Improper disposal of perchloroethylene and trichloroethylene (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-dichloroethylene (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 monoxygenase (pMMO) or soluble methane monoxygenase (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 (k₁) 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 LAT₅₀ 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 monoxygenase 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 (Tₚ) (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 Tₚ 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 Tₚ values that we obtained in our experiments for most compounds are in the same range as those obtained by other.
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...
TABLE 1. Toxicities of cometabolic transformation of chlorinated ethenes by M. trichosporium OB3b expressing pMMO and sMMO

<table>
<thead>
<tr>
<th>Enzyme expressed by cells</th>
<th>Compound</th>
<th>Tc</th>
<th>LAT_{50}</th>
<th>1/2Tc/LAT_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMMO</td>
<td>VC</td>
<td>0.2</td>
<td>&gt;0.5</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td></td>
<td>trans-1,2-DCE</td>
<td>0.6±0.1</td>
<td>3.0±1.0</td>
<td>0.1±0.04</td>
</tr>
<tr>
<td></td>
<td>cis-1,2-DCE epoxide</td>
<td>0.8±0.2</td>
<td>0.05±0.03</td>
<td>8±5.2</td>
</tr>
<tr>
<td></td>
<td>1,1-DCE</td>
<td>0.2</td>
<td>0.01±0.005</td>
<td>10±5</td>
</tr>
<tr>
<td></td>
<td>TCE</td>
<td>4.0±0.9</td>
<td>0.3±0.1</td>
<td>6.7±2.7</td>
</tr>
</tbody>
</table>

sMMO                      | VC                | 108 CFU ml^{-1} | 0.1| 3.0 | 1 | 0.03 | 8 | 5.2 | 10 | 5 | 6.7 | 2.7 |

The values are the averages from experiments performed with cell suspensions containing 0.29 and 0.2 mg ml^{-1}.

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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