180 min, respectively. Supplementing the reaction vials with additional reductant (sodium lactate or sodium butyrate) did not stimulate additional TCE disappearance, suggesting that reductant limitation was not the reason for the time-dependent decrease in TCE disappearance.

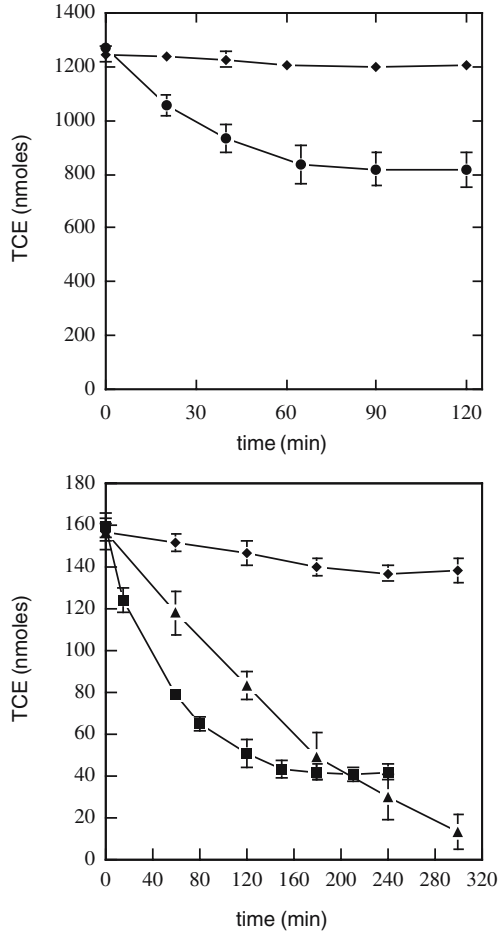

Fig. 1 Time-courses for TCE disappearance by butane-grown *P. butanovora* (●) with 165 μM initial TCE concentration, and *Nocardioides* sp. CF8 (■), *M. vaccae* (▲), and acetylene-inactivated control cells (♦) with 22 μM initial TCE concentration. Cells were grown on butane, harvested, and washed three times prior to incubation with TCE at 30°C

Following exposure to TCE, residual BMO activities for butane-grown *P. butanovora*, *Nocardioides* sp. CF8, and *M. vaccae* were measured and are summarized in Table 1. Incubation in the absence of substrate (0 μM TCE) caused a significant reduction in BMO activity in all three species. The same loss in BMO activity was measured when the air in the vials was replaced with nitrogen. It appears that BMO can be partially inactivated by the mechanical disturbance experienced during wash procedures and shaking. To determine whether BMO activity was affected by TCE transformation, BMO activity was measured before and after incubation of each butane-grown species with different initial concentrations of TCE. Because cellular damage is imparted by a toxic intermediate(s) formed during TCE transformation (most likely TCE epoxide), exposure conditions were selected that allowed maximal TCE transformation (see Fig. 1). Exposure to TCE concentrations as low as 5.5 μM resulted in loss of BMO activity; and the residual BMO activity decreased with increasing TCE concentrations for all three bacteria. While incubation of all three strains in the presence or absence of air resulted in the loss of BMO activity, exposure to TCE consistently resulted in even further reduction of BMO activity. These results showed that BMO activities in *P. butanovora* and *Nocardioides* sp. CF8 were very sensitive to TCE transformation. The fact that BMO activity in *M. vaccae* suffered only a modest decline following TCE transformation is reminiscent of T2MO activity in *B. cepacia* G4, which was also relatively unaffected by TCE transformation (Yeager et al. 2001).

Transformation capacity (T_c) is defined as the mass of a compound that can be degraded prior to enzyme inactivation by a given amount of non-growing cells (Chu and Alvarez-Cohen 1998). *P. butanovora* (1.0 mg total protein) was exposed to an initial TCE concentration of 165 μM (1,265 nmol of TCE) for 90 min and *Nocardioides* sp. CF8 (1.0 mg total protein) was exposed to an initial TCE concentration of 165 μM for 180 min. Because only 4% of the initial BMO activities remained in *P. butanovora* and *Nocardioides* sp. CF8 under these exposure conditions, their transformation capacities were determined. T_c for *P. butanovora* was 475±30 nmol mg^{-1} total protein, and T_c for *Nocardioides* sp. CF8 was 214±27 nmol mg^{-1} total protein. We could not determine a transformation capacity for *M. vaccae*, because it retained over 30% of its initial BMO activity following TCE exposure.

TCE degradation follows Michaelis–Menten enzyme kinetics

Whole-cell kinetics for TCE cooxidation have been determined for a number of physiologically diverse bacteria, including ammonia-, phenol-, methane-, and toluene-oxidizing bacteria (Arp et al. 2001). Here, we contribute information about the TCE degradation kinetics of pure

Table 1 Residual BMO activity following exposure of cells to TCE. All data expressed are means ±standard deviations of at least three trials. TCE exposure times were selected to ensure maximal TCE transformation (*P. butanovora* 90 min, *M. vaccae* 300 min, *Nocardioides* sp. CF8 180 min)

	Initial BMO activity (nmol min^{-1} mg^{-1} protein)	Residual BMO activity (%)							
		Following wash steps	Butane-protected	N_2-exposed	TCE concentration				
					0 μM	5.5 μM	22 μM	55 μM	165 μM
P. butanovora	15.5±0.8	71±1.9	65±2.1	49±2.2	50±1.1	35±1.5	27±1.6	13±1.1	4.3±1.2
M. vaccae	41.1±1.9	86±2.0	65±2.5	61±1.0	62±2.1	58±2.2	39±1.7	34±1.5	34±2.1
Nocardioides sp. CF8	12.0±0.4	85±0.9	75±1.3	63±1.6	64±0.7	31±0.7	11±0.2	6.0±0.3	3.6±0.1

cultures of butane oxidizers. For all three species of bacteria, initial rates of degradation were plotted against TCE concentration and fitted to the Michaelis–Menten model of enzyme velocity versus substrate concentration (Fig. 2), and the corresponding V_{max} and K_s values were determined.

The maximal rate of TCE transformation by *P. butanovora* was over five times faster than the rates of TCE transformation by *Nocardioides* sp. CF8 and *M. vaccae*. K_s values ranged from a low of 6 μM for *M. vaccae* to 22 μM for *Nocardioides* sp. CF8. Despite the phylogenetic and biochemical diversity of these three butane-oxidizing species and their respective BMOs, the kinetic parameters for TCE transformation were comparable.

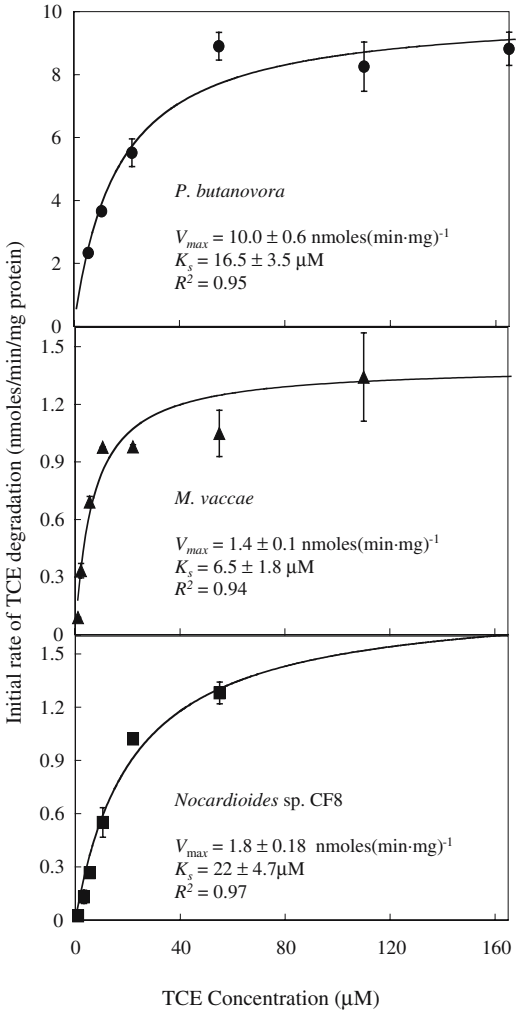

Fig. 2 K_s and V_{max} determinations for TCE degradation by *P. butanovora*, *M. vaccae*, and *Nocardioides* sp. CF8. Initial rates of TCE degradation for butane-grown *P. butanovora* (●), *M. vaccae* (▲), and *Nocardioides* sp. CF8 (▪) are plotted against TCE concentration. The *symbols* represent the means for three experiments and the *lines* represent the Michaelis–Menten model fit for each species tested

Effect of TCE cometabolism on cellular respiration and viability

Although BMO inactivation during TCE cometabolism occurred with all three butane-grown bacteria, there remained the possibility of other adverse cellular responses. Residual substrate-dependent oxygen uptake rates and the viability of cells exposed to two different concentrations of TCE were determined. Eighty-three percent of *P. butanovora* cells lost viability when exposed to 165 μM initial TCE concentration, whereas only 32% of butyrate-dependent oxygen uptake was lost (Table 2). In contrast, no loss in viability was detected at an initial TCE concentration of 22 μM. Cells of *Nocardioides* sp. CF8 and *M. vaccae* that were exposed to 22 μM and 165 μM TCE did not appear to lose viability, but 1-butanol-dependent oxygen-uptake rates decreased about 32% in *Nocardioides* sp. CF8 cells exposed to 165 μM initial TCE concentration. The substrate-dependent oxygen-uptake rates for cells of *M. vaccae* following incubation with TCE were similar to the oxygen-uptake rates of cells incubated in the absence of TCE. Substrate-dependent oxygen-uptake rates for acetylene-treated cells of all three strains were intermediate to cells incubated in the absence of TCE and TCE-treated cells.

Because significant transformation-dependent TCE toxicities, including severe loss of sBMO activity and loss of viability, were detected in *P. butanovora*, we determined whether the TCE transformation-dependent toxicities observed in *P. butanovora* were manifested in neighboring cells not expressing sBMO. Yeager et al. (2001) showed that *B. cepacia* G4 reduces the viability of neighboring T2MO-inactivated *B. cepacia* TCS-100 cells when incubated with TCE. When wild-type *P. butanovora* was mixed 9:1 with citrate-grown *P. butanovora bmoX::lacZ::kan* and exposed to 165 μM TCE for 90 min, the BMO-deficient mutant remained viable (Table 3). In contrast, when toluene-grown *B. cepacia* G4 was mixed 9:1 with *P. butanovora bmoX::lacZ::kan*, 97% of the BMO-deficient mutant cells lost viability. To determine whether the viability of neighboring *P. butanovora bmoX::lacZ::kan* cells were sensitive to lower levels of TCE transformation, toluene-grown *B. cepacia* G4 was mixed 6:1 with *P. butanovora* mutant cells and exposed to 165 μM TCE for 90 min. While the amount of TCE transformed by *B. cepacia* G4 was equivalent to that transformed by wild-type *P. butanovora*, 87% of *P. butanovora bmoX::lacZ::kan* cells were no longer viable. Either wild-type *P. butanovora* does not release a toxic product extracellularly, or the toxic product(s) do not accumulate to concentrations that could cause damage to other bacterial community members. These results emphasize the intriguing possibility that T2MO and sBMO produce or release different products or different ratios of products during the transformation of TCE.

Discussion

Toxicity resulting from TCE cometabolism can be viewed as a continuum from specific damage (to the transforming

Table 2 Remaining viability and substrate dependent oxygen-uptake rates of TCE-exposed *P. butanovora*, *M. vaccae*, and *Nocardioides* sp. CF8. Viability is reported as the mean ± standard deviation of at least three samples. The substrate used for determination of oxygen-uptake rates was butyrate for *P. butanovora* and 1-butanol for *Nocardioides* sp. CF8 and *M. vaccae*

	Acetylene-treated prior to TCE exposure	Exposure time (min)	Initial [TCE] (μM)	TCE transformed (nmol)	Number of viable cells ($\times 10^7$ CFU ml^{-1})	Substrate-dependent oxygen-uptake rate (nmol O_2 mg^{-1} min^{-1})
P. butanovora	No	90	22	165±0	76±4	15.0±2.0
	No	90	165	451±62	13±3	12.9±1.1
	Yes	90	165	38±8	76±5	16.6±0.8
	No	90	0	0	75±6	19.3±1.9
M. vaccae	No	300	22	155±12	49±17	18.0±1.7
	No	300	165	346±40	44±14	17.1±1.7
	Yes	300	165	49±10	47±12	18.5±0.4
	No	300	0	0	46±10	19.8±1.5
Nocardioides sp. CF8	No	180	22	125±10	73±12	35.6±7.3
	No	180	165	236±30	67±12	28.6±4.9
	Yes	180	165	42±8	75±13	37.2±1.3
	No	180	0	0	77±16	42.1±2.0

Table 3 Loss of viability of BMO-deficient cells (*P. butanovora bmoX::lacZ::kan*) in the presence of TCE-transforming cells. Viability is reported as mean ±standard deviation of at least three samples

TCE-transforming strain	Acetylene-treated prior to TCE exposure	Ratio of TCE transforming strain to BMO-deficient strain	TCE transformed (nmol)	Number of viable BMO-deficient cells ($\times 10^6$ CFU ml^{-1})
P. butanovora	No	9:1	530±25	39±5
P. butanovora	Yes	9:1	35±12	41±2
B. cepacia G4	No	9:1	1065±61	1.0±0.2
B. cepacia G4	Yes	9:1	41±8	40±4
B. cepacia G4	No	6:1	482±21	5.5±4
B. cepacia G4	Yes	6:1	30±11	42±2

enzyme itself) to general damage (affecting cellular respiration, viability, or the bacterial community). The results of this study present a somewhat different picture of toxicity associated with TCE degradation than that emerging from studies with methanotrophs and the toluene-oxidizing bacterium, *B. cepacia* G4. Whereas the predominant TCE transformation-dependent toxicity measured in MMO- and T2MO-expressing cells is loss of cellular viability, the three butane-oxidizing bacteria studied here cometabolize TCE with toxic consequences that are either modest or severe and are either specific or broad-based, depending on the parameters considered.

Our results also contribute kinetic constants for three more bacteria; and these allow some general trends to be noted. Interestingly, the kinetic parameters for butane-oxidizing bacteria are similar to those determined for other organisms that carry out TCE cometabolism. As with the characterized BMOs, the closely related MMOs can be expressed as either a soluble MMO (when copper is limiting) or as a particulate MMO. Other soluble monooxygenases include T2MO from *B. cepacia* G4 and alkene monooxygenase from *Xanthobacter* Py2. The maximal rates reported for organisms expressing these soluble monooxygenases range from 2.4 nmol to 580 nmol of TCE transformed min^{-1} mg^{-1} protein, with the majority of measurements falling between 8 nmol and 38 nmol min^{-1} mg^{-1} protein. The corresponding K_s values range from 3 μM to 225 μM (for a review, see Arp et al. 2001). The kinetic constants determined for *P. butanovora* compare most favorably with those of *B. cepacia* G4, which has a maximum TCE oxidation rate of 10 nmol min^{-1} mg^{-1} protein (Landa et al. 1994). The half-saturation constant (K_s) for *B. cepacia* G4 is 6 μM, compared with 16.5 μM for *P. butanovora*. Organisms expressing membrane-associated, copper-containing monooxygenases appear to have slightly lower kinetic constants: V_{max} values range from 4.1 nmol min^{-1} mg^{-1} protein to 10.9 nmol min^{-1} mg^{-1} protein and K_s values range from 7.9 μM to 30 μM in *Nitrosomonas europaea* expressing AMO and *Methylosinus trichosporium* expressing pMMO (for a review, see Arp et al. 2001). The kinetic constants determined for *Nocardioides* sp. CF8 expressing pBMO and *M. vaccae* are similar to those of bacteria expressing copper-containing monooxygenases. Although the BMO in *M. vaccae* has not been shown to be copper-containing, its partial sensitivity to ATU and low V_{max} towards TCE suggests it may have some properties that are similar to the pBMO of *Nocardioides* sp. CF8.

The primary toxic event resulting from TCE transformation by all three of these butane-oxidizing bacteria appears to be loss of BMO activity. The reduction of BMO

activity in *Nocardioides* sp. CF8 and *P. butanovora* was particularly striking. Both of these bacteria maintained less than 5% of their initial BMO activity after exposure to 165 μM TCE, as described in Table 1. We considered the possibility that TCE acts as a mechanism-based inactivator. Indeed, many of the requirements for describing TCE as a mechanism-based inactivator are met (Silverman 1988): inactivation of BMO by TCE is irreversible, an active catalyst (BMO) is required for inactivation, the presence of substrate (butane) protects against inactivation (data not shown), and the rate of inactivation is proportional to the concentration of TCE at low concentrations and approaches a maximum at higher concentrations. Attempts to determine first-order rates of inactivation were confounded by loss of activity due to washing procedures and mechanical disturbance and (in the case of *P. butanovora*) demonstrable secondary toxicities (i.e., reduced cellular viability) at high TCE concentrations. Regardless of whether or not all the criteria were met for classifying TCE as a mechanism-based inactivator of the BMOs, the data are consistent with the idea that TCE transformation by BMO leads to the loss of BMO activity.

While loss of BMO activity was the primary toxic event in butane-grown cells, secondary toxicity was only measured in *P. butanovora*. Both toluene-grown *B. cepacia* G4 and methane-grown *M. trichosporium* OB3b lose culturability following TCE exposure (Yeager et al. 2001). In fact, in both cases, overall cellular damage is considered to be the principal toxic effect associated with TCE oxidation, while damage to the monooxygenases is secondary. Considering the severe loss of BMO activity incurred during TCE transformation coupled with the modest decrease in cellular respiratory activity, we did not expect viability to be significantly reduced. However, the number of viable *P. butanovora* cells was markedly decreased when exposed to high concentrations of TCE. Presumably, at the higher initial TCE concentration, *P. butanovora* attained the toxicity threshold introduced by Chu and Alvarez-Cohen (1999), which directly correlates T_c with general cellular damage. In this case, the T_c achieved (478 nmol TCE degraded mg^{-1} protein) resulted in sufficient cumulative cellular damage to render the cells irreparable. The toxicity threshold was also achieved by *Nocardioides* sp. CF8 (as predicted by the amount of TCE transformed per milligram of protein). However, the corresponding loss in viability was not detected. It is curious that no loss in viability was measured in *Nocardioides* sp. CF8 or *M. vaccae* cells following TCE exposure. There are several possible explanations for these disparate results. First, the maximal rates of TCE degradation for both *Nocardioides* sp. CF8 and *M. vaccae* are lower than those of *P. butanovora*, *M. trichosporium* OB3b, and *B. cepacia* G4. Therefore, it is possible that repair of general cellular damage keeps pace with toxicity imparted by a reactive intermediate, such as TCE epoxide. This idea would suggest that bacteria with higher V_{max} values experience a "dosage effect" resulting from the formation of high concentrations of a toxic intermediate in a short time period. Second, the BMOs of *M. vaccae* and *Nocardioides* sp. CF8 may catalyze the formation of different, non-toxic products during TCE transformation. Or, alternatively, different ratios of the same products (including the toxic intermediates) may be formed, resulting in less accumulation of the more destructive intermediates.

It is also conceivable that the partition ratio of TCE transformation products by pBMO in *Nocardioides* sp. CF8 is smaller than that of strains exhibiting losses in cellular viability. The transformation of a mechanism-based inactivator (I) into its activated form (I') is described by this equation:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} E \cdot I \xrightarrow{k_2} E \cdot I' \xrightarrow{k_4} E\text{–}I''$$
$$E \cdot I' \xrightarrow{k_3} E + P$$

The partition ratio, k_3/k_4, is an indicator of the efficiency of the inactivator (Silverman 1988). A low partition ratio describes a system in which most inactivator molecules lead to enzyme inactivation (E–I'') and fewer molecules are converted and released as (potentially damaging) product (P). TCE transformation by *Nocardioides* sp. CF8 results in a lack of general respiratory damage, maintenance of full cellular viability, and a striking sensitivity of pBMO. Taken together, these results suggest that there is minimal release of toxic intermediates from the $E{\cdot}I'$ complex and support the presumption of a low partition ratio.

It appears that higher rates of TCE cometabolism can cause the rapid accumulation of cellular damage. The maintenance of BMO activity and overall cellular health in *M. vaccae* following TCE transformation encourages consideration of this and other strains that have slow rates of TCE degradation. Unfortunately, these "plodding" bacterial strains are often overlooked in favor of speedier but potentially less robust strains. Interestingly, several aerobic, vinyl chloride-degrading *Mycobacterium* strains and one *Nocardioides* strain were recently isolated from sites contaminated with chlorinated ethenes (Coleman et al. 2002), indicating the importance of these particular genera in remediation of xenobiotic compounds in the environment.

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