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Degradation of chlorinated and brominated hydrocarbons by *Methylobacterium album* BG8

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Abstract The degradation kinetics of ten halogenated hydrocarbons by *Methylobacterium album* BG8 expressing particulate methane monooxygenase (pMMO) and the inhibitory effects of these compounds on microbial growth and whole-cell pMMO activity were measured. When *M. album* BG8 was grown with methane, growth was completely inhibited by dichloromethane (DCM), bromoform (BF), chloroform (CF), vinyl chloride (VC), 1,1-dichloroethylene (1,1-DCE), and *cis*-dichloroethylene (*cis*-DCE). Trichloroethylene (TCE) partially inhibited growth on methane, while dibromomethane (DBM), *trans*-dichloroethylene (*trans*-DCE), and 1,1,1-trichloroethane (1,1,1-TCA) had no effect. If the cells were grown with methanol, DCM, BF, CF, and 1,1-DCE completely inhibited growth, while VC, *trans*-DCE, TCE, and 1,1,1-TCA partially inhibited growth. Both DBM and *cis*-DCE had no effect on growth with methanol. Whole-cell pMMO activity was also affected by these compounds, with all but 1,1,1-TCA, DCM, and DBM reducing activity by more than 25%. DCM, DBM, VC, *trans*-DCE, *cis*-DCE, 1,1-DCE, and TCE were degraded and followed Michaelis-Menten kinetics. CF, BF, and 1,1,1-TCA were not measurably degraded. These results suggested that the products of DCM, TCE, VC, and 1,1-DCE inactivated multiple enzymatic processes, while *trans*-DCE oxidation products were also toxic but to a lesser extent. *cis*-DCE toxicity, however, appeared to be localized to pMMO. Finally, DBM and 1,1,1-TCA were not inhibitory, and CF and BF were themselves toxic to *M. album* BG8. Based on these results, the compounds could be separated into four general categories, namely (1) biodegradable with minimal inactivation, (2) biodegradable with substantial inactivation, (3) not biodegradable with minimal inactivation, and (4) not biodegradable but substantial inactivation of cell activity.

Key words Methanotrophs · Bioremediation · Halogenated hydrocarbons · Kinetics

Abbreviations *pMMO* Particulate methane monooxygenase · *AMO* Ammonia monooxygenase · *sMMO* Soluble methane monooxygenase · *DBM* Dibromomethane · *DCM* Dichloromethane · *BF* Bromoform · *CF* Chloroform · *VC* Vinyl chloride · *1,1-DCE* 1,1-Dichloroethylene · *cis-DCE* *cis*-Dichloroethylene · *trans-DCE* *trans*-Dichloroethylene · *TCE* Trichloroethylene · *1,1,1-TCA* 1,1,1-Trichloroethane

Introduction

Halogenated hydrocarbons are common pollutants in the United States and are found in aquifers, landfills, wastewaters, and waste disposal sites (Westrick et al. 1984; Semprini 1997). Once in the environment, these compounds have been shown to undergo a variety of anaerobic and aerobic biotransformations. Under anoxic conditions, biologically mediated dechlorination reactions have been found to be relatively slow and can result in the accumulation of harmful metabolites such as *cis*-dichloroethylene (*cis*-DCE) and the carcinogen vinyl chloride (VC) (Bouwer and McCarty 1983; Vogel and McCarty 1985; DiStefano et al. 1991). Under oxic conditions, however, many of these halogenated hydrocarbons can be cometabolically oxidized by bacteria expressing mono- and dioxygenases when growing on methane, propane, phenol, ammonia, or toluene (Ensley 1991; Arp 1995; Wackett 1995).

Of these microorganisms, methanotrophs, gram-negative bacteria that utilize methane for both carbon and energy, have been extensively studied (Hanson and Hanson 1996). The enzyme responsible for the initial oxidation of methane, methane monooxygenase (MMO), can also oxidize aliphatic and aromatic hydrocarbons including priority pollutants such as trichloroethylene. Modeling and control of methanotrophic degradation of halogenated hy-

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drocarbons, although feasible, is complicated by the fact that multiple forms of the MMO exist. Some methanotrophs can synthesize a cytoplasm-associated MMO, or soluble methane monooxygenase (sMMO). This form of MMO has a relatively broad substrate range and has been extensively characterized (Fox et al. 1990; Cardy et al. 1991; Rosenzweig et al. 1993). Most known methanotrophs, however, can only express a membrane-bound, or particulate methane monooxygenase (pMMO). This form of MMO is found in all known methanotrophs, but its substrate range is narrower than that of sMMO, and its rate of trichloroethylene (TCE) degradation is approximately one order of magnitude less than that of sMMO (Lontoh and Semrau 1998).

Despite the low rates of chlorinated hydrocarbon degradation, pMMO-mediated oxidation of small halogenated hydrocarbons can be useful, particularly for in situ remediation, since most known methanotrophs can only express pMMO. In at least one field demonstration, it has been speculated that pMMO was expressed by natural methanotrophic populations after they had been stimulated through provision of methane and air (McCarty 1997). Based on the fact that most known methanotrophs can only express pMMO, and pMMO expression can apparently be increased in situ, we have examined (1) the oxidation of chlorinated and brominated one- and two-carbon compounds by *Methylomicrobium album* BG8 expressing pMMO, and (2) how these compounds affect methanotrophic activity. Correlation of the kinetics of degradation with assays of microbial growth and activity suggested that the compounds tested could be categorized into several groups, as has been seen for chlorinated methane, ethane, and ethylene cometabolism by the ammonia oxidizer *Nitrosomonas europaea* (Rasche et al. 1991), namely: (1) not biodegradable and little toxicity to *M. album* BG8, (2) biodegradable with little toxicity, and (3) biodegradable with substantial toxicity. Since methanotrophs and ammonia oxidizers have significant similarity, particularly substantial homology between sequences of the genes encoding ammonia monooxygenase and particulate methane monooxygenase (McTavish et al. 1993; Semrau et al. 1995), such a result is not surprising. Unlike the study of Rasche et al. (1991), however, we found two compounds that were best categorized in a fourth group, (4) not significantly biodegradable, but with substantial toxicity.

Materials and methods

Culture conditions

M. album BG8 was grown on nitrate mineral salt (NMS) medium (Whittenbury et al. 1970) at 30°C in batch flasks shaken at 250 rpm in a methane/air atmosphere (1:2 ratio) at 1 atm of pressure. The culture medium was no more than 15% of the total flask volume to prevent mass transfer limitations of methane and oxygen from the head space to liquid medium. Since this strain can express only pMMO, 10 µM was added aseptically as $\text{Cu}(\text{NO}_3)_2 \cdot 2.5(\text{H}_2\text{O})$ after autoclaving and was equilibrated for at least 2 days before the media were inoculated. This concentration of copper was added since previous work had shown that cells expressing pMMO de-

graded TCE at high growth concentrations of copper (Lontoh and Semrau 1998).

Inhibition of cell growth by brominated and chlorinated hydrocarbons

The toxicity of halogenated hydrocarbons on methanotrophs expressing pMMO was examined by measuring the growth of *M. album* BG8 in the presence of these compounds. Cells were initially grown to the late exponential phase (OD_{600} of 0.9), and methane was removed by evacuating the growth flasks five times and allowing air to re-equilibrate after each evacuation. The cells were aseptically diluted 30-fold into 3 ml of NMS medium with 10 µM copper and were transferred to 20-ml serum vials for an initial OD_{600} of 0.023. The vials were capped with Teflon-coated butyl-rubber stoppers (Wheaton) and crimp-sealed. Either 10 mM methanol or 550 µM methane (aqueous concentration) was then added as a growth substrate along with 100 µM aqueous solution of one of the halogenated hydrocarbons. Duplicate vials were prepared for both the positive control (no halogenated hydrocarbon) and samples with a chlorinated or brominated hydrocarbon. The appropriate amount of substrate to add was calculated using the following dimensionless Henry's constants: methane, 27.02; VC, 1.262 (Morel and Hering 1991); bromoform (BF), 0.0281; dibromomethane (DBM), 0.0442; dichloromethane (DCM), 0.125; 1,1-dichloroethylene (1,1-DCE), 1.355; *trans*-dichloroethylene (*trans*-DCE), 0.474; *cis*-dichloroethylene (*cis*-DCE), 0.197; 1,1,1-trichloroethane (1,1,1-TCA), 0.804; TCE, 0.458 (Tse et al. 1992); and chloroform (CF), 0.189 (Gosset 1987). After incubating the serum vials at 30°C and 250 rpm for 60 h, the OD_{600} was again measured to determine if growth was inhibited by any of the halogenated hydrocarbons.

Methane and halogenated hydrocarbon degradation assays

The cells were grown to mid-exponential phase (OD_{600} of 0.8–0.9) and then diluted to an OD_{600} of 0.3 with prewarmed fresh medium. To normalize rates of degradation to cell biomass, protein concentrations were measured using the BioRad protein assay kit with bovine serum albumin as a standard. The cells were digested at 90°C for 30 min in 5 M NaOH. Serial dilutions were prepared to achieve final protein concentrations within the linear range of the assay. The amount of protein was determined by measuring the absorbance at 595 nm after the BioRad assay reagent had been added. In these experiments, the protein concentration of these cell suspensions varied between 0.092 and 0.098 mg/ml. The initial rate of halogenated hydrocarbon degradation was analyzed using cells harvested as described earlier (Lontoh and Semrau 1998) and is briefly described here. After methane was removed from the growth flasks, 3-ml aliquots were aseptically transferred to 20-ml serum vials. The vials were capped and sealed as described above. For each concentration of methane and halogenated hydrocarbon examined in these assays, triplicate samples were created with duplicate controls. Controls were made by adding 50 ml of 5 M NaOH except for CF and BF, which were abiotically degraded at high pH. The controls for CF and BF were made by adding 1% acetylene (v/v), an irreversible inhibitor of methane monooxygenase (Stirling and Dalton 1977; Prior and Dalton 1985).

After addition of substrate, vials were shaken at 250 rpm at 30°C again to prevent mass transfer effects from limiting measured rates of substrate degradation. Formate in the form of sodium formate was added at a concentration of 20 mM. The initial rates of degradation were determined by measuring the amount of halogenated hydrocarbon remaining using a Hewlett-Packard 5890 series II gas chromatograph with an automated head-space sampler, flame ionization detector (FID), and two DB-624 analytical columns (J&W Scientific). The gas chromatograph was operated with a carrier gas flow rate of helium at a flow rate of 5.8 ml min^{-1} and with injector, oven, and detector temperatures set at 160, 80, and 250°C, respectively. Initial rates of substrate degradation

were determined over initial time frames depending on the substrate and extent of degradation. For those compounds readily degradable such as methane, DCM, DBM, and *trans*-DCE, a short time frame from $t = 0$ h to $t = 1$ h was used. The initial rate of TCE degradation was calculated using a 2-h time period from $t = 0$ h to $t = 2$ h since this had previously been found to be appropriate (Lontoh and Semrau 1998). The initial degradation rates of more refractory halogenated hydrocarbons including VC, *cis*-DCE, and 1,1-DCE were measured over 5 h. For BF, CF, and 1,1,1-TCA, the time frame to determine initial rates of degradation was extended to 10 h from $t = 0$ h to $t = 10$ h since these compounds were quite recalcitrant. The measured rates of degradation were normalized to the initial protein concentration, and the average initial degradation rate of triplicate samples is reported here along with the standard deviation. The kinetic parameters of apparent maximal degradation rate, V_{\max} [$\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$] and apparent affinity, K_s (μM), were determined by applying nonlinear regression on the Michaelis-Menten formula using Kaelida Graph 3.0 for Macintosh.

Inhibition of whole-cell methane oxidation by halogenated hydrocarbons

To determine if pMMO activity was inhibited by the chosen brominated and chlorinated hydrocarbons, inhibition of pMMO activity was specifically measured. The cells were prepared as described above for determination of the Michaelis-Menten kinetics of halogenated hydrocarbon degradation. After aseptically transferring the diluted cells to 20-ml serum vials, 100 μM of the halogenated hydrocarbon was added with the exception of 1,1-DCE and *cis*-DCE. Since these substrates showed toxicity at concentrations greater than 10 μM , 5 μM was added to the vials. Triplicate samples were prepared for each halogenated hydrocarbon along with positive controls (no halogenated hydrocarbons) and negative controls (killed cells). The cells were incubated with the halogenated hydrocarbons for 5 h at 30°C while being shaken at 270 rpm. After 5 h, the halogenated substrates were removed by purging the vials with air for 1 min. Killed controls verified that this amount of time was sufficient for complete removal of the halogenated hydrocarbons as determined using head-space gas-chromatographic analysis (data not shown). Methane (50 μM ; aqueous concentration) was then added, and the amount of methane consumed over 1 h was measured using the head-space analytical techniques described above.

Chemicals

All chemicals in the preparation of media were of reagent grade or better. Methane of the highest purity (> 99.99%) was obtained from Matheson (Newark, N.J., USA). Chloroform [99.9% gas chromatography (GC) grade], bromoform (99.5% GC grade), 1,1,1-trichloroethane (99.9% GC grade), 1,1-dichloroethylene (99% GC grade), *trans*-dichloroethylene (98% GC grade), *cis*-dichloroethylene (97% GC grade), dichloromethane (99% GC grade), and dibromomethane (99.5% GC grade) were purchased from Aldrich (Milwaukee, Wis., USA). Vinyl chloride (> 99.5% GC grade) was purchased from Fluka (Ronkonkoma, N.Y., USA). Distilled-deionized water from a Corning Millipore D2 system was used for all experiments. All glassware was washed with detergent and then acid-washed in 2 M HNO_3 overnight to remove trace metals, including copper. The acid was subsequently removed by repeated rinses with distilled-deionized water.

For compounds that are liquid at 25°C (i.e., CF, BF, TCE, 1,1,1-TCA, 1,1-DCE, *trans*-DCE, *cis*-DCE, DCM, and DBM), stock saturated solutions were prepared by the method of Chang and Alvarez-Cohen (1996). Stock solutions were added to sample vials using Hamilton 1700 series gas-tight syringes, with care to exclude non-aqueous-phase liquids. For compounds that are gaseous at 25°C (i.e., VC and methane), samples were added to sample vials using Dynatech A-2 gas-tight syringes. Methanol was added as a 100% solution.

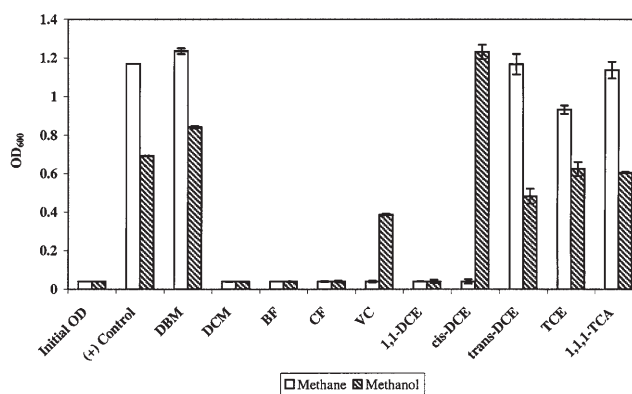


Fig. 1 Growth inhibition of *Methylobacterium album* BG8 by chlorinated and brominated halogenated hydrocarbons. Cells were grown with either 550 μM methane or 10 mM methanol in the presence of 100 μM of the halogenated hydrocarbon for 60 h. Error bars indicate the range of duplicate samples (DBM dibromomethane, DCM dichloromethane, BF bromoform, CF chloroform, VC vinyl chloride, 1,1-DCE 1,1-dichloroethylene, *cis*-DCE *cis*-dichloroethylene, *trans*-DCE *trans*-dichloroethylene, TCE trichloroethylene, and 1,1,1-TCA 1,1,1-trichloroethane)

Results

Toxicity of halogenated compounds on the growth of *M. album* BG8

Since *M. album* BG8 can utilize both methanol and methane as carbon sources (Whittenbury et al. 1970; Bensted et al. 1998), examining growth of *M. album* BG8 in the presence of halogenated hydrocarbons and either methane or methanol will help determine if pMMO is inactivated over time in the presence of the halogenated hydrocarbons. As can be seen in Fig. 1, growth with methane as the sole carbon source was completely inhibited by 100 μM solutions of six of the halogenated compounds tested (DCM, BF, CF, VC, 1,1-DCE, and *cis*-DCE). When methanol was used as the carbon source, inhibition of growth by some of these compounds (DCM, BF, CF, and 1,1-DCE) showed a similar pattern, indicating that the growth inhibition of *M. album* BG8 by these compounds was caused not solely by inactivation of pMMO, but also by the inactivation of other cell components. Interestingly, growth inhibition by VC and TCE in the presence of methanol was less than that in the presence of methane, suggesting that the toxicity associated with VC and TCE degradation selectively targets pMMO activity. Furthermore, *cis*-DCE appeared to stimulate the cell growth with methanol to the degree of growth with methane in the absence of *cis*-DCE, although *cis*-DCE completely inhibited growth on methane. *trans*-DCE and 1,1,1-TCA inhibited growth with methanol (30 and 12%, respectively), but did not substantially affect growth with methane.

Kinetics of substrate degradation by *M. album* BG8

Table 1 reports the values of apparent maximal uptake rate (V_{\max}) and apparent affinity (K_s) using nonlinear re-

Table 1 Michaelis-Menten kinetics for the degradation of methane and halogenated hydrocarbons by *Methylomicrobium album* BG8 expressing particulate methane monooxygenase. Values in parentheses represent the standard deviation of triplicate samples (DCM dichloromethane, DBM dibromomethane, CF chloroform, BF bromoform, VC vinyl chloride, *trans*-DCE *trans*-dichloroethylene, *cis*-DCE *cis*-dichloroethylene, 1,1-DCE 1,1-dichloroethylene, TCE trichloroethylene, 1,1,1-TCA 1,1,1-trichloroethane, and NM no measurable degradation)

Substrate	Apparent V_{\max} [nmol · min ⁻¹ · (mg protein) ⁻¹]	Apparent K_s (μM)	V_{\max}/K_s (ml · min · mg protein) ⁻¹	Concentration range (μM)
Methane	453 (13)	19 (2)	25	0–80
DCM	33 (4)	73 (18)	0.45	0–100
DBM	45 (3)	171 (29)	0.26	0–400
CF	NM	NM	NM	0–100
BF	NM	NM	NM	0–100
VC	7 (0.5)	30 (7)	0.23	0–100
<i>trans</i> -DCE	43 (6)	60 (19)	0.72	0–100
<i>cis</i> -DCE	0.12 (0.01)	0.8 (0.2)	0.15	0–10
1,1-DCE	0.23 (0.03)	2.5 (0.8)	0.092	0–4
TCE	4.3 (0.1)	59 (3)	0.072	0–120
1,1,1-TCA	NM	NM	NM	0–100

gression analysis to fit the Michaelis-Menten equation to the measured initial rates of degradation for each substrate. Most of the compounds tested were degraded by *M. album* BG8, with the exception of BF, CF, and 1,1,1-TCA. DCM, DBM, *trans*-DCE, TCE, and VC were all readily degraded in the presence of formate at a rate of approximately 1–10% of the V_{\max} of methane consumption. 1,1-DCE and *cis*-DCE were also degraded, but the maximal transformation rates of these compounds was less than 0.5% of the maximal rate of methane consumption. Also shown in Table 1, the whole-cell affinity for DCM, DBM, TCE, *trans*-DCE, and VC was much less than that for methane, with affinity typically decreasing as the size of halogen substituents increased. For example, DBM and DCM had relatively similar V_{\max} values, but the K_s value for DBM was more than twice that for DCM. 1,1-DCE and *cis*-DCE had lower K_s values than methane, however, suggesting that the apparent affinity of *M. album* BG8 for these compounds was greater than that for methane.

All halogenated hydrocarbons that were degraded, however, had pseudo-first-order rate constants (V_{\max}/K_s) at least 40-fold less than those for methane (Table 1). Such a large difference indicates that *M. album* BG8 expressing pMMO is highly selective. The remaining compounds tested (CF, BF, and 1,1,1-TCA) were not measurably degraded by *M. album* BG8 and are apparently very poor substrates. To determine if pMMO was responsible for the degradation of those compounds readily degraded (DCM, DBM, *trans*-DCE, *cis*-DCE, 1,1-DCE, TCE, and VC), acetylene, a potent inhibitor of MMO (Prior and Dalton 1985), was added at 1% (v/v) to cell suspensions. In the presence of acetylene, no degradation was observed for any of the compounds for over 5 h. Therefore, pMMO

was solely responsible for halogenated hydrocarbon degradation (data not shown).

Table 1 also reports the concentration range used to determine the apparent V_{\max} and K_s values for the degradation of the halogenated hydrocarbons and methane. Of the compounds that were degraded, standard hyperbolic Michaelis-Menten kinetics were observed when aqueous concentrations were increased up to 100 μM (120 and 400 μM for TCE and DBM, respectively). The notable exceptions, however, are 1,1-DCE and *cis*-DCE. For these two substrates, the Michaelis-Menten kinetic parameters were determined using concentration ranges of 0–4 μM and 0–10 μM, respectively. These substrates apparently are toxic at relatively low concentrations (< 100 μM). The degradation of CF, BF, and 1,1,1-TCA was also monitored, but since no substrate disappearance was observed at concentrations of up to 100 μM, it was assumed that these compounds were not biodegradable by *M. album* BG8.

Inactivation of methane oxidation by halogenated hydrocarbons

The effect of these halogenated hydrocarbons on pMMO activity is reported in Fig. 2 and Table 2. 1,1-DCE and *cis*-DCE showed almost complete inhibition of pMMO activity after incubating *M. album* BG8 for 5 h in the presence of 5 μM of these chlorinated ethylenes. CF also completely inhibited pMMO activity, and VC and TCE reduced pMMO activity by approximately 85%, but at much higher initial concentrations of 100 μM. Of the remaining compounds, BF inhibited methane oxidation by approximately 50%, while *trans*-DCE, DCM, DBM, and 1,1,1-TCA reduced pMMO activity by 20–30%. These

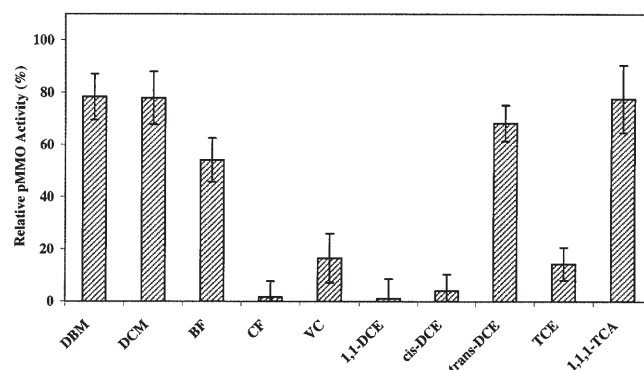


Fig. 2 Inhibition of particulate methane monooxygenase (pMMO) activity in whole cells of *Methylomicrobium album* BG8 after exposure to chlorinated and brominated hydrocarbons for 5 h. The concentration of the halogenated hydrocarbon was 100 μM with the exception of 1,1-DCE and *cis*-DCE, which were added at a concentration of 5 μM. Error bars indicate the standard deviation of triplicate samples (DBM dibromomethane, DCM dichloromethane, BF bromoform, CF chloroform, VC vinyl chloride, 1,1-DCE 1,1-dichloroethylene, *cis*-DCE *cis*-dichloroethylene, *trans*-DCE *trans*-dichloroethylene, TCE trichloroethylene, and 1,1,1-TCA 1,1,1-trichloroethane)

Table 2 Effect of halogenated hydrocarbons on methanotrophic and ammonia-oxidizer activity. Compounds are classified as either I not biodegradable with little inactivation, II biodegradable with little inactivation, III biodegradable with substantial inactivation, or IV not biodegradable but with substantial inactivation. Values in parentheses represent the standard deviation of triplicate sam-

ples (1,1,1-TCA 1,1,1-trichloroethane, DBM dibromomethane, DCM dichloromethane, VC vinyl chloride, 1,1-DCE 1,1-dichloroethylene, *trans*-DCE *trans*-dichloroethylene, *cis*-DCE *cis*-dichloroethylene, TCE trichloroethylene, BF bromoform, CF chloroform, pMMO particulate methane monooxygenase, and ND not determined)

Halogenated hydrocarbon	<i>Methylobacterium album</i> BG8 (This study)			<i>Nitrosomonas europaea</i> (Rasche et al. 1991)		
	Amount added (μM)	pMMO activity (%)	Compound classification	Amount added (μM) ^a	O ₂ uptake (%)	Compound classification
1,1,1-TCA	100	78 (13)	I	600	53	III
DBM	100	78 (9)	II	ND	ND	ND
DCM	100	78 (10)	II	1,900	60	III
VC	100	17 (9)	III	16	30	III
1,1-DCE	5	1 (7)	III	7.5	3	III
<i>trans</i> -DCE	100	68 (7)	III	121	50	III
<i>cis</i> -DCE	5	4 (6)	III	125	4	III
TCE	100	14 (6)	III	109	3	III
BF	100	54 (8)	IV	ND	ND	ND
CF	100	2 (6)	IV	740	1	III

^aConverted from reported units of μmol using the Henry constants reported in Materials and methods

activity assays were performed after the halogenated hydrocarbons had been removed to minimize the possibility of the substrate(s) themselves causing a reduction in pMMO activity. It is possible, however, that some substrates remained strongly bound to the pMMO, causing an apparent decrease in activity.

The results of the effect of these compounds on growth, pMMO activity, and the measured Michaelis-Menten parameters of apparent V_{max} and K_s suggest that these compounds can be divided into four groups similar to the ones proposed by Rasche et al. (1991). 1,1,1-TCA was found neither to be toxic nor to be degraded by *M. album* BG8, and could be considered as a single category of halogenated substrates. DCM and DBM also had little effect on either growth or pMMO activity, but were readily degraded and thus were considered to be a second group of substrates. A third group consisting of the chlorinated ethylenes (*trans*-DCE, VC, 1,1-DCE, *cis*-DCE, and TCE) were also substrates of the pMMO, but their oxidation significantly affected both growth and pMMO activity. Finally, we discovered a fourth group of halogenated hydrocarbons (CF and BF) that were not measurably oxidized by pMMO-expressing cells but that had significant toxicity to growth on both methane and methanol and also substantially reduced whole-cell pMMO activity.

Discussion

The growth of *M. album* BG8 with methane as a sole carbon source was completely inhibited by many of halogenated hydrocarbons tested. i.e., DCM, CF, BF, *cis*-DCE, VC, and 1,1-DCE. *trans*-DCE was not found to inhibit growth on methane. These results are in agreement with the findings of growth inhibition of a mixed methan-

otrophic culture with methane as a growth substrate in the presence of DCM, CF, *trans*-DCE, *cis*-DCE, and TCE at concentrations varying from 30 to 80 μM (Janssen et al. 1988). VC was not tested in that study. Interestingly, 1,1,1-TCA was also found to inhibit growth of this mixed culture on methane, but 1,1,1-TCA had little effect on the growth of *M. album* BG8 on methane. It is possible that in the mixed culture, sMMO was being expressed. This form of the MMO has been shown to degrade 1,1,1-TCA, while the pMMO does not (Oldenhuis et al. 1989). Since *M. album* BG8 can only express pMMO, toxicity of the products of 1,1,1-TCA oxidation was minimized in the experiments presented here.

The inhibition of growth on methane could have been due to competition between the halogenated hydrocarbon and methane for binding sites in pMMO, product toxicity, or both. To determine if competition for pMMO binding sites was responsible for the measured growth inhibition on methane, *M. album* BG8 was also grown on methanol in the presence of these halogenated hydrocarbons. As shown in Fig. 1, growth of *M. album* BG8 on methanol was clearly affected by many these compounds. DCM and 1,1-DCE completely inhibited growth on methanol, while VC and TCE reduced the extent of growth over 60 h by 44 and 10%, respectively. Furthermore, DCM, 1,1-DCE, VC, and TCE were degraded and followed hyperbolic kinetics, indicating that product toxicity and not substrate toxicity was responsible for growth inhibition. Such a conclusion is supported by the finding of Dolan and McCarty (1995) that the transformation product of 1,1-DCE is highly toxic to a mixed methanotrophic culture. This finding is supported by assays of reduced whole-cell pMMO activity after incubation of *M. album* BG8 with these compounds for 5 h and subsequent removal (Table 2). Since growth on both methanol and methane was affected, apparently

the transformation products can inhibit other enzymatic processes as well as the pMMO. The products of VC and TCE oxidation, however, appear to predominantly inhibit pMMO activity since growth on methane was more strongly affected.

Of the other compounds, CF and BF also completely inhibited growth on both methane and methanol and also substantially inhibited pMMO activity. These compounds, however, were not measurably degraded by *M. album* BG8. Thus, it is likely that these compounds themselves were toxic to the cells. DBM had no effect on growth with methane or methanol, suggesting that neither DBM nor its oxidative products were toxic to *M. album* BG8. 1,1,1-TCA reduced growth on methanol by 12%, but had no effect on growth on methane. It is unclear why growth on methanol was affected by 1,1,1-TCA since 1,1,1-TCA was not seen to be degraded. *trans*-DCE was not found to inhibit growth on methane after 60 h, but the products of oxidation did inhibit pMMO activity as shown in Table 2, and also growth on methanol was reduced by approximately 30% in the presence of *trans*-DCE. It is possible that the products of *trans*-DCE oxidation are inhibitory, but not as significantly as the products of TCE, VC, and 1,1-DCE oxidation. Finally, *cis*-DCE was toxic to cells grown on methane, but had little effect on cells grown with methanol. This suggests that *cis*-DCE or its products selectively inhibit pMMO activity. This result is supported by the finding that after incubating *M. album* BG8 for 5 h with 5 μ M *cis*-DCE, no methane consumption could be measured, indicating that pMMO was inactivated.

Not surprisingly, the apparent V_{\max} values found for halogenated hydrocarbons degraded readily by pMMO in *M. album* BG8 in this study were smaller than the V_{\max} measured for methane (Table 1). Comparison of the K_s values to the value for methane, however, reveals a different pattern. The apparent K_s values of all compounds with

the exception of 1,1-DCE and *cis*-DCE were greater than the apparent K_s value for methane by *M. album* BG8. These two compounds apparently are bound much better to pMMO than the primary substrate, methane. Although this result is unusual, it is not unprecedented. The ammonia monooxygenase (AMO), an enzyme structurally and genetically similar to pMMO, also was found to bind some chlorinated hydrocarbons greater than its primary substrate, ammonia. Using whole cells of *Nitrosomonas europaea*, Ely et al. (1997) have discovered that the relative affinity of whole cells expressing AMO is greater for both 1,1-DCE and TCE than it is for ammonia. Furthermore, kinetic analysis of whole-cell degradation of chlorinated hydrocarbons by *M. trichosporium* OB3b expressing sMMO indicates that whole-cell affinity for both 1,1-DCE and *cis*-DCE, and CF and DCM is better than that for methane (Oldenhuis et al. 1991). Although some chlorinated hydrocarbons apparently bind more strongly to pMMO in whole cells, the pseudo-first-order rate constant (V_{\max}/K_s) for all of the degraded compounds was much less than that measured for methane (Table 1), indicating that in whole cells the preferred substrate is methane. Furthermore, the lower pseudo-first-order rate constant and larger K_s for DBM degradation relative to that of DCM suggests that the larger size of bromine interferes with the effective transformation of DBM.

Although the authors are aware of no previous studies that have measured the Michaelis-Menten kinetic parameters of V_{\max} and K_s for many of the chlorinated and brominated hydrocarbons examined here, one earlier study has measured the pseudo-first-order rate constants of whole-cell degradation of chlorinated methanes and ethanes by *M. trichosporium* OB3b expressing pMMO (Van Hylckama Vleig et al. 1996). As shown in Table 3, values of pseudo-first-order constants for halogenated compounds by pMMO in *M. album* BG8 found here were lower than those reported in *M. trichosporium* OB3b ex-

Table 3 Comparison of pseudo-first-order constants of halogenated hydrocarbon degradation by methanotrophs known to be expressing soluble methane monooxygenase (sMMO) or particulate methane monooxygenase (pMMO) (DCM dichloromethane, DBM dibromomethane, CF chloroform, BF bromoform, VC vinyl chlo-

ride, *trans*-DCE *trans*-dichloroethylene, *cis*-DCE *cis*-dichloroethylene, 1,1-DCE 1,1-dichloroethylene, TCE trichloroethylene, 1,1,1-TCA 1,1,1-trichloroethane, NM no measurable degradation, and ND not determined)

Compound	<i>Methylosinus trichosporium</i> OB3b expressing sMMO (Van Hylckama Vleig et al. 1996) [ml min ⁻¹ (mg protein) ⁻¹]	<i>Methylosinus trichosporium</i> OB3b expressing pMMO (Van Hylckama Vleig et al. 1996) [ml min ⁻¹ (mg protein) ⁻¹] ^a	<i>Methylomicrobium album</i> BG8 expressing pMMO (This study) [ml min ⁻¹ (mg protein) ⁻¹] ^a
DCM	16	1.4	0.45
DBM	ND	ND	0.26
CF	2.6	0.06	NM
BF	ND	ND	NM
VC	15.2	3.8	0.23
<i>trans</i> -DCE	6.6	1.8	0.72
<i>cis</i> -DCE	9.8	0.12	0.15
1,1-DCE	6.4	< 0.06	0.092
TCE	6.2	< 0.06	0.072
1,1,1-TCA	ND	ND	NM

^aConverted from reported units of ml min⁻¹ (mg cells)⁻¹ assuming cell mass is 50% protein

pressing pMMO. It is unknown why the rates for *M. trichosporium* OB3b were so much greater, in some cases over an order of magnitude greater, but for the commonly tested substrates, both strains showed faster rates of transformation for DCM, VC, and *trans*-DCE. Regardless of the difference in rates found between methanotrophs expressing pMMO, it is clear from the data presented here and elsewhere that pMMO rates of degradation of halogenated hydrocarbons are slower than the rate in cells expressing sMMO. The affinity of pMMO for many of these compounds (particularly the chlorinated ethylenes), however, is typically much better than that measured for methanotrophs expressing sMMO (Van Hylckama Vlieg et al. 1996). This supports the use of cells expressing pMMO in certain situations where concentrations may be low although the overall rate of degradation will be low. Based on the kinetics of chlorinated ethylene degradation at a field demonstration site at Moffett Naval Air Station (Mountain View, CA, USA), it has now been suggested that pMMO was actually being expressed by the in situ methanotrophic community (McCarty 1997). This indicates that whole-cell pMMO-mediated degradation can be stimulated with the provision of limiting growth substrates.

The results of the effects of these compounds on growth, on pMMO activity, and on the measured Michaelis-Menten parameters of apparent V_{\max} and K_s indicate that the halogenated hydrocarbons can be divided into four groups: (I) not biodegradable with little inactivation, (II) biodegradable with little inactivation, (III) biodegradable with substantial inactivation, and (IV) not biodegradable but with substantial inactivation of cell activity. This characterization was first proposed by Rasche et al. (1991) for the AMO of *N. europaea*. Since AMO and pMMO are quite similar, it is interesting to compare our findings to those of *N. europaea*, as shown in Table 2. The units reported in the AMO study have been converted from μmol to μM to facilitate comparison using the Henry's constants reported earlier in Materials and methods. All five of the chlorinated ethenes assayed in *M. album* BG8 and *N. europaea* were classified into the same group (III). Of the two other common compounds tested, DCM was found to inhibit whole-cell pMMO activity less significantly than whole-cell AMO activity (22 vs 40%, respectively) and, thus, was classified as a group II compound while it is considered a group III compound for *N. europaea*. This may partly be due to the fact that in the experiments performed here, *M. album* BG8 was incubated for 5 h in the presence of 100 μM DCM as opposed to *N. europaea* (exposed to 1900 μM for 1 h). 1,1,1-TCA was considered to be a group I compound for *M. album* BG8 since it was not degraded and showed little inactivation. In *N. europaea*, however, it was classified as a group III compound since it was degraded and significantly affected whole-cell AMO activity. AMO is apparently able to oxidize 1,1,1-TCA while pMMO does not, and the products of oxidation are toxic. Finally, we discovered a fourth group of halogenated hydrocarbons, CF and BF, that were not measurably oxidized by pMMO-expressing

cells but that prevented any growth on both methane and methanol and also significantly reduced whole-cell pMMO activity. This suggests that these compounds themselves are toxic to the cells and that such toxicity is not specific to pMMO, but rather is more general and may affect multiple enzymatic processes. Product toxicity, however, cannot be excluded since some small amount of substrate may have been oxidized into toxic product(s). Such uncertainty is apparent when one considers that previous research is unclear on the ability of cells expressing pMMO to degrade CF. One early study has indicated that CF can be degraded (Oldenhuis et al. 1989), but a more recent one has reported that the rate is less than the detection limit (Van Hylckama Vlieg et al. 1996). It has also been shown that CF is degraded by *N. europaea* and is toxic to these cells, with toxicity not limited to inhibition of AMO (Ely et al. 1997). It is possible that cells expressing pMMO can degrade trihalomethanes with the formation of nonspecific inhibitory products, but the appropriate conditions are uncertain and further research should be performed to determine if and when methanotrophs expressing pMMO can degrade CF and BF.

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References

- Arp DJ (1995) Understanding the diversity of trichloroethylene co-oxidations. *Curr Opin Biotechnol* 6:352–358
- Benstead J, King GM, Williams HG (1998) Methanol promotes atmospheric methane oxidation by methanotrophic cultures and soils. *Appl Environ Microbiol* 64:1091–1098
- Bouwer EJ, McCarty PL (1983) Transformation of 1- and 2-carbon halogenated aliphatic compounds under methanogenic condition. *Appl Environ Microbiol* 45:1286–1294
- Cardy DLN, Laidler V, Salmond GPC, Murrell JC (1991) Molecular analysis of the methane monooxygenase (MMO) gene cluster of *Methylosinus trichosporium* OB3b. *J Gen Microbiol* 5:335–342
- Chang HL, Alvarez-Cohen L (1996) Biodegradation of individual and multiple chlorinated aliphatic hydrocarbons by methane-oxidizing cultures. *Appl Environ Microbiol* 62:3371–3377
- DiStefano TD, Gossett JM, Zinder SH (1991) Reductive dechlorination of high concentrations of tetrachloroethene to ethene by an anaerobic enrichment culture in the absence of methanogenesis. *Appl Environ Microbiol* 57:2287–2292
- Dolan ME, McCarty PL (1995) Methanotrophic chloroethene transformation capacities and 1,1-dichloroethene transformation product toxicities. *Environ Sci Technol* 29: 2741–2747
- Ely RL, Williamson KJ, Hyman MR, Arp DJ (1997) Cometabolism of chlorinated solvents by nitrifying bacteria: kinetics, substrate interactions, toxicity effects, and bacterial response. *Biotech Bioeng* 54:520–534
- Ensley BD (1991) Biochemical diversity of trichloroethylene metabolism. *Annu Rev Microbiol* 45:283–299
- Fox BG, Borneman JG, Wackett LP, Lipscomb JD (1990) Haloalkene oxidation by soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental application. *Biochemistry* 29:6419–6427
- Gossett JM (1987) Measurement of Henry's law constants for C1 and C2 chlorinated hydrocarbon. *Environ Sci Technol* 21: 202–208
- Hanson RS, Hanson TE (1996) Methanotrophic bacteria. *Microbiol Rev* 60:439–471

- Janssen DB, Grobben G, Hoekstra R, Oldenhuis R, Witholt B (1988) Degradation of *trans*-1, 2-dichloroethene by mixed cultures of methanotrophic bacteria. *Appl Environ Biotechnol* 29:392–399
- Lontoh S, Semrau JD (1998) Methane and trichloroethylene degradation by *Methylosinus trichosporium* OB3b expressing particulate methane monooxygenase. *Appl Environ Microbiol* 64: 1106–1114
- McCarty PL (1997) Aerobic cometabolism of chlorinated aliphatic hydrocarbons. In: Ward CH, Cherry JA, Scaif MR (eds) Subsurface restoration. Ann Arbor Press, Chelsea, MI, pp 373–395
- McTavish H, Fuchs JA, Hooper AB (1993) Sequence of the gene coding for ammonia monooxygenase in *Nitrosomonas europaea*. *J Bacteriol* 175:2436–2444
- Morel FM, Hering JG (1991) Principles and applications of aquatic chemistry. Wiley, New York
- Oldenhuis R, Vink RLJM, Janssen DB, Witholt B (1989) Degradation of chlorinated aliphatic hydrocarbon by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. *Appl Environ Microbiol* 55:2819–2826
- Oldenhuis R, Oedzes JY, Van der Waarde JJ, Janssen DB (1991) Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. *Appl Environ Microbiol* 57:7–14
- Prior SD, Dalton H (1985) Acetylene as a suicide substrate and active site probe for methane monooxygenase from *Methylococcus capsulatus* (Bath). *FEMS Microbiol Lett* 29:105–109
- Rasche ME, Hyman MR, Arp DJ (1991) Factors limiting aliphatic chlorocarbon degradation by *Nitrosomonas europaea*: cometabolic inactivation of ammonia monooxygenase and substrate specificity. *Appl Environ Microbiol* 57:2986–2994
- Rosenzweig AC, Frederick CA, Lippard SJ, Nordlund P (1993) Crystal structure of a bacterial non-haem iron hydroxylase that catalyses the biological oxidation of methane. *Nature* 366:537–43
- Semprini L (1997) In situ transformation of halogenated aliphatic compounds under anaerobic conditions. In: Ward CH, Cherry JA, Scaif MR (eds) Subsurface restoration. Ann Arbor Press, Chelsea, Mich., pp 429–450
- Semrau JD, et al (1995) Particulate methane monooxygenase genes in methanotrophs. *J Bacteriol* 177:3071–3079
- Stirling DI, Dalton H (1977) Effect of metal-binding agents and other compounds on methane oxidation by two strains of *Methylococcus capsulatus*. *Arch Microbiol* 114:71–76
- Tse G, Orbey H, Sandler SI (1992) Infinite dilution activity coefficients and Henry's law coefficients of some priority water pollutants determined by a relative gas chromatographic method. *Environ Sci Technol* 26:2017–2022
- Van Hylckama Vlieg JET, De Koning W, Janssen DB (1996) Transformation kinetics of chlorinated ethenes by *Methylosinus trichosporium* OB3b and detection of unstable epoxides by on-line gas chromatography. *Appl Environ Microbiol* 62: 3304–3312
- Vogel TM, McCarty PL (1985) Biotransformation of tetrachloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. *Appl Environ Microbiol* 49:1080–1083
- Wackett LP (1995) Bacterial co-metabolism of halogenated organic compounds. In: Young YL, Cerniglia CE (ed) Microbial transformation and degradation of toxic organic chemicals. Wiley-Liss, New York, pp 217–241
- Westrick JJ, Mello JW, Thomas RF (1984) The groundwater supply survey. *J Am Water Works Assoc* 76:52–59
- Whittenbury RK, Philips KD, Wilkinson JF (1970) Enrichment, isolation and some properties of methane-utilizing bacteria. *J Gen Microbiol* 61:205–218