Cometabolic transformation of *cis*-1,2-dichloroethylene and *cis*-1,2-dichloroethylene epoxide by a butane-grown mixed culture

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Abstract Aerobic cometabolism of *cis*-1,2-dichloroethylene (c-DCE) by a butane-grown mixed culture was evaluated in batch kinetic tests. The transformation of c-DCE resulted in the coincident generation of c-DCE epoxide. Chloride release studies showed ~75% oxidative dechlorination of c-DCE. Mass spectrometry confirmed the presence of a compound with mass-to-charge-fragment ratios of 112, 83, 48, and 35. These values are in agreement with the spectra of chemically synthesized c-DCE epoxide. The transformation of c-DCE required O_2, was inhibited by butane and was inactivated by acetylene (a known monooxygenase inactivator), indicating that a butane monooxygenase enzyme was likely involved in the transformation of c-DCE. This study showed c-DCE epoxide was biologically transformed, likely by a butane monooxygenase enzyme. c-DCE epoxide transformation was inhibited by both acetylene and c-DCE indicating a monooxygenase enzyme was involved. The epoxide transformation was also stopped when mercuric chloride ($HgCl_2$) was added as a biological inhibitor, further support a biological transformation. To our knowledge this is the first report of the biological transform c-DCE epoxide by a butane-grown culture.
Keywords Butane-grown mixed culture; aerobic cometabolism; *cis*-1, 2-dichloroethylene; *cis*-1, 2-dichloroethylene epoxide

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Introduction

Aerobic cometabolism is a potential method for remediating aquifers contaminated with chlorinated aliphatic hydrocarbons (CAHs) (McCarty and Semprini 1993). Microorganisms grown on a variety of substrates express oxygenase enzymes that are capable of transforming CAHs. Transformation of chlorinated ethenes by monooxygenases results in the formation of epoxides (Oldenhuis *et al.*, 1991; Van Hylckama Vlieg *et al.*, 1996). These electrophilic compounds are unstable in aqueous solution. The reactivities of the epoxides and their degradation products often result in covalent modification of cellular components, causing transformation product toxicity (Oldenhuis *et al.*, 1991; Van Hylckama Vlieg *et al.*, 1997). Consequently, the amount of chlorinated ethene that can be transformed is limited due to transformation product toxicity (Alvarez-Cohen and McCarty, 1991). Since the toxicity that is associated with cometabolic transformation products of chlorinated ethenes is a main limiting factor for the application of monooxygenase-expressing organisms, it is desirable to find ways to biologically detoxify transformation products.

c-DCE is an important groundwater pollutant that is generated from perchloroethylene (PCE) and trichloroethylene (TCE) by dechlorination under anaerobic conditions. However, few data on the aerobic degradation of c-DCE are available, compared with the data on other chlorinated ethenes, such as TCE. van Hyclkama Vlieg *et al.* (1996, 1998) reported that c-DCE epoxide formed by transformation of c- DCE was actively transformed by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase

and *Rhodococcus* sp. strain AD45. However, rapid inactivation occurred during this transformation, indicating toxic products were generated. (van Hylckama Vlieg *et al.*, 1996, 1997).

In microcosm studies with aquifer core material from Hanford in Washington, butane was found to be an effective substrate for aerobic cometabolism of chloroform (CF) and 1,1,1-trichloroethane (1,1,1-TCA) (Kim *et al.* 1997). Resting-cell studies with a butane-oxidizing mixed culture isolated from the Hanford DOE site microcosms showed that butane-utilizers effectively transformed a broad range of CAHs, especially 1,1-dichloroethylene (1,1-DCE), c-DCE, 1,1,1-TCA, and 1,1-dichloroethane (1,1-DCA) (Kim *et al.*, 2000, 2002a,b).

In this work, we evaluated: 1) how effectively a butane-grown enrichment culture could transform c-DCE; 2) if c-DCE epoxide was produced from c-DCE transformation; 3) whether a butane monooxygenase was involved in the transformation of c-DCE and c-DCE epoxide; and 4) how the transformation of c-DCE and c-DCE epoxide affected cell activity. Loss of butane uptake ability after exposure to c-DCE was used as a measure of cell inactivation due to the c-DCE transformation. Chloride release was measured as an indicator of the extent of dehalogenation achieved. This study provides the first evaluation of c-DCE and c-DCE epoxide that can be transformed by a butane-grown enrichment culture.

Materials and methods

A butane-utilizing mixed culture

The butane-utilizing enrichment was obtained from Hanford soil microcosms described by Kim *et al.* (1997). The enrichment was batch grown in 750-mL capped-bottles containing 10% butane (v/v) in air and 250 ml of *Xanthobacter* Py2 medium (Wiegant and de Bont 1980) with the pH adjusted to 7.3, except NH_4NO_3 replaced by $NaNO_3$. The bottles were rotary shaken at 200 rpm at 30 °C, and harvested at an optical density (OD_{600}) of 1.3, with a cell yield of 0.8 mg total suspended solid (TSS) per mg butane. Cells were harvested by centrifugation (6,000 × *g* for 15 minutes), washed and resuspended in a chloride-free phosphate buffer (adjusted pH 7.3; 2 mM KH_2PO_4 and 2 mM $Na_2HPO_4{\cdot}7H_2O$) to give a final cell density of 2000 mg/L (on a TSS basis). Resting cell transformation tests were performed within 2 hours of harvesting. Resting cell activity was stable for 30 hours after harvesting, based on butane uptake activity. Cell activities for different batches of cells were measured by determining butane uptake and 1,1-DCE transformation rates and the transformation capacity (T_c) of 1,1-DCE. 1,1-DCE was used to examine cell activities, since it was rapidly transformed, and its transformation led to complete cell inactivation (Kim *et al.*, 2000).

Chemicals

A saturated aqueous stock solution of c-DCE (97%, Aldrich Chemical Co., Milwaukee, WI) was prepared at room temperature by adding specific amounts of the liquid to 125-mL serum bottle containing autoclaved deionized water. This procedure eliminated the use of carrier solvents, such as methanol. The bottles were shaken for 6 hours prior to use to ensure saturation, and then allowed to settle for 6 hours before use. Butane (≥ 99%, Liquid Carbonic Inc., Chicago, IL), was directly transferred to the batch reactors.

Analysis

Gaseous concentrations of c-DCE were determined by a headspace analysis. The total compound mass in each test bottle was calculated using the headspace and solution volumes and published Henry's constants (Mackay and Shiu 1981; Gossett 1987). All the

experiments were conducted with vigorously shaking of the batch reactors to avoid mass transfer limitations. Calibration curves for c-DCE were developed using external standards. Headspace concentrations of butane were determined on a HP5890A series gas chromatograph (GC) using 3.2 mm × 1.2 m HayeSep D80/100-mesh, packed column (Alltech Associates, Deerfield, IL), operated at 130 °C, and a flame ionization detector (FID). c-DCE analysis was conducted by injecting headspace samples onto a HP 5890 series II GC with 0.25 mm × 30-m HP-624 capillary column operated at 140 °C and Model 5220 electrolytic conductivity detector (OI Analytical, College Station, TX). c-DCE epoxide analysis was conducted by injecting 0.5 mL of aqueous samples and 4.5 mL of deionized water onto a Purge & Trap with the same column and GC operating conditions as c-DCE GC analysis. Qualitative analysis of the c-DCE epoxide produced during c-DCE transformation was conducted using solid-phase microextraction of 1-mL aqueous samples with an 85-mm acrylate fiber (Supelco Inc., Bellefonte, PA). The GC equipped with mass spectrometer analysis was conducted as previously reported by Vancheeswaran *et al.* (1999).

Transformation of c-DCE

The transformation of c-DCE in the batch reactors was conducted as follows. Autoclaved phosphate buffer solution (58 mL) was added to autoclaved 125-mL amber serum bottles which were crimp sealed with Teflon™-lined rubber septa (Kimble, Vineland, NJ). The c-DCE was added, and the initial c-DCE concentration was determined after 15 minutes of shaking. Washed and resuspended cells (4–6 mg on a TSS basis) were then added, and bottles were shaken at 180 rpm. Temporal samples of the reactor headspace were taken for c-DCE analysis, and of the aqueous phase for chloride and c-DCE epoxide analysis.

Acetylene blocking studies were performed on c-DCE and cell amended bottles. Acetylene inhibits activities of methane and ammonia monooxygenases (Bedard and Knowles 1989; Prior and Dalton 1985), and was shown to inhibit butane-utilization and CAH transformation in butane – utilizing pure cultures (Hamamura *et al.* 1997). To evaluate the effect of acetylene on the transformations of c-DCE and c-DCE epoxide, acetylene (1% (v/v) in a gas phase) was added into the c-DCE and cell amended bottles in the middle of c-DCE transformation.

To confirm the biological transformations of c-DCE and c-DCE epoxide, mercuric chloride ($HgCl_2$) was used as a biological inhibitor. The same procedures as the acetylene blocking test described above were used to evaluate $HgCl_2$ effect on the transformations of c-DCE and c-DCE epoxide.

Chloride release study

To evaluate the degree of dechlorination of c-DCE, the amount of chloride released was measured. The observed chloride release was compared with stoichiometric release of chloride required for the amount of CAH transformed. The dechlorination extent is presented on a percentage basis. Aqueous chloride concentrations at the beginning and end of the 30 hour incubation were determined using a colorimetric method (Bergnam and Sanik 1957). This method was used to determine CAH dechlorination by *M. trichosporium* OB3b (Oldenhuis *et al.* 1989; van Hylckama Vlieg *et al.* 1996).

Results and discussion

Transformation of c-DCE and the effects of its transformation on cell inactivation

In our previous study the effects of O_2, butane, and acetylene on the transformation of c-DCE were evaluated (Kim *et al.*, 2000). In summary, c-DCE was not transformed in the absence of O_2. Acetylene-treated cells transformed less than 15% of the amount of

c-DCE transformed by untreated cells. Butane highly inhibited c-DCE transformation, and vice versa. Thus, the involvement of a monooxygenase enzyme in the transformation of the c-DCE was indicated by the lack of transformation in the absence of O_2, the inactivation of c-DCE transformation by acetylene, the inhibition of c-DCE transformation by butane, the inhibition of butane degradation by c-DCE. The possible involvement of butane monooxygenase in the transformation of c-DCE is consistent with the results of Hamamura *et al.* (1997), with pure butane-utilizing cultures and an enrichment of the culture tested here.

Transformations of c-DCE and c-DCE epoxide and chloride release

Transformation of c-DCE resulted in the concomitant generation of c-DCE epoxide (Figure 1). Mass spectrometry confirmed the presence of a compound with mass-to-charge-fragment ratios of 112, 83, 48, and 35. These values are in agreement with the spectra of a chemically synthesized c-DCE epoxide (Janssen *et al.* 1988). After transformation of approximately 70% of the c-DCE, c-DCE epoxide started to decrease rapidly. Chloride concentrations slowly increased during the initial transformation of c-DCE, and more rapidly increased during transformation of c-DCE epoxide. Seventy five percentage Cl^- release occurred after 30 h of incubation of c-DCE. The abiotic half-life of c-DCE epoxide is approximately 72 h (Janssen *et al.* 1988). The nearly complete oxidative dechlorination of c-DCE within 30 h of incubation indicates the biological transformation of c-DCE epoxide likely by the butane utilizers. The high degree of inactivation of the butane utilizers potentially resulted from the biotic transformation of the epoxide.

Effects of biological inactivators on the transformation of c-DCE epoxide

To confirm whether the c-DCE epoxide was biologically transformed, $HgCl_2$, a potent inhibitor of biological activity was added in a parallel experiment. $HgCl_2$ ($\sim$25 mg/L) was added after 80% of the c-DCE was transformed (Figure 2A). This immediately stopped the transformation of both c-DCE and c-DCE epoxide, indicating that the

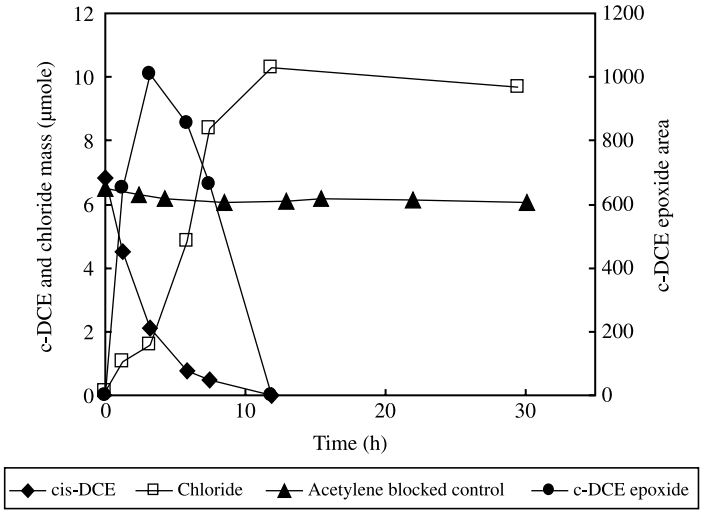

Figure 1 Formation and transformation of c-DCE epoxide during transformation of c-DCE by resting cell suspensions of the butane utilizers (4.3-mg TSS used)

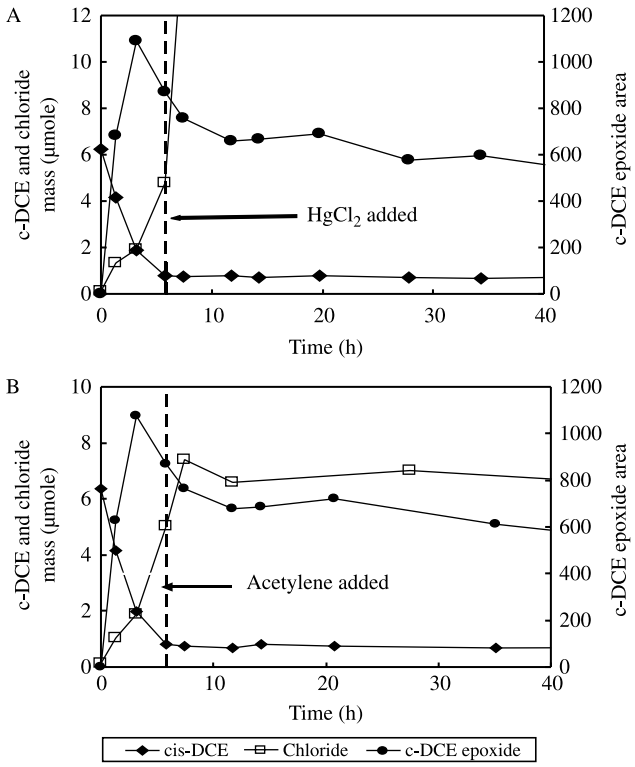

Figure 2 Effects of $HgCl_2$ [25 mg/L] (A) and acetylene [1%, vo./vol.] (B) additions on the transformations of c-DCE and c-DCE epoxide by resting cell suspensions of butane utilizers (4.5-mg TSS used)

epoxide was being biologically degraded. Chloride concentrations dramatically increased due to the chloride present with the $HgCl_2$ addition.

Acetylene is a known to be an irreversible inactivator of methane monooxygenase (MMO) from *Methylococcus capsulatus* (Bath) (Prior and Dalton, 1985), butane monooxygenase (BMO) from butane-grown *Pseudomonas butanovora*, and the environmental isolate, CF8 (Hamamura *et al.*, 1997), and propane monooxygenase (PMO) from propane-grown *Mycobacterium vaccae* JOB5 (Vanderberg and Perry, 1994). Acetylene inhibition has been also observed in mixed cultures grown on methane and propane (Alvarez-Cohen and McCarty, 1991), and the butane enrichment culture used in this study (Kim *et al.*, 2000). Acetylene was used to evaluate the involvement of a monooxygenase enzyme in the transformation of c-DCE epoxide. Acetylene addition completely stopped the transformation both c-DCE and the c-DCE epoxide (Figure 2B). This indicated that c-DCE epoxide was likely transformed by a butane monooxygenase enzyme.

c-DCE inhibition on the transformation of c-DCE epoxide

A final batch experiment was performed to evaluate if the same monooxygenase was likely involved in the transformation of both c-DCE and c-DCE epoxide. c-DCE inhibition of the c-DCE epoxide was evaluated by adding c-DCE into a batch bottle when production and transformation of c-DCE epoxide were occurring (Figure 3). After transformation of approximately 76% c-DCE, c-DCE epoxide concentrations started to decrease. c-DCE was then added into the reactor and c-DCE epoxide began to accumulate and production rate of chloride decreased, indicating c-DCE epoxide transformation was likely inhibited by c-DCE. After the readdition of c-DCE, the rate of c-DCE transformation decreased, and the transformation rate of c-DCE epoxide also decreased. Both

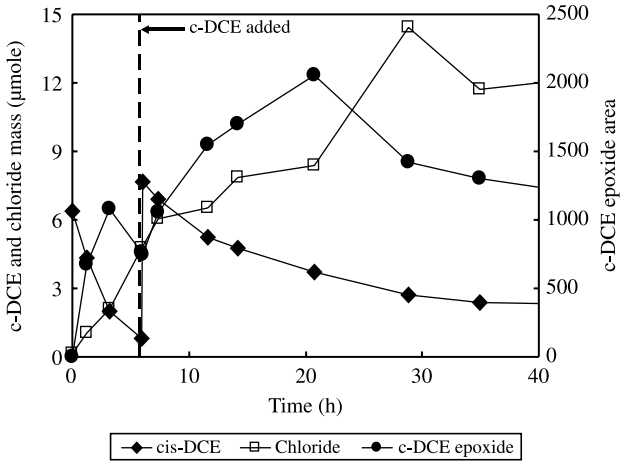

Figure 3 Inhibition of c-DCE on the transformation of c-DCE epoxide

transformations essentially stopped after 40 hours of incubation (data not shown). These results indicate that the same monooxygenase was likely responsible for the transformation of both c-DCE and the c-DCE epoxide.

Conclusions

To our knowledge this is the first report of butane-oxidizers expressing butane monooxygenase biologically transforming c-DCE epoxide. The involvement of a butane monooxygenase in the transformation of both c-DCE and c-DCE epoxide was indicated by: (1) the O_2 requirement for transformation of c-DCE; (2) the inactivation of c-DCE, and c-DCE epoxide transformation by acetylene; (3) the inhibition of c-DCE epoxide transformation by c-DCE; and the inhibition of butane on c-DCE transformation, and (4) the inhibition of $HgCl_2$ on c-DCE epoxide transformation. The transformation of c-DCE epoxide appears to be causing significant toxicity, since its transformation clearly inactivated the cells. This biological transformation of the epoxide needs to be studied in greater detail.

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