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Effect of Chlorinated Ethene Conversion on Viability and Activity of *Methylosinus trichosporium* OB3b

JOHAN E. T. VAN HYLCKAMA VLIEG, WIM DE KONING, AND DICK B. JANSSEN*

Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands

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The effect of transformation of chlorinated ethenes on the cell viability of *Methylosinus trichosporium* OB3b was investigated. A comparison of the loss of viability with the decrease in transformation rates showed that for the monooxygenase-mediated transformation of all chlorinated ethenes except vinyl chloride the decrease in cell viability was the predominant toxic effect.

Improper disposal of perchloroethene and trichloroethene (TCE) has frequently caused contamination of groundwater. Dechlorination reactions occurring in situ under anaerobic conditions have resulted at many sites in the generation of *cis*-1,2-dichloroethene (*cis*-1,2-DCE) and the suspected carcinogen vinyl chloride (VC) (8, 28). Methanotrophic bacteria, which oxidize chlorinated ethenes to the corresponding epoxides, are good candidates for the treatment of groundwater contaminated with chlorinated ethenes (1, 6, 13, 15, 17, 19–21, 25–27). The epoxides formed are electrophilic compounds that are unstable in aqueous solutions, and they may undergo a variety of secondary reactions, such as hydrolysis, rearrangement, or carbon-carbon bond cleavage. The reactivity of the epoxides or their degradation products is assumed to be the cause of the toxicity associated with the cometabolic transformation of chlorinated ethenes (1, 3, 7, 10, 12, 14, 15, 19). Toxicity studies have mainly focused on decreasing transformation rates, suggesting that this is the main toxic effect that limits the use of methanotrophs for removal of chlorinated ethenes. Hardly any attention has been paid to the effect of chlorinated ethenes on cell viability (15). However, due to the reactive electrophilic nature of the products formed, nonspecific toxic effects may occur (1, 7, 15, 19).

The goals of this study were to quantify the effect of transformation of chlorinated ethenes on the cell viability of *Methylosinus trichosporium* OB3b and to compare this effect with the inhibiting effect on transformation rates, which we refer to as activity below. *M. trichosporium* OB3b expressing particulate methane monooxygenase (pMMO) or soluble methane monooxygenase (sMMO) was grown continuously in a 3-liter fermentor in mineral medium (MMF) at a dilution rate of 0.028 h^{-1} with methane as the sole carbon source as described previously (27). The effect of repeated additions of chlorinated ethenes to resting cell suspensions on both parameters was investigated to determine whether the transformation of TCE mainly affected cell viability or activity. Freshly harvested cell suspensions (0.2 to 0.3 mg ml^{-1}), to which 20 mM sodium formate was added, were incubated in 120-ml conical flasks, and TCE was added to a concentration in the liquid phase of 18 μM. The concentration in the headspace was monitored on-line by gas chromatography (Fig. 1A), and first-order degradation rate constants (k_1) were determined as described previously (27). After approximately 90% of the substrate was transformed, a new pulse of substrate was added. During the experiments, five 50-μl samples were withdrawn to determine the cell viability by plating a 10-fold dilution series on MMF plates. The plates were incubated at 30°C in a desiccator containing 25% natural gas in air. All plate counts were performed in triplicate. Viable cells were defined as cells that were able to form colonies. Control experiments showed that the abiotic loss was less than 5% and that the viability and activity of suspensions to which no TCE was added did not decrease significantly during 2.5 h under the conditions used.

The viability of the cultures decreased exponentially with the amount of substrate converted, and a 50% loss of viability was observed after degradation of 0.3 μmol of TCE mg of $cells^{-1}$ (in our experiments LAT_{50} was defined as the amount of chlorinated ethene transformed per unit of cell mass that resulted in a 50% decrease in the number of viable cells) (Fig. 2). When the cell viability of a TCE-transforming suspension was determined on plates containing methanol instead of methane as the growth substrate, the results were similar to the results obtained with methane, indicating that the loss of viability was not caused by decreased monooxygenase activities. The TCE-degrading activity of the cells was found to decrease linearly with the amount of substrate that was converted (Fig. 2), with a transformation capacity (T_c) (the maximum amount of chlorinated ethene that could be transformed per unit of cell mass) of 4.0 ± 0.9 μmol mg of $cells^{-1}$.

The toxicities of transformation of 1,1-DCE, VC, and *trans*-1,2-DCE were studied in experiments analogous to the experiments described above for TCE. The activity rapidly decreased due to the transformation of 1,1-DCE and VC (Fig. 2; Table 1). The cell viability decreased during transformation of 1,1-DCE, while it was much less affected during the transformation of VC (Fig. 2; Table 1). Since VC is an important contaminant and a good substrate for pMMO, we also tested the toxicity of transformation of VC for cells expressing this enzyme. In this case, activity also decreased, and the T_c was somewhat lower than that with sMMO-containing cells, whereas the cell viability was not affected (Fig. 2; Table 1).

trans-1,2-DCE epoxide is relatively stable in aqueous solutions, with a reported half-life of 30 h (17), and thus it accumulated upon transformation of *trans*-1,2-DCE. The transformation of *trans*-1,2-DCE resulted in relatively small toxic effects; a decrease in cell viability was the predominant effect (Fig. 2; Table 1).

The T_c values that we obtained in our experiments for most compounds are in the same range as those obtained by other

* Corresponding author. Mailing address: Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands. Phone: 31-50-3634209. Fax: 31-50-3634165.

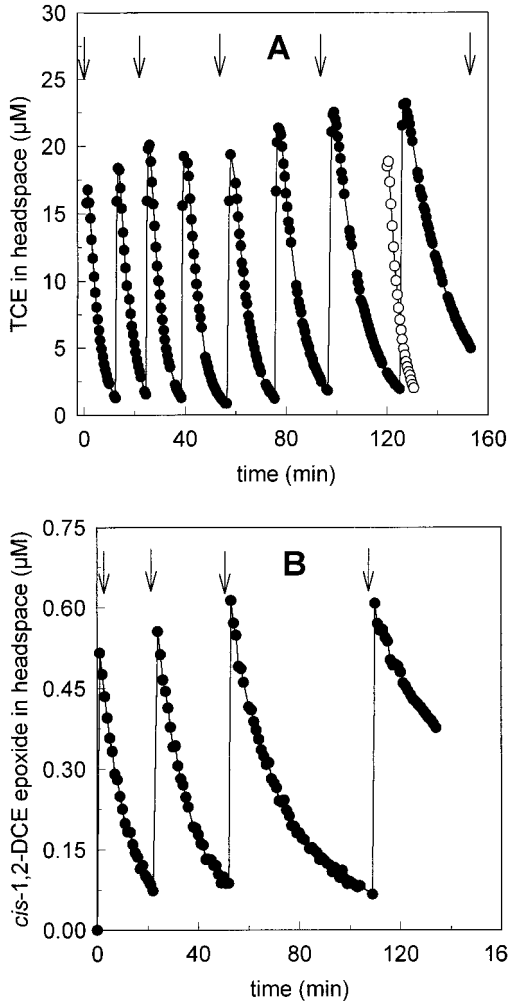

FIG. 1. Transformation of TCE (A) and *cis*-1,2-DCE epoxide (B) by cell suspensions of *M. trichosporium* OB3b (0.29 mg ml^{-1}) expressing sMMO (●). Headspace concentrations were monitored by on-line gas chromatography, and the k_1 of each substrate depletion curve was determined. As a control, transformation by a suspension to which TCE was added for the first time after 120 min was monitored (○). The arrows indicate the time points at which samples were withdrawn for viability measurements.

workers in studies of methanotrophs. In these studies pure and mixed cultures of methanotrophs grown under various conditions were used, which may explain some of the differences between our data and some previously published values, such as the fourfold-lower T_c for TCE transformation and the fourfold-higher T_c for VC transformation obtained by Dolan and McCarthy (10). An 18-fold-higher T_c for VC transformation by *M. trichosporium* OB3b expressing sMMO was obtained by Chang and Alvarez-Cohen (7).

cis-1,2-DCE is more toxic for *M. trichosporium* OB3b expressing sMMO than *trans*-1,2-DCE is (7, 10). This is probably due to the fact that *cis*-1,2-DCE epoxide is actively converted by this organism (27). Therefore, we determined the toxic effects caused by transformation of this epoxide (Fig. 1B and 2; Table 2). Both the activity and the viability of cells rapidly decreased during transformation of *cis*-1,2-DCE epoxide. Previously, we found that when a cell suspension was incubated with *cis*-1,2-DCE, all of the *cis*-1,2-DCE was converted, but conversion of the epoxide ceased after part of it was converted (27). This corresponded to a T_c of 0.5 μmol mg of $cells^{-1}$. Combined with the T_c of 0.8 μmol mg of $cells^{-1}$ that we obtained when *cis*-1,2-DCE epoxide was added as a substrate, this indicates that the toxicity of *cis*-1,2-DCE is mainly caused by the transformation of the primary oxidation product, *cis*-1,2-DCE epoxide, making comparisons with previously published T_c values for *cis*-1,2-DCE difficult.

Acetylene is a potent inhibitor of sMMO (23). We tested the influence of transformation of this compound on the viability of cells. A cell suspension was inactivated by incubating the cells with 1% acetylene in the headspace for 10 min. The k_1 for TCE transformation decreased from 2.7 to less than 0.1 ml min^{-1} mg of $cells^{-1}$ after this treatment, whereas the cell viability decreased less than 30%. Thus, acetylene had a much stronger negative effect on activity than on cell viability.

These experiments did not discriminate between a decrease in cell viability due to toxic effects of the chlorinated substrates and a decrease in cell viability due to the reactivity of the

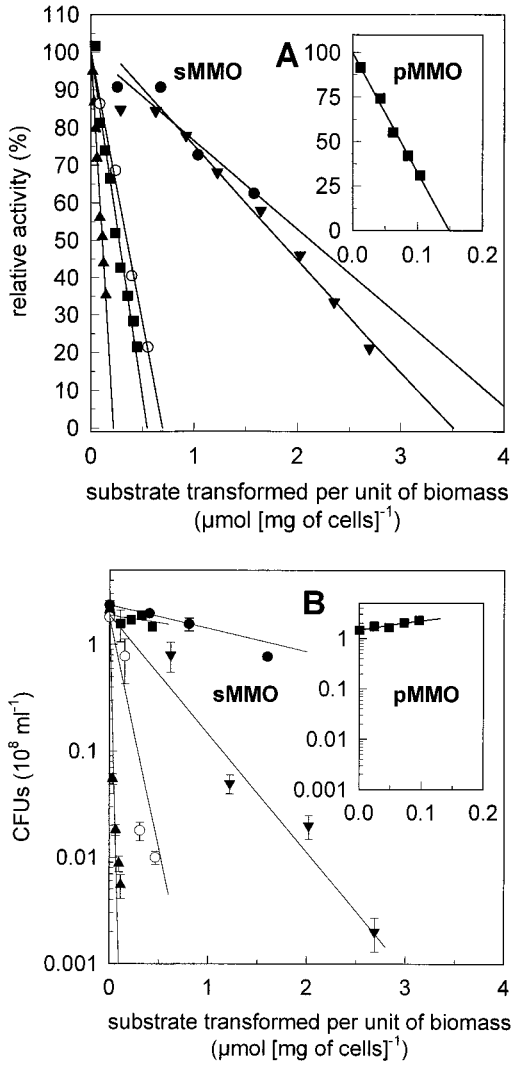

FIG. 2. Inactivation (A) and cell death (B) due to the transformation of chlorinated ethenes by resting cell suspensions of *M. trichosporium* OB3b (0.29 mg ml^{-1}) expressing pMMO or sMMO. The cell suspensions were harvested from a continuous culture grown at a dilution rate of 0.028 h^{-1} in the absence of copper (sMMO expressed) or in the presence of copper (pMMO expressed). Symbols: ▲, 1,1-DCE; ■, VC; ○, *cis*-1,2-DCE epoxide; ▼, TCE; ●, *trans*-1,2-DCE. The amount of substrate transformed represents the cumulative amount transformed up to the time that viability or activity was measured.

TABLE 1. Toxicities of cometabolic transformation of chlorinated ethenes by *M. trichosporium* OB3b expressing pMMO and sMMO[a]

Enzyme expressed by cells	Compound	T_c (μmol mg of cells^{-1})	LAT_{50} (μmol mg of cells^{-1})	$1/2T_c/LAT_{50}$
pMMO	VC	0.2	>0.5	<0.2
sMMO	VC	0.6 ± 0.1	3.0 ± 1.0	0.1 ± 0.04
	trans-1,2-DCE	5.0	1.0	2.5
	cis-1,2-DCE epoxide	0.8 ± 0.2	0.05 ± 0.03	8 ± 5.2
	1,1-DCE	0.2	0.01 ± 0.005	10 ± 5
	TCE	4.0 ± 0.9	0.3 ± 0.1	6.7 ± 2.7

[a] The values are the averages from experiments performed with cell suspensions containing 0.29 and 0.20 mg ml^{-1}.

transformation products. Therefore, acetylene-inactivated cell suspensions (0.14 mg ml^{-1}) were incubated for 2 h with amounts of chlorinated ethene sufficient for complete inactivation of an active cell suspension. In all samples less than 5% of the substrate was transformed. The viable counts for these suspensions were $(1.1 \pm 0.1) \times 10^8$ CFU ml^{-1} (non-acetylene-treated control) and $(0.8 \pm 0.1) \times 10^8$ CFU ml^{-1} (acetylene-inactivated preparation). The viabilities of the acetylene-inactivated suspensions that were incubated with chlorinated ethenes were as follows: $(0.8 \pm 0.1) \times 10^8$ CFU ml^{-1} for VC, $(1.0 \pm 0.1) \times 10^8$ CFU ml^{-1} for *cis*-1,2-DCE, $(1.0 \pm 0.2) \times 10^8$ CFU ml^{-1} for *trans*-1,2-DCE, $(0.9 \pm 0.2) \times 10^8$ CFU ml^{-1} for 1,1-DCE, $(0.7 \pm 0.1) \times 10^8$ CFU ml^{-1} for TCE, and $(0.3 \pm 0.1) \times 10^8$ CFU ml^{-1} for *cis*-1,2-DCE epoxide. Thus, the effects of chlorinated ethenes on acetylene-inactivated cells are much smaller than the effects on cells with high monooxygenase activity. This indicates that the decrease in cell viability is caused by transformation products rather than by the substrates themselves.

In order to compare the toxic effects of chlorinated ethene transformation on fully active cells, the amount of substrate transformed per unit of cell mass that caused a 50% decrease in activity ($1/2T_c$) and the LAT_{50} were calculated (Table 1). The $1/2T_c/LAT_{50}$ ratio was less than 1 for the conversion of VC, both with cells expressing pMMO and with cells expressing sMMO. This indicates that inactivation was the predominant toxic effect. For the other chlorinated ethenes, the ratios were greater than 1, indicating that cell death was the predominant toxic effect. This explains the results of Oldenhuis and Janssen (18), who reported wash-out of a chemostat culture at TCE loads at which 0.3 μmol of TCE was converted per mg of cells produced. Wash-out may have been caused by a decrease in the cell viability since it can be calculated that under these conditions less than 10% of the activity is lost, whereas 50% of the cells are killed.

The linear relationship that was observed between the decrease in activity and the amount of substrate that was converted per unit of biomass suggests that enzyme inactivation is a partitioning process in which most oxidized molecules are released from the active site of the monooxygenase, whereas some molecules inactivate the enzyme. The decrease in cell viability, however, could be described by exponential decay as a function of the amount of substrate that was transformed per unit mass of cells. Thus, the rate of killing was proportional to the number of viable cells present, suggesting that cell viability is affected via a diffusible oxidation product. Previously, we have shown that even unstable epoxides, such as VC epoxide (half-life = 78 s) and TCE epoxide (half-life = 21 s), rapidly diffuse over the membrane and temporarily accumulate in the medium during the transformation of VC and TCE before they disappear due to rearrangement or hydrolysis (27). Acyl chlorides are generated upon the rearrangement or hydrolysis of the epoxides of 1,1-DCE and TCE. Transformation of these compounds had the strongest effect on viability. Chloroaldehydes rather than acyl chlorides are generated from the epoxides of *trans*-1,2-DCE and VC (16). Interestingly, transformation of the latter chlorinated ethenes does not have a strong effect on viability. Therefore, acyl chlorides may be the reactive products that cause the loss of viability. These are highly electrophilic compounds that can alkylate nucleophilic groups in nucleic acids and proteins and thus inhibit essential metabolic processes.

Several types of reactors have been proposed or constructed in which methanotrophs are used for the treatment of waste streams contaminated with chlorinated ethenes. Some of these systems depend on the reactivation of inactivated populations or on the continuous transformation of chlorinated compounds by growing cells (4, 5, 11, 13, 22, 24). Since, at least with *M. trichosporium* OB3b, a decrease in viability is the most important toxic effect with important pollutants such as TCE and *cis*-1,2-DCE, reactivation of the microbial populations may require growth of new cells rather than reactivation of inactivated cells. Our results provide further evidence that a two-stage reactor in which organisms are first grown to high cell densities and then added to the contaminated waste stream may allow maximal exploitation of the cometabolic transformation potential of *M. trichosporium* OB3b (1, 2, 9).

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