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Transformation Kinetics of Chlorinated Ethenes by *Methylosinus trichosporium* OB3b and Detection of Unstable Epoxides by On-Line Gas Chromatography

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

Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9747 AG Groningen, The Netherlands

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A rapid and accurate method for the determination of transformation kinetics of volatile organic substrates was developed. Concentrations were monitored by on-line gas chromatographic analysis of the headspace of well-mixed incubation mixtures. With this method, the kinetics of transformation of a number of C1 and C2 halogenated alkanes and alkenes by *Methylosinus trichosporium* OB3b expressing particulate methane monoxygenase or soluble methane monooxygenase (sMMO) were studied. Apparent specific first-order rate constants for cells expressing sMMO decreased in the order of dichloromethane, vinyl chloride, *cis*-1,2-dichloroethene, *trans*-1,2-dichloroethene, 1,1-dichloroethene, trichloroethene, chloroform, and 1,2-dichloroethane.

During the degradation of trichloroethene, *cis*-1,2-dichloroethene, *trans*-1,2-dichloroethene, and vinyl chloride, the formation of the corresponding epoxides was observed. The epoxide of vinyl chloride and the epoxide of trichloroethene, which temporarily accumulated in the medium, were chemically degraded according to first-order kinetics, with half-lives of 78 and 21 s, respectively. Cells expressing sMMO actively degraded the epoxide of *cis*-1,2-dichloroethene but not the epoxide of *trans*-1,2-dichloroethene. Methane and acetylene inhibited degradation of the epoxide of *cis*-1,2-dichloroethene, indicating that sMMO was involved.

Trichloroethene (TCE), vinyl chloride (VC), 1,2-dichloroethane, and other chlorinated ethenes and ethanes are important environmental pollutants. TCE has been widely used as a degrading agent. VC and 1,1-dichloroethene (1,1-DCE) are used for the synthesis of synthetic resins. Under aerobic conditions, VC (16) and several chlorinated alkanes (19, 22) have been shown to serve as sole sources of carbon and energy for a bacterial culture. For TCE and DCEs, cometabolic degradation by organisms expressing monooxygenases or dioxygenases during growth on methane (18, 28, 35, 37), propane (40), propene (9), isoprene (10), ammonia (2), or toluene (26, 41) has been reported. There is a strong interest in using such organisms for the bioremediation of soil, groundwater, or air contaminated with chlorinated aliphatics. TCE, VC, and *cis*-1,2-DCE are of special importance in this respect since they can also be produced anaerobically in contaminated sites from perchloroethene.

Several factors determine the suitability of organisms for application in a cometabolic remediation process. A broad substrate range is desirable, since polluted groundwater or other waste streams often contain more than one contaminating compound. The kinetics of degradation are also important since high degradation rates may allow high volumetric degradation capacities, and high substrate affinities make it easier to obtain low effluent concentrations. However, the kinetics of cometabolic conversion are often complex and can be influenced by a number of factors. Cometabolic degradation can be regarded as an unavoidable side reaction, and this can be a disadvantage for the organism. Energy may be lost without any benefit to the cell, as, for instance, in the use of NADH in oxygenase reactions which yield products that are not further metabolized. Competitive inhibition between the growth substrate and the pollutant, resulting in a reduction of degradation rates, can occur (1, 23). Furthermore, cometabolic transformation of chlorinated hydrocarbons can result in the generation of reactive compounds that inactivate the cell and thus limit the transformation capacity (1, 27). For important industrial pollutants such as chloroform and TCE, however, cometabolic transformation is the only available possibility for an aerobic bioremediation process.

Methanotrophs are good candidates for such applications, since they are capable of degrading a broad range of halogenated aliphatics (1, 12, 27, 28, 35, 37). The methane monoxygenases (MMO) that are expressed by these organisms can be divided into two classes. All methanotrophs tested are able to form a particulate enzyme (pMMO). Under copper limitation, only some strains such as *Methylosinus trichosporium* OB3b, *Methylcococcus capsulatus* (Bath) (34), and *Methylomonas methanica* 68-1 (35) are able to form a soluble enzyme (sMMO), which has a broader substrate range and is capable of oxidizing TCE and other chlorinated aliphatics. Several bench-scale reactors have been constructed, such as biofilm reactors (3, 11) and plug flow reactors (36), in which the removal of TCE by these organisms has been studied.

The oxidation of chlorinated ethenes by sMMO results in generation of the corresponding epoxides. These reactions are similar to the oxidation of chlorinated ethenes by cytochrome P-450 in eukaryotes. The epoxides are electrophilic compounds, and from studies of eukaryotic systems, it is known that a variety of secondary reactions, such as alkylation of biomolecules or hydrolysis to vic-diols or acylhalides, may occur. The reactivity of the epoxides and their degradation products is considered to be responsible for the toxicity of chlorinated ethenes. The metabolism of VC in mammals has been studied extensively because of its mutagenic and carcinogenic potential (24, 31). From the DNA alkylation products that have been isolated from animals exposed to VC, it could be

* 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 3634108. Fax: 31 50 3634165.

*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 3634108. Fax: 31 50 3634165.
derived that the epoxide generated after the oxidation of VC, chloroepoxyethane (VC epoxide), is the ultimate carcinogenic product of VC. TCE is a suspected carcinogen, but little information is available about the metabolic modifications of DNA due to the metabolism of this compound.

Toxicity related to conversion of chlorinated ethenes has also been observed in bacteria (12, 27). However, little is known about the kinetics of degradation of the epoxides, since their instability in aqueous solutions makes it difficult to perform accurate quantitative analysis.

In this article, we describe the on-line monitoring of volatile substrates and oxidation products by gas chromatography (GC). The method was used to determine the kinetics of degradation of various halogenated aliphatics by *Methylosinus trichosporium* OB3b expressing pMMO or sMMO and made possible the detection of the corresponding epoxides. The results showed that significant amounts of all epoxides, except 1,1-dichloroepoxyethane (1,1-DCE epoxide), leave the cell. Furthermore, it was found that cis,1,2-dichloroepoxyethane (cis,1,2-DCE epoxide) is actively transformed by sMMO, whereas other structurally related epoxides are not substrates for sMMO.

**MATERIALS AND METHODS**

**Nomenclature.** The following parameters are used in this paper: *a*, constant relating peak area to substrate concentration; *C*, the substrate concentration (micromolar); *f*, the fraction of oxidized chlorinated ethene that is converted to epoxide (dimensionless); *H*, Henry's gas-liquid partition coefficient (millilitres of gas milliliter of liquid); *k*, the specific first-order degradation rate constant (millilitres minute milligram of cells); *k*<sub>0</sub>, mass-transfer rate coefficient (minute<sup>-1</sup>); *K<sub>M</sub>, the Michaelis-Menten constant (micromolar); *k<sub>L</sub>*<sub>a</sub>, the Monod constant (micromolar); *r<sub>max</sub>*<sub>sw</sub>, the maximal specific substrate conversion rate (micromoles minute<sup>-1</sup> milligram of cells<sup>-1</sup>); *r*, specific substrate conversion rate (micromoles minute<sup>-1</sup> milligram of cells<sup>-1</sup>); *S*, amount of substrate (micromoles); *p<sub>max</sub>*<sub>sw</sub>, the maximal specific growth rate (minute<sup>-1</sup>); *V<sub>c</sub>*, volume (millilitre); *X*, the concentration of cells (milligram of cells [dry weight] millilitre<sup>-1</sup>); *Y*, growth yield (milligram of cells [dry weight] micromole<sup>-1</sup>). Subscripts denote the following parameters: *g*, gas phase; *l*, liquid phase; *O*, epoxide; *S*, substrate; *X*, biomass; *U*, at time zero.

**Organism and growth conditions.** *Methylosinus trichosporium* OB3b was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom. The organism was grown continuously in a 3-liter fermentor with methane as the sole carbon source at a dilution rate of 0.03 h<sup>-1</sup>. Mineral medium (MMF) was essentially the same as the medium described by Jannsen et al. (18). Only Na<sub>2</sub>HPO<sub>4</sub> was replaced by KH<sub>2</sub>PO<sub>4</sub> and the medium in the supply vessel was acidified to pH 3.0 with H<sub>2</sub>SO<sub>4</sub> to prevent formation of acid by the cells. The dissolved oxygen tension was 25 to 50% air saturation. The sterile air-methane mixture was bubbled through the fermentor. The biomass concentration was 0.3 to 0.4 mg of cells ml<sup>-1</sup>.

**On-line monitoring of substrate concentrations and transformation experiments.** Degradation rates of chlorinated aliphatics were determined by on-line analysis of their concentrations in the headspace of incubation mixtures. The experimental setup consisted of a 120-ml jacketed glass incubation vessel that was temperature controlled at 30°C. Gas was continuously withdrawn from the headspace with a micromembrane pump (model NMP 02LU; KNF Neuberger GmbH, Freiburg-Munzingen, Germany). After passage through a Valco 6 port sampling injector (Vici AG, Schenkon, Switzerland) to which a 35-μl sample loop was connected, the gas was injected back into the mechanically stirred liquid phase in the incubation vessel through an open-ended tube with an internal diameter of 0.08 mm and a flow rate of approximately 200 ml min<sup>-1</sup>. This allowed rapid mass transfer between the gas and liquid phases. Stainless-steel tubing and a glass-embedded magnetic stirrer were used, since sorption was observed with synthetic materials. With time intervals of 0.25 to 1 min, the contents of the sample loop were injected into the GC and analyzed isothermally at 60° to 130°C. The GC (type CP 9001; Chrompack, Middelburg, the Netherlands) was equipped with a CPW 5 CB column (Chrompack; length, 25 m; inner diameter, 0.53 mm; film thickness, 5 μm) and a flame-ionization detector. Helium was used as the carrier gas (26 ml min<sup>-1</sup>) and the flame ionization detector was supplied with hydrogen (30 ml min<sup>-1</sup>), air (270 ml min<sup>-1</sup>), and N<sub>2</sub> (30 ml min<sup>-1</sup>). The high sensitivity of the GC analysis allowed the determination of concentrations as low as 0.05 μM in the headspace. Calibrations were carried out by adding a well-defined amount of substrate to the incubation system. In this way, errors in the estimations of the total mass and transformation rates were minimized.

To evaluate the mass-transfer characteristics, the *k<sub>L</sub>*<sub>a</sub> was determined by adding a pulse of TCE to the liquid phase or to the headspace of a reaction vessel which contained 25 ml of MMF medium. The concentration in the headspace was monitored until equilibrium was reached. The substrate concentration and its dependence on the *k<sub>L</sub>*<sub>a</sub> in time are described by:

\[
\frac{dC}{dt} = -k_L a \frac{V_g}{V_f} \left( \frac{C_g}{V_g} + \frac{S}{V_l} \right)
\]

which can be solved to:

\[
C_{eq} = \frac{H {S_{eq}}}{V_f + H {V_g}} + \frac{C_{eq}}{V_i} \left( \frac{p_{max}}{S_{eq}} - 1 \right)
\]

where *C<sub>eq</sub>* is 0 or $S_{eq} V_f$ when TCE was added to the liquid phase or gas phase, respectively.

The published Henry constants that were used for the calculations are as follows: methane, 33 (29); dichloromethane, 0.11; chloroform, 0.19; VC, 1.26; cis,1,2-DCE, 0.19; trans-1,2-DCE, 0.47; 1,1-DCE, 1.27; TCE, 0.51 (13). Other Henry constants were determined by analyzing a series of closed bottles of equal volume containing various amounts of MMF medium to which a fixed amount of epoxide was added. The Henry constants were calculated from a plot of the reciprocal peak area versus the liquid volume, which gives a straight line from which *H* can be calculated with:

\[
\frac{1}{\text{area}} = \frac{H - 1}{H \text{ area}} + \frac{V_c}{S_{eq}}
\]

In this equation, *a* is a constant relating peak area to concentrations. The resulting values for the Henry coefficient were 0.011, 0.049, and 0.011 for cis,1,2-DCE epoxide, *trans*-1,2-dichloroepoxyethane (*trans*-1,2-DCE epoxide), and epoxyethane, respectively.

In a typical transformation experiment, 25 ml of cell suspension freshly taken from the fermentor was used. Formate and phosphate buffer (pH 6.9) were added to a final concentration of 20 mM. The cell suspension was incubated for 5 min at 30°C to allow the cells to generate enough reducing power to obtain the maximal initial degradation rate. Assays were started by adding 0.1 to 3 μmol of substrate either from a stock solution in water or as a gas. Cells were protected from light, since it was observed that cells rapidly inactivate when illuminated (38a). When cell densities did not exceed 0.5 mg of cells ml<sup>-1</sup>, the activity of the cells remained constant for at least 2 h.

Chloride levels were determined by the colorimetric method of Bergmann and Sank (5).

**Calculation of transformation rates.** At low substrate concentrations, where *S* ≪ *K<sub>M</sub>*<sub>sw</sub>, transformation follows first-order kinetics. In the case of equilibrium, volatile substrates will be distributed over the headspace and liquid phase according to the dimensionless Henry partition coefficient. In this case, the microbial degradation of the substrate can be described with:

\[
\frac{dC}{dt} = -k_L a \frac{V_g}{V_f} \left( \frac{C_g}{V_g} + \frac{S}{V_l} \right)
\]

After integrating over time, this can be rewritten to:

\[
C_{t} = C_{eq} e^{-k_L a \left( \frac{V_g}{V_f} \right)}
\]

These equations are only valid when mass-transfer limitation does not occur. When mass transfer plays a role, the headspace and liquid phase concentration can be described by:

\[
\frac{dC_{HS}}{dt} = -k_L a \frac{V_f}{V_l} (C_G - C_{eq})
\]

\[
\frac{dC_{L}}{dt} = -k_L a \frac{C_l}{V_l} - r_{max} X \left( \frac{C_l}{C_l + K_l} \right)
\]

The *k<sub>L</sub>* and *k<sub>L</sub>*<sub>a</sub> can be obtained by fitting a data set of headspace concentrations to equations 6 and 7. In case transformation proceeds according to Michaelis-Menten kinetics, equation 8 was used instead of equation 7:

\[
\frac{dC_{HS}}{dt} = \frac{C_l}{V_l} \left( \frac{C_l}{C_l + K_l} \right) - r_{max} X \left( \frac{C_l}{C_l + K_l} \right)
\]

The concentration of epoxide over time is a function of the biological oxidation rate of the chlorinated ethene that is described by equations 6 and 7, with an extra factor *f* which is the fraction of oxidized ethene that is converted to epoxide, and a term for the (chemical) first-order degradation rate of the ep-
oxide. Since chemical degradation of the epoxide will take place in the liquid phase according to first-order kinetics, the headspace concentration can be described by an equation analogous to equation 6, and the liquid-phase concentration of epoxide can be described by:

$$\frac{dO}{dt} = k_{a}O \left( \frac{O_{g}}{H} + O_{l} \right) + \beta_{l}XC_{l} - k_{O}O_{l}$$

(9)

in which \(O_{g}\) was determined by fitting the equation to the measured peak areas with a variable that related peak areas to concentrations. In the case of competitive inhibition, the rate of substrate conversion is described by:

$$r = \frac{r_{max}C_{l}}{K_{c} + \frac{C_{l}}{K_{m}} + C_{l}}$$

(10)

If the transformation of a chlorinated substrate follows first-order kinetics and transformation is competitively inhibited by methane, the relation between the observed \(k_{i}\) and the methane concentration simplifies to:

$$k_{ij} = k_{i0} \left( \frac{K_{c}}{K_{m}} + 1 \right)$$

(11)

The \(K_{c}\) of methane on the conversion of cis-1,2-DCE epoxide was obtained by determining the \(k_{c}\) value of cis-1,2-DCE epoxide degradation in the presence of various concentrations of methane. The methane concentrations in the liquid phase were calculated with equations 6 and 8. Since the methane concentration changed less than 5% as a result of bacterial consumption, it was assumed to be constant.

Data were fitted to analytical equations by nonlinear regression analysis in Sigma Plot (Jandel Scientific Software, San Rafael, Calif.) or by numerical integration by use of the episode routine in Scientist for Windows 2.0 (MicroMath Scientific Software, Salt Lake City, Utah).

Preparation of cis- and trans-1,2-DCE epoxide. The compounds cis- and trans-1,2-DCE epoxide are not commercially available, and their half-lives in aqueous solutions are 72 and 30 h, respectively. Chemical synthesis is time-consuming and gives rather poor yields (14). We synthesized these epoxides with Methylobacterium trichosporium OB3b. For this, cells were harvested from a continuous culture, centrifuged at 10,000 × g for 5 min, and resuspended in 25 ml of MMF medium containing 40 mM phosphate to a cell density of 20 mg of cells ml\(^{-1}\). The suspension was transferred to the incubation vessel, and 5 to 10 pulses of 5 μl of cis- or trans-1,2-DCE were added. Degradation of the substrate and generation of the epoxide were monitored continuously. Repeated addition of formate and \(O_{2}\) enabled optimal transformation. Transformation was stopped by the addition of \(HgCl_{2}\) (0.1 mg ml\(^{-1}\)). The epoxides were removed from the suspension by bubbling \(N_{2}\) gas at a flow rate of 20 ml min\(^{-1}\) through the suspension and subsequently trapped in a glass U-tube kept in liquid nitrogen.

The identity and purity of the isolated compound were checked with \(1H\) nuclear magnetic resonance. Therefore, 10 μl of the isolated compound was dissolved in 1 ml of CDCl\(_{3}\), and 20 μl of \(TCE\) was added as an internal standard.

The \(1H\) nuclear magnetic resonance spectrum showed two singlets at 5.07 and 6.46 ppm, corresponding to \(trans\)-1,2-DCE epoxide. The productsof the oxidation of \(cis\) and \(trans\)-1,2-DCE were assumed to be equal. In all experiments, calibration with \(O_{2}\) enabled optimal transformation. Transformation was stopped by the addition of \(HgCl_{2}\) (0.1 mg ml\(^{-1}\)). The epoxides were removed from the suspension by bubbling \(N_{2}\) gas at a flow rate of 20 ml min\(^{-1}\) through the suspension and subsequently trapped in a glass U-tube kept in liquid nitrogen.

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Chemicals. All gases were obtained from AGA Gas B.V. (Amsterdam, The Netherlands). Organic chemicals used in this study were obtained from Acros Chimica (Gael, Belgium), from Sigma Chemical Co. (St. Louis, Mo.), or Aldrich (Milwaukee, Wis.).

RESULTS

On-line headspace analysis. On-line headspace analysis is an attractive method for the determination of cometabolic transformation kinetics of volatile compounds. However, it is essential to evaluate to what extent the course of headspace and liquid-phase concentrations are influenced by mass-transfer limitation. The rate of gas-liquid mass transfer is determined by the product of the Henry coefficient and the rate of gas-liquid mass transfer (12). The extinction coefficient of PNBP adducts used for quantitation are 24 mM\(^{-1}\) cm\(^{-1}\) at 565 nm and 24 mM\(^{-1}\) cm\(^{-1}\) at 540 nm for VC epoxide and 1,1,2-trichloro-epoxythane (TCE epoxide), respectively (12, 15).

The \(k\) values of the inoculation vessel was measured for TCE by adding a pulse of TCE to the liquid phase or the headspace (Fig. 1). The data were fitted with equation 2, and the \(k\) values for TCE were calculated to be 3.8 and 3.4 min\(^{-1}\) from fits of pulses added to the liquid phase and headspace, respectively.

The \(k\) values of a compound is dependent on its diffusibility and can be calculated for other compounds by multiplying the \(k\) obtained for TCE with the third root of the molecular weight of TCE relative to the molecular weight of the compound of interest (29). The \(k\) values that could thus be calculated for the other compounds used in this study are as follows: methane, 7.3 min\(^{-1}\); epoxynitrene, 5.2 min\(^{-1}\); epoxynitrene, 4.7 min\(^{-1}\); 1,2-epoxybutane, 4.4 min\(^{-1}\); cis-2,3-epoxybutane, 4.4 min\(^{-1}\); trans-2,3-epoxybutane, 4.4 min\(^{-1}\); VC, 4.6 min\(^{-1}\); 1,1-DCE, 4.4 min\(^{-1}\); cis-1,2-DCE, 4.0 min\(^{-1}\); trans-1,2-DCE, 4.0 min\(^{-1}\); 1,2-dichloroethane, 4.0 min\(^{-1}\); 1,2-dichloroethane, 3.8 min\(^{-1}\); trans-1,2-DCE epoxide, 3.8 min\(^{-1}\); TCE epoxide, 3.5 min\(^{-1}\).
The course of headspace and liquid-phase concentrations also depends on the Henry coefficient. A higher Henry coefficient will result in a larger influence of mass transfer on the course of the headspace concentration (i.e., a larger deviation from equilibrium). This influence was studied for various compounds with high or low Henry coefficients. For this, substrate depletion curves with a $k_1$ value of 0.25 min$^{-1}$ and a biomass concentration of 1.0 mg ml$^{-1}$, assuming equilibrium, were simulated with equation 5. The resulting simulated datum points were fitted with equations 6 and 7 assuming different $k_{La}$ values.

In Fig. 2, the $k_1$ values resulting from these fits are plotted as a function of the $k_{La}$. For the compound used in this study with the lowest Henry coefficient, cis-1,2-DCE epoxide ($H = 0.011$), the error in the $k_1$ value that is derived from the fit is less than 10% different from the $k_1$ that is derived assuming equilibrium, even if the $k_{La}$ was as low as 0.1 min$^{-1}$ (0.4 times the value of $k_1$). For a compound with a very high Henry coefficient, such as VC ($H = 1.26$), the $k_{La}$ value resulting in an error that is smaller than 10% is 2.0 min$^{-1}$ (8 times the value $k_1$). This illustrates that equilibrium may be assumed if the $k_{La}$ is about 10-fold larger than the value of $k_1$.

Experimental data were routinely fitted by numerical integration by use of the equations in which mass transfer was implemented. Whenever possible, transformation experiments were carried out with cell densities resulting in first-order transformation rates at which mass-transfer limitation did not significantly influence the course of the headspace concentrations. Thus, problems due to inaccurate estimation of the $k_{La}$ were minimized.

**Transformation of halogenated compounds by Methylosinus trichosporium OB3b.** The transformation kinetics of several C$_1$ and C$_2$ chlorinated aliphatics with cells expressing pMMO and cells expressing sMMO were determined by on-line GC analysis. Except for dichloromethane conversion with cells expressing sMMO, transformation of all compounds followed first-order kinetics at concentrations up to 30 μM.

As an example, substrate depletion curves obtained with cells expressing pMMO or sMMO are shown in Fig. 3. The conversion of dichloromethane with cells expressing pMMO followed first-order kinetics (Fig. 3A) and was fitted to equations 6 and 7. From this fit, a specific first-order transformation rate constant ($k_1$) of 0.67 ml min$^{-1}$ mg of cells$^{-1}$ was calculated. Transformation of dichloromethane with cells expressing sMMO did not follow first-order kinetics (Fig. 3B). By fitting headspace data to equations 6 and 8, describing Michaelis-Menten kinetics, the data were fitted with equations 6 and 8. Input parameters: $H = 0.11; k_{La}, 4.2$ min$^{-1}; V_g = 94.8$ ml; $V_l = 25.5$ ml; $X = 0.63$ mg of cells ml$^{-1}$. An optimal fit was obtained with a $k_1$ of 0.67 ml min$^{-1}$ mg of cells$^{-1}$ and a $K_m$ of 12 μM and an $r_{max}$ of 0.093 μmol mg of cells$^{-1}$ min$^{-1}$ (solid line). The broken line indicates the fit obtained by assuming first-order kinetics. Inset, effect of the $k_{La}$ on the estimation of the $r_{max}$ ($\ddot{\underline{\nu}}$) and $K_m$ ($\ddot{\underline{\nu}}$).
The presence of a compound with the structure of VC was observed (Fig. 4A). Mass spectrometry showed an unstable intermediate with a retention time longer than the other products of the reaction with PNBP, and GC-mass spectrometry were identified by comparison of retention times, analysis of the products of the reaction with PNBP, and GC-mass spectrometry. The kinetics of formation and degradation were studied.

During transformation of VC, the concomitant generation of an unstable intermediate with a retention time longer than that of VC was observed (Fig. 4A). Mass spectrometry showed the presence of a compound with m/z 58 (([M + H]+), 50 (100)(M – 28, C₂H₄), and 43 ([M + H]+), C₂H₃O). Mass spectra for VC epoxide have not been reported. The spectrum is similar to the spectrum published for chloracetaldehyde (33), but this compound clearly had a different retention time. These data indicate that the intermediate is VC epoxide. The fragment with m/z 50 may have been generated after rearrangement that is likely to occur during mass spectrometric analysis. To confirm that the accumulating intermediate was the epoxide and not one of its degradation products, the compound was trapped with PNB, as described in Materials and Methods. The adduct showed an

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cells expressing pMMO</th>
<th>Cells expressing sMMO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dichloromethane</td>
<td>0.7 ± 0.1</td>
<td>8.0 ± 0.2</td>
</tr>
<tr>
<td>Chloroform</td>
<td>&lt;0.03</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>1,2-Dichloroethane</td>
<td>&lt;0.03</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>VC</td>
<td>1.9 ± 0.2</td>
<td>7.6 ± 0.4</td>
</tr>
<tr>
<td>cis-1,2-DCE</td>
<td>0.06 ± 0.02</td>
<td>4.9 ± 0.1</td>
</tr>
<tr>
<td>trans-1,2-DCE</td>
<td>0.90 ± 0.00</td>
<td>3.3 ± 0.1</td>
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<tr>
<td>1,1-DCE</td>
<td>&lt;0.03</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>TCE</td>
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</tr>
<tr>
<td>cis-1,2-DCE epoxide</td>
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</tr>
<tr>
<td>trans-1,2-DCE epoxide</td>
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<tr>
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<tr>
<td>Epoxynitrene</td>
<td>ND</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>cis-2,3-Epoxybutane</td>
<td>ND</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>trans-2,3-Epoxybutane</td>
<td>ND</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

* Specific first-order rate constants (k₁) were determined in the presence of 20 mM formate. Values given are averages of duplicate measurements.
* ND, not determined.

The k₁ values for dichloromethane in these experiments was 4.2 min⁻¹. The insets in Fig. 3 show the kinetic parameters that were derived by assuming different k₁ values. The results indicated that the terms describing mass transfer in equations 6, 7, and 8 were not significantly influencing the outcome of these experiments. Consequently, it can be concluded that concentrations in the headspace and liquid phase were close to equilibrium.

The values of the specific first-order rate constants that were obtained for various chlorinated compounds are summarized in Table 1. Cells expressing pMMO only showed significant activity with dichloromethane, VC, and trans-1,2-DCE. The highest rate was found with VC. The other chlorinated alkanes and alkenes were poorly transformed or not transformed by these cells.

All halogenated alkanes and alkenes tested were transformed by cells expressing sMMO (Table 1). The specific first-order rate constants decreased in the order of dichloromethane, VC, cis-1,2-DCE, trans-1,2-DCE, 1,1-DCE, TCE, chloroform, and 1,2-dichloroethane.

Formation and degradation of epoxides. Formation of intermediates was observed during transformation of chlorinated ethenes by Methylosinus trichosporium OB3b. The intermediates were identified by comparison of retention times, analysis of the products of the reaction with PNBP, and GC-mass spectrometry. The kinetics of formation and degradation were studied.

During transformation of VC, the concomitant generation of an unstable intermediate with a retention time longer than that of VC was observed (Fig. 4A). Mass spectrometry showed the presence of a compound with m/z 58 ([M + H]+), 50 (100)(M – 28, C₂H₄), and 43 ([M + H]+), C₂H₃O). Mass spectra for VC epoxide have not been reported. The spectrum is similar to the spectrum published for chloracetaldehyde (33), but this compound clearly had a different retention time. These data indicate that the intermediate is VC epoxide. The fragment with m/z 50 may have been generated after rearrangement that is likely to occur during mass spectrometric analysis. To confirm that the accumulating intermediate was the epoxide and not one of its degradation products, the compound was trapped with PNB, as described in Materials and Methods. The adduct showed an
absorption maximum ($\lambda_{max}$) at 570 nm, which is in close agreement with the value of 560 nm for the PNBP-VC adduct reported by Fox et al. (12).

The Henry coefficient for VC epoxide could not be determined easily and calibration of the GC is difficult, since the compound is unstable in water. However, the epoxide concentrations could be derived by fitting the experimental data (Fig. 4A) with equations 6, 7, and 9 with the following assumptions: (i) conversion of VC results in 100% generation of the corresponding epoxide (12); (ii) the value of the Henry coefficient is between 0.01 and 0.05 (based on the value for related epoxides); and (iii) degradation is a chemical process. From this, it was calculated that the half-life of VC epoxide was approximately 78 s ($k = 0.53$ min$^{-1}$). A Henry coefficient of VC epoxide of 0.03 was used in the fits shown in Fig. 4A. A higher Henry coefficient means that a greater fraction of the epoxide is present in the headspace and thus is less susceptible to hydrolysis by water. Changing the value of the Henry coefficient to between 0.01 and 0.05 resulted in less than 3% deviation of the half-life giving the optimal fit, indicating that with this range the obtained half-life is not significantly influenced by the presence of the headspace, since essentially all epoxide is present in the liquid phase. Neglecting of mass-transfer limitation would have influenced the outcome of this experiment since the $k_1$ of VC transformation would have been significantly underestimated. The low Henry coefficient in combination with a $k_{f,\alpha}$ value of 4.3 min$^{-1}$ ensured that epoxide concentrations were close to equilibrium.

The concentration of epoxide derived from these fits will be valid only when the half-life of epoxide is not influenced by the presence of cells due to either aspecific reactions with biomolecules or enzymatic activity, and the diffusion over the membrane is not rate limiting. Concentrations in the liquid phase were determined with PNBP by quenching incubations at different time points with benzene (Fig. 4A). The concentrations obtained with this method were about 40% lower than the concentrations derived from the GC data.

An unstable intermediate was also detected in the headspace during the conversion of TCE (Fig. 4B). Mass spectrometry of this compound yielded fragments with $m/z$ (relative intensity) 111 (4)$\times$(M$^+$-35, C$_2$HClO$_2$), 82 (100)$\times$(M$^+$-64, CCl$_2$), 63 (16)$\times$(M$^+$-83, CCIO), 48 (32)$\times$(M$^+$-98, CHCI), and 35 (12)$\times$(M$^+$-111, CI), which is in agreement with published spectra of TCE epoxide (21). The $\lambda_{max}$ of the complex with PNBP was 560 nm. Previously, Fox et al. (12) reported a $\lambda_{max}$ of 520 nm for the complex of PNBP and TCE epoxide. The data were fitted by use of equations 6, 7, and 9, as with VC and VC epoxide, with the exception that oxidation of TCE results in 94% formation of epoxide (12) (Fig. 4B), and thus the half-life of the epoxide was estimated to be 21 s ($k = 2.0$ min$^{-1}$). The determination of liquid-phase concentrations with PNBP yielded similar values as the ones derived from headspace determination of liquid-phase concentrations with PNBP.

During transformation, epoxide concentrations in the headspace and liquid phase were close to equilibrium because of the low Henry coefficient combined with a $k_{f,\alpha}$ of 3.5 min$^{-1}$.

Conversion of cis-1,2-DCE resulted in a stoichiometric formation of trans-1,2-DCE epoxide (data not shown). Mass spectrometry showed the presence of a compound with $m/z$ (relative intensity) 112 (13)$\times$(M$^+$), 83 (7)$\times$(M$^+$-29, CHCl$_2$), 63 (3)$\times$(M$^+$-49, CCIO), 48 (100)$\times$(M$^+$-64, CHCI), and 35 (6)$\times$(M$^+$, CI). The values are in agreement with published spectra of chemically synthesized trans-1,2-DCE epoxide (14, 18). When 99% of the trans-1,2-DCE was degraded, the concentration of trans-1,2-DCE epoxide did not decrease significantly for at least 15 min, which was expected since Janssen et al. (18) determined that the half-life of this compound is 31 h.

During conversion of 1,1-DCE, no intermediate accumulated in the headspace. The epoxide that is presumably generated by oxidation by sMMO is very unstable and to our knowledge has never been detected in biological systems. Conversion of cis-1,2-DCE resulted in the concomitant generation of cis-1,2-DCE epoxide (Fig. 5). Mass spectrometry showed the presence of a compound with $m/z$ (relative intensity) 112 (10)$\times$(M$^+$), 83 (7)$\times$(M$^+$-29, CHCl$_2$), 63 (3)$\times$(M$^+$-49, CCIO), 48 (100)$\times$(M$^+$-64, CHCI), and 35 (7)$\times$(M$^+$, CI). The values are in agreement with published spectra of chemically synthesized cis-1,2-DCE epoxide (14, 18).

After degradation of approximately 80% of the cis-1,2-DCE, the concentration of cis-1,2-DCE epoxide started to decrease again. This suggested that the epoxide was actively degraded, since it is known to be relatively stable in aqueous solutions, with a half-life of approximately 72 h (18). In a parallel experiment HgCl$_2$, a potent inhibitor of biological activity was added 3.5 min after the conversion of cis-1,2-DCE was started. This immediately stopped the conversion of both cis-1,2-DCE and cis-1,2-DCE epoxide, confirming that the epoxide was degraded biologically. Inhibition of sMMO activity with acetylene, a specific inhibitor of MMO (30, 34), also completely stopped the degradation of these compounds (Fig. 5). This indicated that cis-1,2-DCE epoxide was converted by sMMO.Biologically synthesized cis-1,2-DCE epoxide was added to a cell suspension freshly harvested from the fermentor to determine the transformation kinetics. The specific first-order rate constant of this conversion was 0.45 ml min$^{-1}$ mg of cells$^{-1}$.

We tested whether other epoxides were also a substrate for cells expressing sMMO. The transformation rate of trans-1,2-DCE epoxide was below the detection limit of 0.03 ml min$^{-1}$ mg of cells$^{-1}$. Of the nonchlorinated epoxides that were tested,
only epoxysthane was transformed with a $k_1$ of 0.8 ml min$^{-1}$ mg of cells$^{-1}$. For the structurally related epoxides epoxopropane, cis-2,3-epoxystiane, and trans-2,3-epoxystiane, the $k_1$ values were below the detection limit of 0.03 ml min$^{-1}$ mg of cells$^{-1}$.

Addition of the natural substrate of sMMO, methane, lowered the degradation rate of cis-1,2-DCE epoxide, indicating competition for the same enzyme by the two compounds. The kinetics of inhibition of the conversion of cis-1,2-DCE epoxide by methane was studied by determining the $k_1$ values at various methane concentrations (Fig. 6). The data were fitted with equation 11, and an optimal fit was obtained with a $K_m$ of 56 µM. Values of $K_s$ and $K_m$ for methane degradation found are 40 µM (6a) and 92 µM (27), respectively.

Conversion of cis-1,2-DCE epoxide appeared to inactivate the cells, resulting in decreasing degradation rates. This indicates that the oxidation product of cis-1,2-DCE epoxide is a highly reactive compound (Fig. 7). A limitation in formate or $O_2$ is not the cause of this decrease, since cells did not lose activity in control incubations after 2 h (data not shown). Chloride levels were determined during the degradation of cis-1,2-DCE and cis,1,2-DCE epoxide (Fig. 7), since this is one of the possible products of the conversion of cis-1,2-DCE epoxide. Chloride was indeed liberated during the conversion of cis-1,2-DCE epoxide. At the end of the experiment, 57% of the chlorine added as cis-1,2-DCE was present as inorganic chloride. At this time, degradation rates of cis-1,2-DCE epoxide had almost stopped, and 28% of the chloride was present as cis-1,2-DCE epoxide. The remaining 15% might be present in nonvolatile chlorinated compounds or in adducts to cellular components.

**DISCUSSION**

Sorption, laborious extraction procedures, and high volatility may interfere with the reliable estimation of microbial transformation rate constants for halogenated aliphatic compounds. The on-line monitoring of headspace concentrations with GC described in this paper enabled the rapid determination of conversion rates at practically relevant low substrate concentrations and allowed identification of unstable intermediates. Mass-transfer limitation and sorption may influence the measurements and therefore these factors were carefully analyzed to ensure that kinetic parameters were determined accurately.

The transformation rates with cells expressing pMMO or sMMO were determined. The $k_1$ values found for 1,2-dichloroethane, TCE, cis-1,2-DCE, and trans-1,2-DCE of cells expressing sMMO are similar to those reported by Oldenhuis et al. (27). The value of $k_1$ for 1,1-DCE reported previously was threefold lower than the value of 3.2 ml min$^{-1}$ mg of cells$^{-1}$ that we found, which may be due to rapid inactivation of the cells (38a). The value of $k_1$ for chloroform of 1.3 ml min$^{-1}$ mg of cells$^{-1}$ is much lower than the value of 16 min$^{-1}$ mg of cells$^{-1}$ determined previously (28). Few data are available about the transformation kinetics of chlorinated compounds by cells expressing pMMO. Complete degradation of 0.2 mM 1,2-dichloroethane and chloroform was observed previously during 24 h of incubation with cell suspensions of *Methylosinus trichosporium* OB3b expressing pMMO (0.3 to 0.4 mg of cells ml$^{-1}$) (28). However, in this study, we found that the transformation rates of these compounds were below the detection limit of 0.03 ml min$^{-1}$ mg of cells$^{-1}$.

Strain OB3b, grown either in the presence or absence of copper, is capable of transformation of VC. Under anaerobic conditions, the degradation rate of chlorinated ethenes decreases with increasing number of chlorine substituents (8, 39). Hence, cis-1,2-DCE and VC often accumulate during anaerobic degradation of perchloroethylene under field conditions, and these compounds are frequently detected in groundwater that was originally contaminated with perchloroethylene. *Mycobacterium* sp. strain L1 is the only strain described to date which utilizes VC as a sole source of carbon and energy (16), but the instability of enzymes involved in VC metabolism in this strain does not favor its application in a bioremediation process. Removal of VC with activated carbon is also less efficient compared with removal of higher chlorinated ethenes, making
aerobic methods using methanotrophs for the treatment of waste streams contaminated with VC an attractive option. The transformation rates obtained with Methylosinus trichosporium OB3b are among the highest observed for cometabolism. Dichloromethane, 1,2-dichloroethane, and VC can also serve as sole sources of carbon and energy for specialized strains. The $k_1$ for organisms growing on these compounds can be derived from the specific growth rate, the Monod constant, and the yield by using $k_1 = \frac{\mu_{max} Y_{k_1}}{s}$. This allows the comparison of the degradation rates of these organisms with the cometabolic degradation rates determined for Methylosinus trichosporium OB3b. The values for $k_1$ for 1,2-dichloroethane that can thus be calculated for Xanthobacter autotrophicus GJ10 and Anoxybacillus aquaticus AD25, 0.3 and 3.0 ml min$^{-1}$ mg of cells$^{-1}$, respectively (38), are similar to the value that we found for Methylosinus trichosporium OB3b. Mycobacterium sp. strain L1 degraded VC with an approximately twofold-higher $k_1$ (16) as that found by us for cometabolic transformation with Methylosinus trichosporium OB3b. The higher $k_1$ value, 53 ml min$^{-1}$ mg of cells$^{-1}$, that can be calculated for dichloromethane degradation by Hyphomicrobium sp. strain GJ21 (7) in comparison with the cometabolic degradation rate of Methylosinus trichosporium OB3b expressing sMMO or pMMO is due mainly to the low-affinity constant, 10 $\mu$M, of strain GJ21.

The on-line analysis of headspace concentrations also made it possible to study the formation and degradation of the corresponding epoxides. The accumulation of epoxides was observed during transformation of all chlorinated ethenes except 1,1-DCE. The somewhat different $\mu_{max}$ values that we found for the PNBP adducts of the epoxides in this study compared with the values reported by Fox et al. (12) may be due to the use of benzene in the previous study for extraction of the epoxides whereas we directly trapped the epoxide in ethylene glycol in which PNBP was dissolved. The half-lives that we determined for VC epoxide, 78 s, and TCE epoxide, 21 s, are in good agreement with the values of 1.5 min (20) and 12 s (25), respectively, found in the absence of cells, indicating that these compounds are mainly chemically degraded. Our results show that during transformation of pulses of VC and TCE, the corresponding epoxides accumulate in the medium at significant concentrations before they disappear as a result of spontaneous hydrolysis, rearrangement reactions, or reactions with biomolecules.

To our knowledge, this is the first time that TCE epoxide has been detected in a microbial whole-cell system. Previously, TCE epoxide formation has only been reported as occurring during oxidation of TCE in vitro by purified sMMO by Fox et al. (12), who found that 94% of the TCE that was oxidized was converted to TCE epoxide. Both TCE epoxide and some of its degradation products, such as acyl chlorides, are highly reactive compounds that rapidly react with nucleophiles (21). The covalent modification of cellular components by these compounds limits the transformation capacity of cell suspensions (1, 27). Experiments using $^{14}$C-labelled TCE revealed that TCE conversion products reacted nonspecifically with cellular components but not with methanol dehydrogenase, which suggested that the epoxide may not reach the periplasm (27). The results found here indicate that the epoxide must leave the cell and diffuse through the periplasm. Recently, it was reported that glucose fermentation by yeast cells was inhibited in the presence of Xanthobacter Py2 cells degrading TCE, indicating that toxic metabolites occurred in the medium (32).

cis-1,2-DCE is an important groundwater pollutant that is generated from perchloroethene and TCE by dechlorination under anaerobic conditions. However, few data about the aerobie degradation of cis-1,2-DCE are available compared with the data about degradation of other chlorinated ethenes. We found that cis-1,2-DCE epoxide formed by oxidation of cis-1,2-DCE is actively degraded by cells expressing sMMO. The effect of methane and acetylene and the competitive inhibition of cis-1,2-DCE epoxide degradation by methane strongly indicated that sMMO was involved. Surprisingly, epoxymethane was also degraded, while related epoxides such as epoxyp propane cis-2,3-epoxybutane, trans-2,3-epoxybutane, and trans-1,2-DCE epoxide were not substrates for these cells, suggesting that unlike for halogenated alkenes and alkanes, the substrate range of sMMO for epoxides is rather small. The nature of the reaction is not clear. Lewis acid-like interactions catalyzed by the heme iron in its Fe(III) state have been postulated to cause the rearrangement of TCE epoxide to chloral in cytochrome P-450 (17). It is unlikely that a similar mechanism holds for the conversion of cis-1,2-DCE epoxide by the diferric cluster in sMMO, since a rearrangement of the epoxide to dichloroacetaldelyde is inconsistent with the liberation of chloride that was observed.

Castro et al. (6), using $^{13}$C and $^{14}$C nuclear resonance suggested that the VC epoxide that is formed after the oxidation of VC by Methylosinus trichosporium OB3b is further degraded via three different pathways. The major pathway was reduction to epoxymethane and a subsequent hydrolysis to ethylene glycol. They postulated that both reactions are catalyzed by the organism. However, the epoxides were not detected, probably because the steady-state concentrations were too low. VC epoxide might also be hydrolyzed biologically or chemically, yielding glycolaldehyde, or chemically rearranged to chloroacetaldelyde.

The toxicity of chlorinated ethene epoxides and their degradation products suggests that active conversion to nonreactive compounds may reduce or prevent such toxic effects. However, no activity of microbial enzymes that are involved in the metabolism of epoxides with chlorinated ethene epoxides has been reported. The conversion of cis-1,2-DCE epoxide catalyzed by sMMO does not seem to reduce toxic effects, since the conversion clearly inactivated the cells.

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