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Transformation Yields of Chlorinated Ethenes by a Methanotrophic Mixed Culture Expressing Particulate Methane Monooxygenase

JAMES E. ANDERSON AND PERRY L. MCCARTY*

Department of Civil Engineering, Stanford University, Stanford, California 94305-4020

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Transformation yields for the aerobic cometabolic degradation of five chlorinated ethenes were determined by using a methanotrophic mixed culture expressing particulate methane monooxygenase (pMMO). Transformation yields (expressed as moles of chlorinated ethene degraded per mole of methane consumed) were 0.57, 0.25, 0.058, 0.0019, and 0.00022 for *trans*-1,2-dichloroethylene (t-DCE), vinyl chloride (VC), *cis*-1,2-dichloroethylene (c-DCE), trichloroethylene (TCE), and 1,1-dichloroethylene (1,1-DCE), respectively. Degradation of t-DCE and VC was observed only in the presence of formate or methane, sources of reducing energy necessary for cometabolism. The t-DCE and VC transformation yields represented 35 and 15%, respectively, of the theoretical maximum yields, based on reducing-energy availability from methane dissimilation to carbon dioxide, exclusive of all other processes that require reducing energy. The yields for t-DCE and VC were 20 times greater than the yields reported by others for cells expressing soluble methane monooxygenase (sMMO). Transformation yields for c-DCE, TCE, and 1,1-DCE were similar to or less than those for cultures expressing sMMO. Although methanotrophic biotreatment systems have typically been designed to incorporate cultures expressing sMMO, these results suggest that pMMO expression may be highly advantageous for degradation of t-DCE or VC. It may also be much easier to maintain pMMO expression in treatment systems, because pMMO is expressed by all methanotrophs whereas sMMO is expressed only by type II methanotrophs under copper-limited conditions.

Aerobic biodegradation of all chlorinated ethenes except tetrachloroethylene (PCE) can result through cometabolism by bacteria that produce oxygenase enzymes. Effective oxygenase-inducing substrates for this purpose include methane (38), propane (36), phenol and toluene (26), and ammonia (32). The cometabolic activity of methane-oxidizing bacteria (methanotrophs) has been studied perhaps the most extensively. All methanotrophic bacteria produce the copper-containing particulate methane monooxygenase (pMMO). However, under copper-limited conditions, some methanotrophs express instead the soluble MMO (sMMO). Both pMMO and sMMO are capable of chlorinated aliphatic hydrocarbon (CAH) oxidation, but sMMO is much more nonspecific with respect to potential substrates and can rapidly oxidize all the chlorinated ethenes except PCE (18). On the other hand, pMMO has a 10- to 100-fold-higher specificity for methane (25) but generally oxidizes trichloroethylene (TCE) and *cis*-1,2-dichloroethylene (c-DCE) at a much lower rate than sMMO does (13, 14, 29). *trans*-1,2-Dichloroethylene (t-DCE), however, is oxidized at rates similar to that by sMMO (6, 23, 29).

Because both CAHs and methane are oxidized by MMO (Fig. 1), competitive inhibition of methane utilization by CAHs (8, 34) and inhibition of CAH oxidation by methane (8–10, 22, 28, 34) have been observed. The products of CAH oxidation are toxic to the organisms (2, 21, 28, 37), with such transformation product toxicity varying greatly among CAHs (14). Oxidation of both methane and CAH by MMO requires molecular oxygen and reducing energy from the cofactor NADH (24). The extent of observed CAH cometabolism is thus finite (2, 28) and is limited by any or all of these processes (12).

The transformation yield (T_y, expressed as moles of CAH per mole of methane) has been used to characterize this finite value and is defined as the mass of CAH degraded per unit mass of growth substrate consumed. As used here, the latter includes both the methane initially used to grow the organisms and that consumed (if any) during the CAH degradation phase. Upper values for T_y of various CAHs for methanotrophs as estimated from information provided in the literature or reported are summarized in Table 1. With sMMO, T_y was generally higher when formate was added, because this ensured that CAH cometabolism was not reducing-energy limited.

Cultures believed to have expressed pMMO exhibited significantly greater transformation yields for c-DCE, t-DCE, and vinyl chloride (VC) than did cultures expressing sMMO (Table 1), even when the latter had formate present. Although pMMO expression was not explicitly determined in these studies, the experimental conditions (high copper-to-biomass ratio) would be expected to induce pMMO expression (6, 29, 35).

By using methane-stimulated soil column microcosms in our laboratory (15), T_y values of 0.26 and 0.90 mol of VC/mol of methane were observed. In mixed-culture experiments, we also found T_y of up to 0.63 mol of t-DCE/mol of methane (3). These results contradict the general assumption that sMMO expression is better for CAH oxidation than is pMMO expression. Indeed, these T_y values appear to approach a theoretical upper limit to T_y as governed by reducing-energy availability. As indicated in Fig. 1, of the 2.64 mol of NADH formed from methane oxidation, 1 mol is used for methane oxidation, leaving only 1.64 mol as a maximum that might be used for CAH oxidation. More definitive studies on T_y with pMMO and their relationship to the theoretical yields are presented here.

MATERIALS AND METHODS

Chemicals. The methane used was 99.0% pure (Liquid Carbonic Specialty Gas Co., San Carlos, Calif.). Saturated solutions of CAHs in deionized water were used for addition of all CAHs except VC. TCE (≥99% pure), t-DCE (98%), and

* Corresponding author. Mailing address: Department of Civil Engineering, Stanford University, Stanford, CA 94305-4020. Phone: (415) 723-4131. Fax: (415) 725-9474.

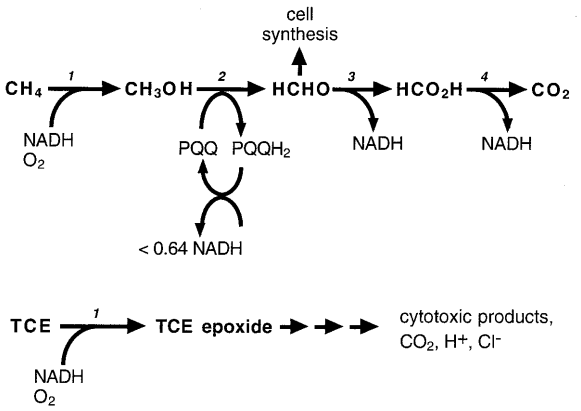

FIG. 1. Methane and TCE oxidation pathways in methane-oxidizing bacteria. Enzymes: *1*, MMO; *2*, methanol dehydrogenase; *3*, formaldehyde dehydrogenase; *4*, formate dehydrogenase.

c-DCE (97%) were obtained from Aldrich Chemical Co., Milwaukee, Wis. Dilutions of 1,1-DCE (neat standard for Environmental Protection Agency methods [Sigma Chemical Co., St. Louis, Mo.]) saturated solutions were prepared with mineral medium. VC gas (99.5% [Fluka Chemicals, Buchs, Switzerland]) was added directly to the bottles.

Mineral medium and sample bottle preparation. Mineral medium, prepared as described elsewhere (5), contained inorganic nutrients including 0.074 μM copper (17), with the addition of 1.0 mg of sodium thiosulfate per liter and 1.0 mM sodium bicarbonate, with the latter added after autoclaving and cooling. Glass bottles (250 ml) contained inverted 15-ml test tubes (Kimax) for methane addition. The bottles were partly filled with mineral medium and autoclaved for 25 min at 121°C and 2 atm. The liquid volume was then adjusted with autoclaved mineral medium to achieve the desired headspace volume.

The bacterial inoculum was added just prior to the start of an experiment, with controls receiving either 220 mg of sodium azide per liter or no inoculum. Methane and VC were added by means of a Pressure-Lok valved gas-tight syringe (Precision Sampling, Baton Rouge, La.) with a specially designed J-shaped needle to dispense gas up into the medium-filled test tube. CAH solution was injected immediately before the bottles were sealed either with two Teflon-lined septa and an open-hole closure or with a Teflon-lined Mininert cap. The bottles were then incubated in the dark at 20°C with rotary shaking at 150 rpm.

Bacterial inoculum. The source of the bacterial inoculum was frozen aliquots (5) of a methanotrophic mixed culture from a continuous-culture growth reactor (1). The dominant methanotrophic organism in this mixed culture showed 92% 16S rRNA homology to *Methylosinus trichosporium* OB3b and expressed sMMO under the copper-limited conditions maintained in the growth reactor (1). Several days prior to an experiment, an aliquot was thawed and enriched with methane in dilute culture and mineral medium containing copper to induce pMMO expression and repress sMMO (29, 35).

Small-inoculum experiments. A bottle containing 230 ml of mineral medium and 16 ml of headspace was autoclaved, cooled overnight, and inoculated with 0.5 ml of a 0.3% dilution of the frozen mixed culture and 0.5 ml of methane. After the cells multiplied and completely removed the methane (ca. 7 days), 0.5 ml of a 6% dilution of these pregrown cells, now expressing pMMO, was added to each sample bottle. Due to expected variability in T_y for different CAHs, two different procedures for sample bottle preparation were followed. For CAHs found to have low T_y values in preliminary experiments (TCE and 1,1-DCE), sample bottles were prepared with 100 ± 1 ml of medium, 146 ± 1 ml of headspace, 5 to 15 ml of methane, and relatively low CAH concentrations. Of the total mass of each compound in the bottle, 2.3% of the methane, 70% of the TCE, and 44% of the 1,1-DCE were present in solution in equilibrium with the headspace. For CAHs with higher T_y values (t-DCE, c-DCE, and VC), sample bottles contained 230 ± 1 ml of medium, 16.0 ml ± 0.1 ml of headspace, 0.5 to 1.5 ml of methane, and higher CAH concentrations. With the smaller headspace volume, 34% of the methane and 94 to 99% of the CAHs were in solution. Resulting initial aqueous methane and CAH concentrations are listed in Table 2.

A few duplicate bottles were sealed with Mininert valves and periodically sampled to monitor methane utilization and CAH removal. Approximately 14 days after cessation of activity in these bottles (82 days), all the remaining bottles were sampled for analysis of methane, CAH, t-DCE epoxide in the case of t-DCE, and oxygen. Transformation yields were calculated as the mass of CAH removed divided by the mass of methane removed. Values were calculated only for bottles satisfying the following criteria: (i) final methane and CAH concen-

TABLE 1. Reported chlorinated T_y values

CAH	Maximum observed T_y (mol of CAH/mol of methane)		Maximum aqueous concn of:		Reference
	No formate	Formate[a]	Methane (μM)	CAH (μM)	
Cultures expressing sMMO					
TCE	0.0015	0.0031		120	2
TCE		0.017		45	28
TCE	0.0020	0.0041		120	10
TCE	0.0040	0.0048		270	14
TCE[b]	0.0053		75	150	16
TCE[b]	0.0075		60	150	31
TCE		0.022		48	11[c]
1,1-DCE	0.00044	0.00074		8.1	14
1,1-DCE		0.0020		8.0	11
c-DCE	0.0050	0.020		480	14
c-DCE		0.031		72	11
t-DCE	0.0051	0.027		620	14
t-DCE		0.042		51	11
VC	0.0072	0.013		240	14
VC[b]	0.0066		6.3	2.2	27
VC		0.058		16	11
Cultures expressing pMMO[d]					
TCE[b]	0.0041		4.7	7.0	6
c-DCE[b]	0.025		4.7	28	6
t-DCE[b]	0.039		4.7	0.6	6
t-DCE	0.063		40	100	23
t-DCE	0.63		3.1	20	3
VC[e]	0.26, 0.90		250	280	15

[a] Initial formate concentrations were 10 or 20 mM.
[b] Obtained with an attached-film bioreactor.
[c] Values from reference 11 are the maximum value of four methanotrophic cultures.
[d] Not expressly determined, but believed the case due to the conditions used.
[e] Obtained with small-column soil microcosms.

TABLE 2. Summary of chlorinated ethene transformation yields and experimental conditions

CAH	Maximum observed T_y (mol of CAH/mol of methane)	Aqueous concn (μM)[a] of:	
		Methane	CAH
Small-inoculum experiments			
TCE	0.0019	**50,** 100, 150	3.1, 6.3, **13**
1,1-DCE	0.00022	**50,** 100, 150	0.11, 0.22, **0.56**
c-DCE	0.058	**30,** 60, 90	21, **86,** 170
t-DCE	0.57	**30,** 60, 90	20, 79, **160**
VC	0.20	**30,** 60, 90	**17,** 86, 340
Large-inoculum experiments			
t-DCE	0.45 ± 0.03[b]	0,[c] 15,[c] 30	0, 160
		0,[c] 3,[c] 15	51
		0,[c] 15, 30,[c] **60,** 90	**160**
VC	0.25	30, 60, 90	34, 86, 260
		0,[c] 90, **180**	**260**
Theoretical maximum[d]	1.64		

[a] Initial equilibrium aqueous concentrations used in the experiments. Values in boldface type are conditions under which the maximum observed T_y was obtained.
[b] 95% confidence interval calculated from triplicate live samples and controls.
[c] A subset of these bottles also received 0.1 and 1 mM sodium formate.
[d] See text.

trations significantly lower (with 95% confidence) than in controls, (ii) more than 5% of the initial CAH and oxygen remaining (14), and (iii) an insignificant t-DCE epoxide level present. The last two criteria ensure that CAH transformation was not limited by a deficiency of CAH, oxygen, or incubation time.

Large-inoculum experiments. All sample bottles, containing 230 ml of medium and 16 ml of headspace, autoclaved and cooled overnight, were inoculated with 0.5 ml of a 0.3% dilution of the frozen mixed culture and 0.25 ml of methane. After complete methane removal (about 8 days), the contents of all bottles were combined and gently mixed. The resulting solution was then redistributed to the bottles so that 16.0 ± 0.1 ml of headspace (and 230 ± 1 ml of medium) would remain in each. The initial active biomass concentration was estimated as 0.3 mg/l from comparison to model results that included growth and decay (5). Methane and CAHs were added to establish the initial concentrations shown in Table 2. Sodium formate was added (0.1 or 1 mM) to a subset of bottles.

Some bottles were sealed with Mininert valves and sampled daily. Initial methane and CAH removal rates were calculated from the first two data points, spanning an interval of 1 or 2 days, after normalizing concentrations to those in controls. Other bottles were sealed with two septa and an open-hole closure and were sampled only after 25 days. Transformation yields were calculated as the CAH mass removed divided by the total methane mass consumed, both during the pregrowth period and during CAH degradation. Criteria for reporting T_y values were the same as for the small-inoculum experiments, except that significant methane removal was not required during CAH degradation.

In the experiments reported here, excess oxygen was always present. At its lowest (i.e., with 180 μM methane), the initial ratio of total oxygen mass to total methane mass was approximately 2.9 mg of oxygen/mg of methane. This value is in the range of reported oxygen yield requirements for methanotrophic cultures (e.g., 1.4 to 3.2 mg of oxygen/mg of methane [16, 27]).

sMMO assay. Similar to the small-inoculum experiments, sample bottles containing 230 ml of medium and 16 ml of headspace, autoclaved and cooled overnight, were inoculated with 0.5 ml of a 0.3% dilution of the frozen mixed culture and 0.5 ml of methane. After the organisms grew and completely removed the added methane (approximately 6 days), the contents of each bottle were filtered through glass fiber filter paper. This yielded 200 μg of biomass. The filter paper was placed in a 1.5-ml clear-glass vial containing 0.50 ml of mineral medium and four crystals of solid naphthalene. The vial was vortexed for 30 s, and 0.10 ml of 2-mg/ml tetrazotized *o*-dianisidine was added. The vial was then vortexed for an additional 5 s. The resulting color of the filter paper was faint yellow, comparable to zero-sMMO controls.

Positive sMMO controls were obtained by filtering 0, 10, 20, 50, 100, or 200 μg of active methanotrophic mixed-culture cells from the chemostat described previously (1). Based on a prior analysis (4), only 20% of the culture was assumed to be active. The sMMO assay positive-control results were as follows: faint yellow for 0 μg of cells, light pink for 10 μg of cells, pink for 20 μg of cells, and light to dark purple for 50, 100, and 200 μg of cells, respectively. These results indicate that organisms expressing sMMO, if present, represented less than 5% of the total biomass in the experiments reported here, far too little to have had a significant effect on the experimental results obtained.

Analytical procedures. All samples were taken from the headspace (0.1 ml) with a 0.25-ml Pressure-Lok gas-tight syringe (Precision Sampling) with a side-port needle. Methane was analyzed on a Hewlett-Packard 5730A gas chromatograph with a 5-ft Supelco 60/80 Carbosieve 1/8-in.-diameter packed column at an oven temperature of 100°C with a flame ionization detector. TCE, t-DCE, the t-DCE oxidation product, and oxygen (0.1 ml of headspace) were analyzed on a Tracor MT-220 gas chromatograph with a 40 or 60°C packed column containing 10% squalene on Chromosorb A/AW and a linearized electron capture detector. VC, c-DCE, and 1,1-DCE were analyzed with a Carlo Erba Fractovap 2900 Series gas chromatograph with a photoionization detector and 60°C capillary column (J&W Scientific; length, 30 m; inner diameter, 0.53 mm) with a 3.0-μm DB-624 film. Solution concentrations were calculated with dimensionless 20°C Henry constants of 28.5 and 30.1 for methane and oxygen, respectively (30), and 0.299, 0.305, 0.124, 0.862, and 0.908 for TCE, t-DCE, c-DCE, 1,1-DCE, and VC, respectively (19). The t-DCE epoxide concentration, quantified by using the t-DCE response factor, was expressed as a fraction of the highest epoxide concentration measured over the course of a given experiment. Mass changes of CAHs and methane were determined from total changes in liquid and headspace masses.

RESULTS

Small-inoculum experiments. Initially, T_y values were determined with a small inoculum of cells. The quantity of methane added (21 to 630 μmol) was much greater than that used to grow the inoculum (0.0026 μmol), so that CAH degradation was due principally to the methane added at the time of CAH addition.

Methane and t-DCE aqueous concentration changes over a 82-day incubation period are shown in Fig. 2. Similar data were obtained for the other chlorinated ethenes. In general, methane removal began after 1 to 2 weeks and ceased after an additional period of a few days to a few weeks. CAH removal was evident only during the period of methane removal. A product of t-DCE oxidation, presumably t-DCE epoxide (14, 23), accumulated during t-DCE removal and then gradually disappeared.

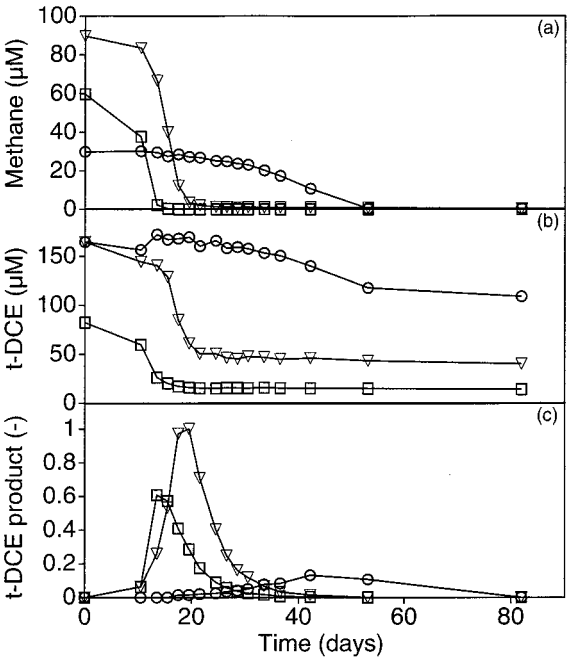

FIG. 2. Aqueous methane (a), aqueous t-DCE (b), and headspace t-DCE product (c) concentrations plotted against time in a small-inoculum experiment. Symbols: ○, 30 μM methane; □, 60 μM methane; ▽, 90 μM methane. Product concentrations are normalized to the highest peak area recorded for the t-DCE product.

The fractions of t-DCE remaining relative to controls (C/C_0) in bottles sampled only after day 82 are shown in Fig. 3a. Methane removal was greater than 99% in all bottles except that initially containing 30 μM methane and 160 μM t-DCE (91% removal). Because less than 5% t-DCE remained in bottles with 20 μM t-DCE, T_y values were thought to be CAH limited and thus were not reported. In general with all CAHs, the percent removals of methane and CAH increased with increasing methane concentration or decreasing CAH concentration.

The maximum T_y values obtained for all CAHs are summarized in Table 2. The highest T_y measured was 0.57 mol of t-DCE/mol of methane, obtained with 30 μM methane and 160 μM t-DCE. The maximum T_y values for all CAHs were obtained at the lowest methane and highest CAH concentrations used. This suggests that competitive inhibition by higher methane concentrations may have slowed CAH transformation rates and thus reduced T_y. On the other hand, CAH transformation rates increased with increasing CAH concentration, as expected from enzyme kinetics, and this may have increased the observed T_y as well.

At high VC concentrations (86 and 340 μM), neither methane nor VC was removed, perhaps because of competitive inhibition, reducing power availability and/or transformation product toxicity. However, with 17 μM VC, both methane and VC were completely removed. Overall, the highest observed T_y for VC was 0.20 mol of VC/mol of methane, obtained with 17

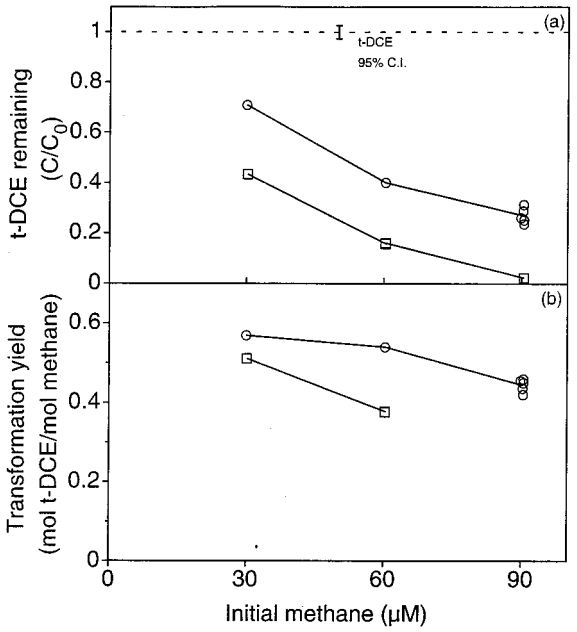

FIG. 3. t-DCE remaining as a fraction of controls (a) and t-DCE transformation yields (b) versus initial methane concentration after 82 days for small-inoculum experiments. Symbols: □, 80 μM initial t-DCE; ○, 160 μM initial t-DCE. Error bars indicate 95% confidence intervals (C.I.) for controls.

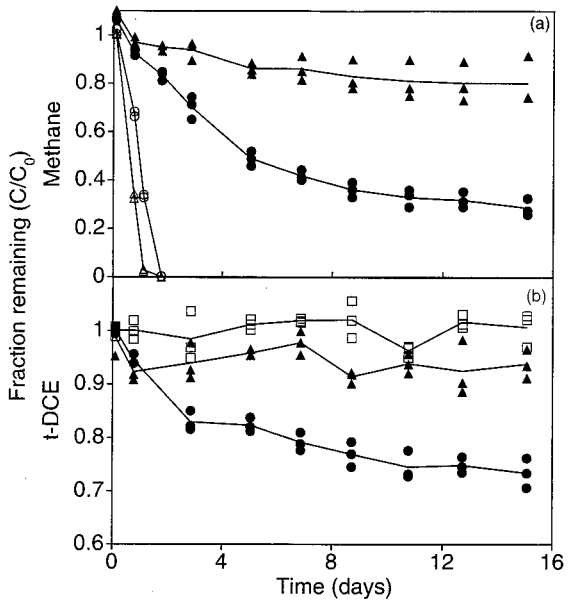

FIG. 4. Methane (a) and t-DCE (b) remaining as a fraction of controls plotted against time in a large-inoculum experiment. Initial concentrations: △, 15 μM methane and no t-DCE; ○, 30 μM methane and no t-DCE; ▲, 15 μM methane and 160 μM t-DCE; ●, 30 μM methane and 160 μM t-DCE; □, no methane and 160 μM t-DCE. Lines connect mean values.

μM VC and 30 μM methane. The low transformation yields with TCE and 1,1-DCE were as found previously (14).

Large-inoculum experiments. The large-inoculum experiments were used to obtain confirmation of the high yields for t-DCE and VC found in the small-inoculation experiments but under different growth conditions. However, with the large inoculum, the methane removal rate was greatly reduced in the presence of CAHs (Table 3). Here, while significant methane utilization was required to provide the reducing power needed for CAH oxidation, such methane removal was reduced significantly due to competitive inhibition at high CAH concentrations. This effect can be clearly seen in Fig. 4 with 160 μM t-DCE, where samples for analyses were obtained over the course of the experiment. Here, fractional removals of both methane and t-DCE were higher at the higher methane concentration of 30 μM compared with the lower concentration of 15 μM. It appears that there exists some combination of methane and CAH at which T_y would be maximal.

TABLE 3. Initial rates of methane and CAH removal in large-inoculum experiments

Methane concn[a] (μM)	Methane or CAH utilization rate (μmol/mg-day)[a]				
	No CAH	VC[b]			160 μM t-DCE
		34 μM	86 μM	260 μM	
Methane utilization					
15	152 ± 4				12 ± 1
30	155 ± 13	51 ± 1	24 ± 6	0 ± 3	40 ± 3
90	85 ± 3		15 ± 2	0 ± 6	
CAH utilization					
0		1 ± 1[c]	4 ± 7	0 ± 8	0 ± 14
15					60 ± 13
30		15 ± 1	16 ± 3	2 ± 5	44 ± 8
90			19 ± 1	19 ± 9	

[a] See column 1 for information on whether methane or CAH utilization is meant. Mean values and standard deviations for duplicate and triplicate bottles are shown.

[b] Initial equilibrium aqueous concentration.

Various combinations of methane and either VC or t-DCE were then used to determine conditions yielding the highest values for T_y. The combinations tried and the results obtained are listed in Table 2. Figure 5 is a summary of results illustrating how T_y as well as methane and CAH fractional degradation varied with the initial methane concentration. The maximum T_y of 0.45 mol/mol for t-DCE was found at an intermediate concentration of 60 μM methane, while that of 0.25 mol/mol for VC was found at the highest methane concentration of 180 μM.

To further investigate the various factors affecting transformation yield, a comparison of results with and without formate addition was made. The results are illustrated in Fig. 6. Formate addition increased T_y very little if methane was not present; apparently, methane is needed to induce pMMO production. However, with methane present, formate increased the T_y considerably, especially at the highest added concentration of 1 mM. At the lower concentration of 0.1 mM, formate increased the T_y at the lower methane concentration of 3 μM but offered little improvement when methane was present at 15 μM. Clearly, the results indicate that reducing-power availability is an important aspect in the extent of CAH oxidation but that enzyme induction is also required.

Comparison between large- and small-inoculum experiments. The maximum T_y values for both t-DCE and VC were essentially the same for the small- and large-inoculum experiments (Table 2). However, the combination of CAHs and methane concentrations under which the maximum values were obtained differed markedly. With the small inoculum, maximum values were found with the lowest methane concentrations (30 μM), while with the large inoculum, it was found

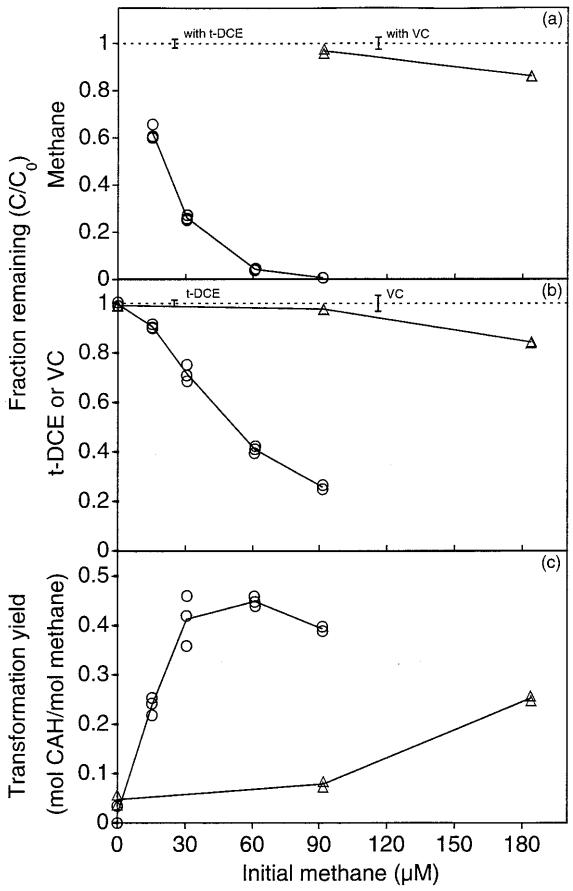

FIG. 5. Methane (a) and CAH (b) remaining as a fraction of controls and transformation yields (c) plotted against the initial methane concentration. Data for 160 μM initial t-DCE (○) or 260 μM initial VC (△) with a 25-day incubation for the large inoculum are shown. Error bars indicate 95% confidence intervals for controls.

with much higher methane concentrations (60 μM for t-DCE and 180 μM for VC). With VC, the lowest concentration gave higher T_y in the small-inoculum experiments, but the highest concentration worked best in the large-inoculum experiments. With t-DCE, the highest concentration worked best in both cases. To obtain maximum values for T_y, the balance between the factors of reducing-energy availability, competitive inhibition, and transformation product toxicity apparently needs to be correct. Perhaps the reason why others have not found such high values for T_y is that experimental conditions were not suitable for obtaining it.

DISCUSSION

The pMMO-expressing methanotrophic mixed culture used here exhibited transformation yields of 0.57, 0.25, 0.058, 0.0019, and 0.00022 mol of CAH/mol of methane for t-DCE, VC, c-DCE, TCE, and 1,1-DCE, respectively. The exceptionally high transformation yields for t-DCE and VC are 20 times greater than those reported for cultures expressing sMMO, even though these CAHs were rapidly degraded by cultures expressing either pMMO or sMMO (14, 23, 28, 29). However, CAH transformation by cultures expressing sMMO continued only for a few hours (14), suggesting a rapid loss of enzyme activity in the organisms as observed by others (2, 21). It is unlikely that the differences in T_y were due to a greater transformation product toxicity in cells expressing sMMO, because both pMMO and sMMO would be expected to produce the epoxide product. Also, excess reducing energy, as formate, was always present in T_y estimates for cultures expressing sMMO (14).

The question that remains is that of which factor(s) determines how large the transformation yields may be. Ultimately, reducing-energy availability, in the form of NADH, required for CAH transformation, must limit T_y to a finite value. Such a value can be estimated.

Criddle (12) suggested that the biochemistry and stoichiometry of electron flow during concurrent substrate utilization and cometabolism should be considered in cometabolism models. Chang and Alvarez-Cohen (9) explicitly accounted for reductant consumed via CAH oxidation and regenerated from endogenous and exogenous sources in a non-steady-state model but did not consider the NADH requirement for methane oxidation by MMO. Four electrons are required for the reduction of the oxygen used in the MMO reaction; two are obtained from NADH, and two are obtained from the methane or CAH transformed (33). Six electrons are recovered in subsequent steps of methane dissimilation (Fig. 1). Methanol dehydrogenase reduces pyrroloquinoline quinone (PQQ) to $PQQH_2$ (24), whereas formaldehyde dehydrogenase and formate dehydrogenase each regenerate NADH.

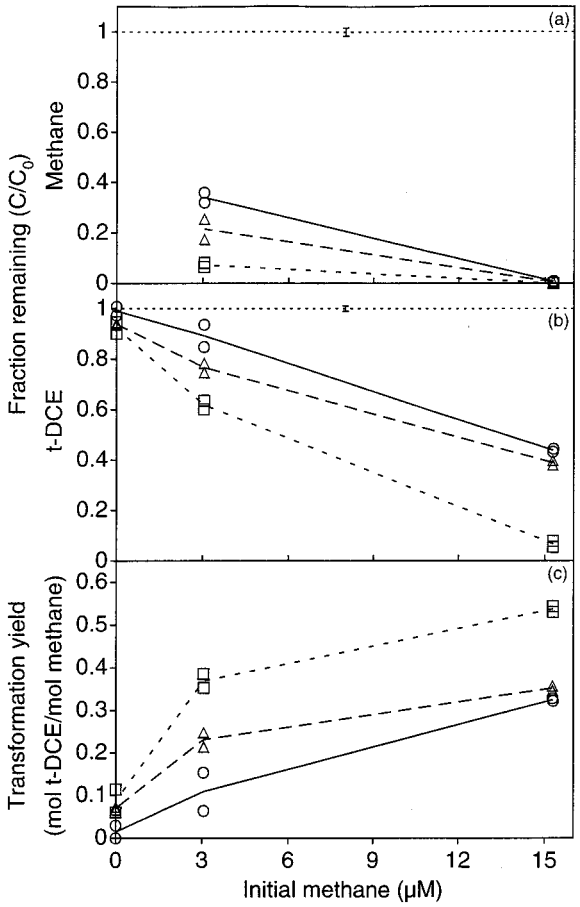

FIG. 6. Methane (a) and t-DCE (b) remaining as a fraction of controls and transformation yields (c) plotted against the initial methane concentration. Data for 50 μM initial t-DCE with a 25-day incubation for the large inoculum are shown. Symbols: ○, no formate; △, 0.1 mM formate; □, 1 mM formate. Error bars indicate 95% confidence intervals for controls.

Oxidation of $PQQH_2$ to PQQ yields approximately 140 kJ/mol, or less than 64% of the 220 kJ/mol resulting from the oxidation of NADH to NAD^+ (20). The true energy yield depends on prevailing physiological conditions and is not known. However, based on this estimate, a cell could theoretically recover at most 0.64 NADH equivalent from $PQQH_2$, for a net gain of 1.64 mol of NADH per mol of methane oxidized to carbon dioxide. Note that if the transfer of electrons from $PQQH_2$ to NADH is less than 100% efficient, which it undoubtedly is, less than 1.64 mol of NADH/mol of methane would be available. On the other hand, if methanol dehydrogenase could shuttle its two electrons directly to MMO (24), this value may be as high as 2 mol of NADH/mol of methane.

Assuming that methane oxidation yields a net of 1.64 mol of NADH, 1.64 mol of CAH could theoretically be oxidized per mol of methane consumed based solely on the NADH requirement for CAH oxidation, exclusive of all other cell processes. The measured T_y of 0.57 mol of t-DCE/mol of methane reported here thus equals 35% of this theoretical maximum, implying that 35% of the methane consumed was used to regenerate the NADH needed by MMO for t-DCE oxidation. The remainder of the methane may have been used as carbon for biosynthesis or to generate reducing energy needed for biosynthesis and respiration. Because such a large fraction of available NADH was shunted into t-DCE transformation, the resulting biomass yield should have decreased significantly.

The importance of reducing-energy limitation is further illustrated by calculation of NADH consumption and regeneration rates observed in the large-inoculum experiments. For example, for bottles incubated with 15 μM methane in the absence and presence of 160 μM t-DCE (Table 3), assuming 1.64 mol of NADH produced per mol of methane consumed, the rate of NADH consumption by t-DCE transformation would be three times greater than the rate of NADH production. As NADH is depleted, the rates of CAH and methane oxidation would decline proportionately (7, 9) and eventually would become zero (Fig. 6), because reducing energy is required for oxidation of both the growth substrate and the cometabolite. This phenomenon has also been reported by others with carbon monoxide as the cometabolite (22). Competitive inhibition by t-DCE would also be expected to reduce the methane utilization rate and thus further limit NADH availability. Finally, in the absence of methane, and thus without NADH production, t-DCE was not removed.

In earlier studies from our laboratory (15), we reported T_y values of 0.26 and 0.9 mol of VC/mol of methane in methane-fed microcosms. These high yields were thought to perhaps result from the presence of organisms having unusually high VC-cometabolizing abilities. However, the comparable yields, now found with quite a different laboratory mixed culture expressing pMMO, suggest that the results were due not to an exceptional culture but, rather, to the efficient conversion by pMMO under suitable environmental conditions of CAH and methane concentrations.

Past interests in the methanotrophic cometabolism of CAHs has generally been with organisms expressing sMMO as they degrade TCE at much higher rates than those expressing pMMO. This study demonstrates that pMMO can be more effective than sMMO for t-DCE and VC cometabolism. VC is often found as a groundwater contaminant and is of major concern because it is a known human carcinogen. Its presence generally results from the reductive dehalogenation of PCE and TCE, a formation that is generally accompanied by the production of high concentrations of methane. The results obtained here, as well as in the laboratory microcosms, indicate that such conditions are ideal for VC cometabolism if oxygen is introduced into the groundwater either by planned engineering or by natural diffusion from the atmosphere or from oxygen-bearing groundwater.

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