ELSEVIER

FEMS Microbiology Letters 186 (2000) 109–113

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Identification of intermediates of in vivo trichloroethylene oxidation by the membrane-associated methane monooxygenase

Sonny Lontoh [a], James A. Zahn [b,1], Alan A. DiSpirito [b], Jeremy D. Semrau [a,*]

[a] *Department of Civil and Environmental Engineering, The University of Michigan, 1351 Beal Avenue, Ann Arbor, MI 48109-2125, USA*
[b] *Department of Microbiology, Iowa State University, Ames, IA 50011-3211, USA*

Received 10 February 2000; received in revised form 8 March 2000; accepted 8 March 2000

Abstract

The rate and products of trichloroethylene (TCE) oxidation by *Methylomicrobium album* BG8 expressing membrane-associated methane monooxygenase (pMMO) were determined using ^{14}C radiotracer techniques. [^{14}C]TCE was degraded at a rate of 1.24 nmol (min mg protein)$^{-1}$ with the initial production of glyoxylate and then formate. Radiolabeled CO_2 was also found after incubating *M. album* BG8 for 5 h with [^{14}C]TCE. Experiments with purified pMMO from *Methylococcus capsulatus* Bath showed that TCE could be mineralized to CO_2 by pMMO. Oxygen uptake studies verified that *M. album* BG8 could oxidize glyoxylate and that pMMO was responsible for the oxidation based on acetylene inactivation studies. Here we propose a pathway of TCE oxidation by pMMO-expressing cells in which TCE is first converted to TCE-epoxide. The epoxide then spontaneously undergoes HCl elimination to form glyoxylate which can be further oxidized by pMMO to formate and CO_2.

Keywords: Trichloroethylene; Particulate methane monooxygenase; Bioremediation; Methanotroph

1. Introduction

Trichloroethylene (TCE), a suspected carcinogen [1,2], is one of the most common ground water pollutants in the USA from its extensive use as a solvent and degreaser [3]. Due to its prevalence in the environment and the health risk it poses, a great deal of research has been performed to enhance methods for TCE removal, including biodegradation. Anaerobic biotransformation of TCE proceeds through reductive dechlorination and produces the toxic agents dichloroethylene (DCE) and vinyl chloride (VC) [4,5]. Several aerobic bacteria including toluene oxidizers [6,7], ammonia oxidizers [8,9] and methane oxidizers [10–17] have also been shown to degrade TCE, but without the production of DCE or VC, and therefore can be useful alternatives. Since methane is non-toxic and methanotrophs are ubiquitous in the environment [18], these bacteria have been extensively examined for TCE bioremediation.

A cytoplasmic or soluble methane monooxygenase (sMMO) is expressed by some methanotrophs in copper-limited environments and degrades TCE with rates relatively fast compared to other bacteria [11,16]. The mechanism of TCE degradation has been well-studied and it is believed to be similar to that by rat liver microsomal cytochrome P-450 [11,19]. The initial products of TCE oxidation by sMMO are TCE-epoxide, a very reactive intermediate, and a small amount of chloral, a controlled substance and mutagen [11,14,18,20]. TCE-epoxide has a very short life of approximately 10–20 s, and can spontaneously undergo hydrolysis to form acyl halides that will further decompose into formate, CO, glyoxylic acid and dichloroacetic acid depending on the pH [11,19,21]. TCE-epoxide and acyl halides, however, can covalently bind to sMMO, causing inhibition [11,15]. Also, chloral is considered more toxic than TCE [14]. Therefore, the fate of TCE when oxidized by sMMO-expressing methanotrophs must be carefully monitored to optimize bioremediation.

Most methanotrophs cannot express sMMO, and instead constitutively express a membrane-associated or par-

---

* Corresponding author. Tel.: +1 (734) 764-6487; Fax: +1 (734) 763-2275; E-mail: jsemrau@engin.umich.edu

[1] Present address: Chemistry Research Technologies and Protein, Lilly Corporate Center, DC1533, Eli Lilly and Company, Indianapolis, IN 46285, USA.

0378-1097/00/$20.00 © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.
PII: S0378-1097(00)00127-0

ticulate methane monooxygenase (pMMO) [18,22] that can also degrade TCE [10,13,23]. However, the mechanism of TCE degradation either by the purified pMMO or in whole cells known to be expressing pMMO has not been examined. Therefore, in this study, the products of TCE oxidation by whole cells of *Methylomicrobium album* BG8 were determined to assess the usefulness of pMMO-expressing cells for TCE bioremediation. From these studies and experiments using purified pMMO from *Methylococcus capsulatus* Bath, a pathway of TCE oxidation by pMMO is proposed.

2. Materials and methods

2.1. *Materials*

All chemicals used in medium preparation were of reagent grade or better. Highest purity methane (> 99.99%, Matheson) was used for cell growth. [1,2-^{14}C]TCE (99.8%) was obtained from New England Nuclear. Milli-Q water from a Corning Millipore D2 system was used for all experiments.

2.2. *[^{14}C]TCE oxidation by M. album BG8 and product identification*

All procedures were performed aseptically. *M. album* BG8 (ATCC 33003), a methanotroph that can only express pMMO, was grown in nitrate mineral salts (NMS) medium [24] with 20 μM copper at 30°C as described previously [13]. After harvesting the cells at $OD_{600} = 0.8$, methane was removed and the cells then were diluted with prewarmed NMS containing 20 μM copper. 3-ml aliquots were transferred to 20-ml vials resulting in a final cell concentration of 0.08 mg protein ml^{-1}. 20 mM of sodium formate was then added and the vials capped with Teflon-coated butyl-rubber septa (Wheaton). Radiolabeled TCE was added to the vials from a bottle of TCE-saturated water stock solution (12.3 μCi l^{-1}) to obtain an aqueous concentration of 40 μM TCE. The vials were shaken at 30°C and 270 rpm. 20 μl of liquid samples were analyzed every hour for 5 h using an HPLC system connected to a Dionex 400 UV-Vis spectrophotometer and also an on-line Ramona-92 radioisotope detector in series with an Aminex HPX-87H column (Bio-Rad) using the protocol developed by Fox et al. [11]. A frit filter column was installed before the HPLC column to separate the cells from the eluent and also to stabilize the baselines of the detectors. The peak, background and monitoring smoothing times of the Ramona-92 were set at 60, 60 and 5 s respectively. The peaks of the radioisotopegram were identified by comparison with the UV chromatogram of authentic unlabeled standards based on retention times. The retention times of the standards based on absorbance at 210 nm were: trichloroethylene (12 min), dichloroacetic acid (16 min), glyoxylic acid (18 min), formate (25 min) and chloral hydrate (31 min). The radioisotope peaks were also used to quantify the amounts of TCE disappearance as well as product appearance.

2.3. *[^{14}C]CO$_2$ production and cell-associated ^{14}C*

One vial in the TCE degradation assay was used to trap [^{14}C]CO$_2$. At 5 h, 100 μl of 1 M NaOH was added into an inner glass tube through the septum. 15 min after adding the NaOH, the vial was unsealed and NaOH was analyzed with a Rackbeta 1219 scintillation counter (LKB Wallac). To determine if any products of TCE oxidation remained in the cells, cells were collected by centrifugation, washed three times and resuspended in phosphate buffer. Cell samples were then analyzed for cell-associated ^{14}C using the scintillation counter.

2.4. *Oxidation of intermediates of TCE degradation by M. album BG8*

To determine if any possible intermediates of TCE oxidation could be further oxidized by pMMO in whole cells, oxygen uptake assays were performed on acetylene-treated and untreated *M. album* BG8 as described previously in well-mixed isothermal oxygen uptake reactors [25]. Oxygen uptake was monitored for up to 10 min for both acetylene-treated and untreated cells in the presence of either 100 μM glyoxylate, dichloroacetic acid, or chloral.

2.5. *TCE oxidation by purified pMMO from M. capsulatus Bath*

pMMO was purified and TCE assays were performed as described previously [10,26]. Reaction mixtures contained 34 mg protein ml^{-1} with 800 nmol (1.57 μCi) [^{14}C]TCE in 10 mM MOPS (3-[*N*-morpholino]propanesulfonic acid) for an initial concentration of 268 μM. The pH was buffered at 7.4 and 35 mM duroquinol was added as the reductant. Vials were incubated at 35°C for up to 200 min. At pre-set times, the reaction was stopped by injection of 1 ml of pentane into the vials. Negative controls to monitor abiotic TCE disappearance were created by adding 1 ml acetylene (7.55 mM) to some vials.

2.6. *SDS–PAGE and phosphoimaging*

SDS-polyacrylamide slab gel electrophoresis was carried out on 14% gels. Reductants were not added to the buffers and the samples were incubated at room temperature 10–30 min prior to loading. Timed exposures to [^{14}C]TCE were carried out as described above using duroquinol as a reductant. Gels were dried and exposed for 3.5 days on a Molecular Imager System GS-363 (Bio-Rad, Hercules, CA).

3. Results

3.1. [14C]TCE oxidation by *M. album* BG8

As can be seen in Fig. 1, [^{14}C]TCE disappearance concomitantly occurred with the appearance of first [^{14}C]glyoxylate and then [^{14}C]formate as found using HPLC with coupled UV/Vis and radioisotope detectors. In the presence of 40 μM TCE, the initial rate of TCE degradation by *M. album* BG8 was 1.24 nmol (min mg protein)$^{-1}$, similar to previous results [23]. After 5 h of incubation, 17% of the added [^{14}C]TCE was degraded and a mass balance was performed to determine the fate of the oxidized TCE. Of the degraded [^{14}C]TCE, 28% of the radiolabeled carbon was found as glyoxylate, 7% as formate, 19% as CO_2 and 46% in the washed cell pellet for a complete mass balance on ^{14}C. Chloral was not found in these experiments with either the UV/Vis or radioisotope detectors.

3.2. Oxidation of possible intermediates of TCE degradation by *M. album* BG8

Oxygen uptake assays were performed to determine if any possible intermediates from TCE degradation could be further oxidized by pMMO in vivo. In the absence of any substrate, endogenous respiration was measured at 6.8 ± 2.3 nmol O_2 (min mg protein)$^{-1}$. In the presence of 100 μM of glyoxylate, the rate of oxygen uptake by *M. album* BG8 was 31 ± 1.0 and 4.6 ± 0.3 nmol O_2 (min mg protein)$^{-1}$ in the absence and presence of acetylene respectively. In the presence of either 100 μM dichloroacetic acid or chloral, oxygen uptake rates were 9.8 ± 0.2 and 8.9 ± 0.5 nmol O_2 (min mg protein)$^{-1}$ respectively in the absence of acetylene. If the cells were treated with acetylene, the rate of oxygen uptake was found to be 10 ± 0.3 and 9.2 ± 0.9 nmol O_2 (min mg protein)$^{-1}$ in the presence of dichloroacetic acid and chloral respectively. As acetylene is a specific inhibitor of pMMO, these results show that pMMO could further oxidize one of the initial products of TCE oxidation, i.e., glyoxylate. Other possible products of TCE degradation, however, were not appreciably oxidized in vivo by either acetylene-treated or untreated cells of *M. album* BG8.

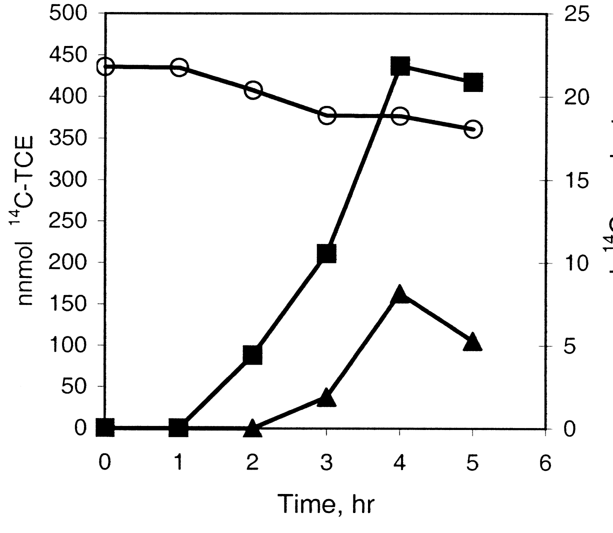

Fig. 1. Disappearance of [^{14}C]TCE (○) with appearance of [^{14}C]glyoxylate (■) and [^{14}C]formate (▲) in whole cell incubations of *M. album* BG8 with 40 μM [^{14}C]TCE.

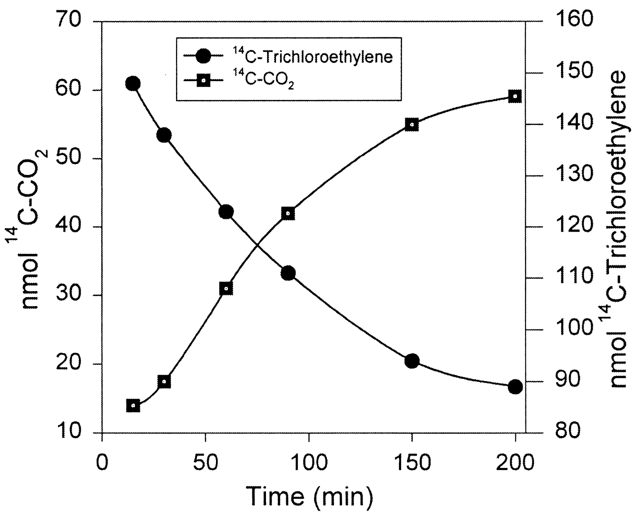

Fig. 2. [^{14}C]TCE disappearance and [^{14}C]CO_2 appearance in incubation of purified pMMO from *M. capsulatus* Bath with 268 μM [^{14}C]TCE.

3.3. TCE oxidation by purified pMMO from *M. capsulatus* Bath

To verify the whole cell experiments of TCE oxidation, TCE degradation by purified pMMO was also examined. As shown in Fig. 2, [^{14}C]TCE disappearance correlated with [^{14}C]CO_2 appearance, indicating that pMMO itself can completely mineralize TCE. After 200 min, 36.6% of TCE was oxidized with 20% of the oxidized TCE found as CO_2. Acetylene-treated pMMO did not degrade TCE (data not shown). SDS–PAGE and phosphoimaging of the purified pMMO incubated with [^{14}C]TCE for up to 200 min indicated that none of the pMMO polypeptides were radiolabeled (data not shown).

4. Discussion

Glyoxylate and formate were the only aqueous intermediates found from TCE oxidation by *M. album* BG8 expressing pMMO. Similar results were found in assays using *Pseudomonas mendocina* KR-1 expressing toluene monooxygenase [7] and *Pseudomonas putida* F1 expressing toluene dioxygenase [6]. From the observed product distribution and time of appearance, a hypothetical pathway of TCE oxidation is shown in Fig. 3. This pathway is

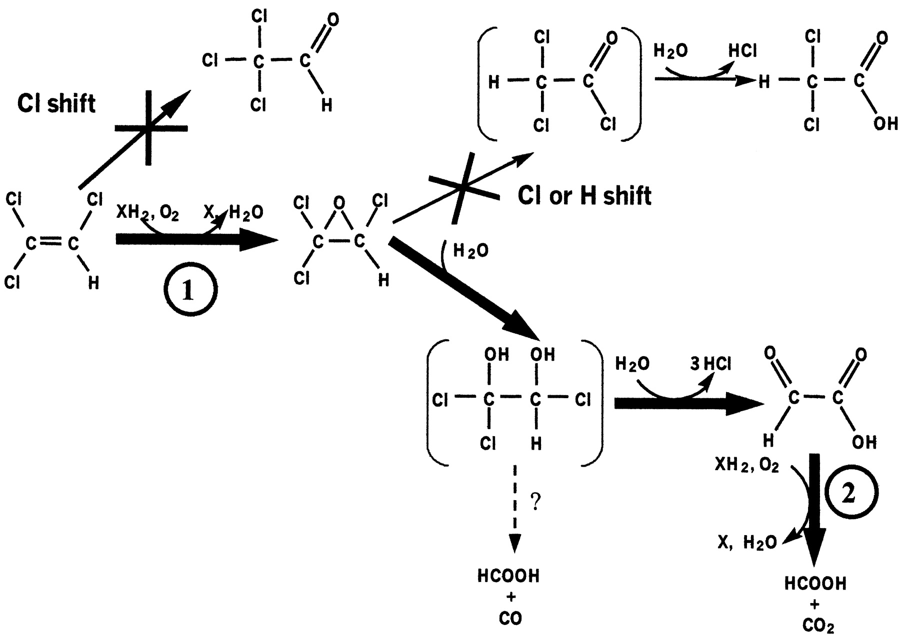

Fig. 3. A proposed pathway of TCE degradation by *M. album* BG8 expressing pMMO. Transformation steps 1 and 2 are catalyzed by pMMO. The predominant pathway is marked in bold, although C–C fission of the gem-halohydrin acyl halide to CO and formate is also possible. XH_2, X = reduced and oxidized forms of in vivo reductant, respectively.

similar to that proposed earlier for TCE oxidation by rat liver cytochrome P-450 [19] with some exceptions noted below.

Unlike studies both on purified sMMO and in whole cells expressing sMMO [11,14], chloral was not found as a product of TCE oxidation by *M. album* BG8, indicating that a chloride shift did not occur during pMMO oxidation of TCE. Oxygen uptake rates in whole cells of *M. album* BG8 incubated with chloral were slightly above that measured in the absence of any substrate, and these rates were not affected by the addition of acetylene. This suggests enzymes other than pMMO can oxidize chloral, but such rates are very slow and chloral could be expected to accumulate over time. Therefore, it appears that chloral is not a product of pMMO-mediated TCE oxidation. The absence of chloral may make TCE oxidation by pMMO attractive since, as noted earlier, chloral is considered to be more toxic than TCE.

The primary pathway of TCE oxidation by pMMO appears to be through the TCE-epoxide. The epoxide is unstable in solution and can form a variety of products. It has been suggested that a chloride or hydrogen shift can occur followed by the elimination of HCl to form dichloroacetic acid [19], and dichloroacetic acid has been discovered as a minor product of sMMO-mediated oxidation of TCE [11]. As found for cytochrome P-450 oxidation of TCE [19], in vivo pMMO oxidation of TCE resulted in no dichloroacetic acid formation, indicating that this pathway was not followed. As with chloral, slight rates of oxygen uptake were observed in *M. album* BG8 in the presence of dichloroacetic acid, and such rates were not affected by the addition of acetylene. It appears that enzymes other than pMMO can oxidize dichloroacetic acid, but such rates are slow. Thus, if dichloroacetic acid was being formed, it would be expected to accumulate over time. Therefore, the absence of dichloroacetic acid was most likely due to it not being produced from the oxidation of TCE.

Based on the observed product distribution, it appears that the TCE-epoxide was hydrated to form a gem-halohydrin acyl halide. This can undergo carbon–carbon fission and hydrolysis to form CO and formate [19]. This pathway cannot be ignored, but it appears that the gem-halohydrin acyl halide predominantly underwent extensive HCl elimination to form glyoxylate as this was the first and most abundant aqueous product. It also appears that glyoxylate was further oxidized to formate and CO_2 as formate appeared after glyoxylate. Unlike previous studies using whole cells, we were able to detect formate as a second product of TCE oxidation due to the addition of 20 mM formate to the incubations. Addition of such a high concentration of formate allowed the detection of any $[^{14}C]$formate as the formate dehydrogenase would preferentially oxidize the added formate to CO_2. This effectively trapped any formate made from $[^{14}C]$TCE oxidation. Oxygen uptake studies of acetylene-inhibited and uninhibited cells confirmed that pMMO in vivo can oxidize glyoxylate. Furthermore, experiments using purified pMMO showed that $[^{14}C]CO_2$ is made from $[^{14}C]$TCE, indicating that pMMO itself can completely mineralize TCE.

Approximately half of the added TCE in whole cell experiments with *M. album* BG8 was found associated with the cells. TCE-epoxide and acyl halides can covalently bind to cell components and labeling of several proteins was observed when *Methylosinus trichosporium* OB3b expressing sMMO was incubated with [^{14}C]TCE, including the α-subunit of the hydroxylase component of sMMO [15]. Studies with purified sMMO also showed that all polypeptides of sMMO were labeled when incubated with [^{14}C]TCE [11]. No labeling of the purified pMMO was observed, however, when pMMO was incubated with [^{14}C]TCE for up to 200 min. It is possible that the TCE-epoxide or acyl halide intermediate covalently binds to other macromolecules in the cell. Further work should be done to determine the cellular location of the products of TCE oxidation to understand more completely how TCE affects whole cell methanotrophic activity when expressing pMMO.

In conclusion, this study indicates that methanotrophs expressing pMMO can oxidize TCE and the predominant products formed differ from those found for sMMO-expressing cells. As most known methanotrophs can only express pMMO, the potential of pMMO-expressing cells for the degradation of TCE in situ, whether through natural attenuation or biostimulation, should be examined more closely.

Acknowledgements

Research support from the Department of Energy 0296ER20237 (A.A.D.) and the National Science Foundation MCB-9708557 (J.D.S.) is gratefully acknowledged.

References

[1] Banerjee, S. and van Duuren, B.L. (1978) Covalent binding of the carcinogen trichloroethylene to hepatic microsomal proteins and to exogenous DNA in vitro. Cancer Res. 38, 776–780.

[2] Herren-Freund, S.L., Pereira, M.A., Khoury, M.D. and Olson, G. (1987) The carcinogenicity of trichloroethylene and its metabolites, trichloroacetic acid and dichloroacetic acid, in mouse liver. Toxicol. Appl. Pharmacol. 90, 183–189.

[3] Westrick, J.J., Mello, J.W. and Thomas, R.F. (1984) The groundwater supply survey. J. Am. Water Works Assoc. 76, 52–59.

[4] Bouwer, E.J., Rittman, B.E. and McCarty, P.L. (1981) Anaerobic degradation of halogenated 1- and 2-carbon organic compounds. Environ. Sci. Technol. 15, 596–599.

[5] Vogel, T.M. and McCarty, P.L. (1985) Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. 49, 1080–1083.

[6] Wackett, L.P. and Householder, S.R. (1989) Toxicity of trichloroethylene to *Pseudomonas putida* F1 is mediated by toluene dioxygenase. Appl. Environ. Microbiol. 55, 2723–2725.

[7] Winter, R.B., Yen, K-M. and Ensley, B.D. (1989) Efficient degradation of TCE by a recombinant *E. coli*. BioTechnology 7, 282–285.

[8] Ely, R.L., Hyman, M.R., Arp, D.J., Guenther, R.B. and Williamson, K.J. (1995) A cometabolic kinetics model incorporating enzyme inhibition, inactivation, and recovery: II. Trichloroethylene degradation experiments. Biotechnol. Bioeng. 46, 232–245.

[9] Vannelli, T., Logan, M., Arciero, D.M. and Hooper, A.B. (1990) Degradation of halogenated aliphatic compounds by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Appl. Environ. Microbiol. 56, 1168–1171.

[10] DiSpirito, A.A., Gulledge, J., Shiemke, A.K., Murrell, J.C., Lidstrom, M.E. and Krema, C.L. (1992) Trichloroethylene oxidation by the membrane-associated methane monooxygenase in type I, type II, and type X methanotrophs. Biodegradation 2, 151–164.

[11] Fox, B.F., Boneman, J.G., Wackett, L.P. and Lipscomb, J.D. (1990) Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. Biochemistry 29, 6419–6427.

[12] Little, C.D., Palumbo, A.V., Herbes, S.E., Lidstrom, M.E., Tyndall, R.L. and Gilmer, P.J. (1988) Trichloroethylene biodegradation by a methane-oxidizing bacterium. Appl. Environ. Microbiol. 54, 951–956.

[13] Lontoh, S. and Semrau, J.D. (1998) Methane and trichloroethylene degradation by *Methylosinus trichosporium* OB3b expressing the particulate methane monooxygenase. Appl. Environ. Microbiol. 64, 1106–1114.

[14] Newman, L.M. and Wackett, L.P. (1991) Fate of 2,2,2-trichloroacetaldehyde (chloral hydrate) produced during trichloroethylene oxidation by methanotrophs. Appl. Environ. Microbiol. 57, 2399–2402.

[15] Oldenhuis, R., Oedzes, J.Y., Waarde, J.J.V.D. and Janssen, D.B. (1991) Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. Appl. Environ. Microbiol. 57, 7–14.

[16] Oldenhuis, R., Vink, R.L.J.M., Janssen, D.B. and Witholt, B. (1989) Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. 55, 2819–2826.

[17] Uchiyama, H., Nakajima, T., Yagi, O. and Nakahara, T. (1992) Role of heterotrophic bacteria in complete mineralization of trichloroethylene by *Methylocystis* sp. strain M. Appl. Environ. Microbiol. 58, 3067–3071.

[18] Hanson, R.S. and Hanson, T.E. (1996) Methanotrophic bacteria. Microbiol. Rev. 60, 439–471.

[19] Miller, R.E. and Guengerich, F.P. (1982) Oxidation of trichloroethylene by liver microsomal cytochrome P-450: evidence for chlorine migration in a transition state not involving trichloroethylene oxide. Biochemistry 21, 1090–1097.

[20] Nakajima, T., Uchiyama, H., Yagi, O. and Nakahara, T. (1992) Novel metabolite of trichloroethylene in a methanotrophic bacterium, *Methylocystis* sp. M, and hypothetical degradation pathway. Biosci. Biotechnol. Biochem. 56, 486–489.

[21] Henschler, D., Hoos, W.R., Fetz, H., Dallmeier, E. and Metzler, M. (1979) Reactions of trichloroethylene epoxides in aqueous systems. Biochem. Pharmacol. 28, 543–548.

[22] Stainthorpe, A.C., Salmond, G.P.C., Dalton, H. and Murrell, J.C. (1990) Screening of obligate methanotrophs for soluble methane monooxygenase genes. FEMS Microbiol. Lett. 70, 211–216.

[23] Han, J.-I., Lontoh, S. and Semrau, J.D. (1999) Degradation of chlorinated and brominated hydrocarbons by *Methylomicrobium album* BG8. Arch. Microbiol. 172, 393–400.

[24] Whittenbury, R., Phillips, K.C. and Wilkinson, J.F. (1970) Enrichment, isolation, and some properties of methane-utilizing bacteria. J. Gen. Microbiol. 61, 205–218.

[25] Lontoh, S., DiSpirito, A.A. and Semrau, J.D. (1999) Dichloromethane and trichloroethylene inhibition of methane oxidation by the membrane-associated methane monooxygenase of *Methylosinus trichosporium* OB3b. Arch. Microbiol. 171, 301–308.

[26] Zahn, J.A. and DiSpirito, A.A. (1996) Membrane-associated methane monooxygenase from *Methylococcus capsulatus* Bath. J. Bacteriol. 178, 1018–1029.