Novel Metabolite of Trichloroethylene in a Methanotrophic Bacterium, \textit{Methylcystis} sp. M, and Hypothetical Degradation Pathway

Toshiaki Nakajima,† Hiroo Uchiyama,*, Osami Yagi, and Tadaatu Nakahara†

National Institute for Environmental Studies, 16–2 Onogawa, Tsukuba-shi, Ibaraki 305, Japan
†Institute of Applied Biochemistry, University of Tsukuba, 1–1–1 Tennoudai, Tsukuba-shi, Ibaraki 305, Japan

Received September 11, 1991

Previously, a new type II methanotrophic bacterium, \textit{Methylcystis} sp. M (strain M), was isolated in our laboratory [H. Uchiyama et al., \textit{Agric. Biol. Chem.}, 53, 2903–2907 (1989)]. In this paper, metabolites resulting from the degradation of trichloroethylene (TCE) by strain M were studied with gas chromatography-mass spectrometry. Trichloroacetic acid, dichloroacetic acid, and a small amount of 2,2,2-trichloroethanol were detected in the water-soluble fraction of the reaction mixture. These results suggest that the conversion of TCE to trichloroacetaldehyde via a C1-shift reaction, followed by the formation of dichloroacetic acid and 2,2,2-trichloroethanol, as well as a spontaneous breakdown of TCE oxide, with subsequent formation of dichloroacetic acid, etc., is involved in the TCE degradation pathway of the methanotrophic bacterium.

Trichloroethylene (TCE), which is a volatile chlorinated compound and a commonly used solvent, is responsible for serious groundwater contamination. It is a suspected carcinogen and generally resists biodegradation in the environment. 1) Numerous studies on the biodegradation of TCE have been conducted. Under anaerobic conditions, 2)–4) TCE was degraded by anaerobic consortia, following the formation of dichloroethylene and vinyl chloride, which are also toxic metabolites. Under aerobic conditions, several aerobic bacteria were able to degrade TCE. For example, methane-utilizing bacteria (methanotrophs), 5)–10) toluene-oxidizing bacteria, 11)–14) and ammonia-oxidizing bacteria 15) 16) degraded TCE. Previously, we also reported that a new type II methanotroph, \textit{Methylcystis} sp. M (strain M), could grow and degrade TCE at relatively high concentrations. 17)–18) It is anticipated that these aerobic bacteria will be used for \textit{in situ} bioremediation of TCE and other chlorinated compounds. 6) 12)–14)

For the environmental use of these bacteria, it is important to gain information about the types and quantities of the degradation products, as well as about their toxicities. At present, however, information about the end-products of TCE degradation by methanotrophs is limited. Henry and Gribiec-Galic 19) reported the formation of dichloroacetic acid, glyoxylic acid, formic acid, and CO in a TCE-degrading culture of a methanotroph isolated from a contaminated aquifer. Little \textit{et al.} 6) detected glyoxylic acid and dichloroacetic acid in water-soluble TCE breakdown products in pure cultures of a type I methanotroph, strain 46-1. Furthermore, Oldenhuis \textit{et al.} 17) observed that the type II methanotroph \textit{Methylosinus trichosporium} OB3b produced only traces of 2,2,2-trichloroethanol and trichloroacetaldehyde (chloral) during the degradation of TCE. Recently, in addition to these whole-cell studies, Fox \textit{et al.} 20) have reported that the soluble methane monoxygenase from \textit{Methylosinus trichosporium} OB3b catalyzed the formation of CO, formic acid, chloral, glyoxylic acid, and dichloroacetic acid from TCE. Thus, these results indicate that the degradation pathway of TCE in methanotrophs still remains to be identified.

We report here the detection of trichloroacetic acid, in addition to dichloroacetic acid and 2,2,2-trichloroethanol, as a TCE metabolite in a pure culture of a type II methanotroph, strain M. Additionally, on the basis of these data, we propose a hypothetical pathway of TCE degradation by strain M.

Materials and Methods

\textit{Microorganism and culture conditions.} \textit{Methylcystis} sp. M which was previously isolated in our laboratory, had been designated as strain M. 18) It is nonmotile, oxidase-negative, and lacks exospores (for details, see ref. 18). Based on these criteria, it was tentatively assigned to the genus \textit{Methylcystis} according to Whittenbury. 21) Cells used for the detection and identification of the TCE metabolites were grown on mineral salt medium (pH 7.4) under a methane-air atmosphere (1:1, v/v) at 30°C, as previously described. 18)

\textit{TCE degradation study.} Cells were harvested by centrifugation and resuspended in the medium described above to give an optical density of 25 at 580 nm. A 35 ml sample of the cell suspension (about 455 mg dry weight) was transferred into a 155-ml (total volume) serum bottle that was crimp-sealed with a Teflon-lined silicone septum. After addition of 7 mg of TCE, the sealed bottle was incubated at 30°C on a shaker table operating at 120 rpm for 18 hr.

\textit{Identification of TCE breakdown products.} To isolate and identify the water-soluble TCE metabolites, the reaction mixture was centrifuged for 20 min at 8,000 × g. The supernatant was acidified to pH 2 and extracted three times with the same volume of diethyl ether. The organic phase was concentrated to about 2 ml at reduced pressure, and methylated with diazomethane, followed by gas chromatography-mass spectrometry (GC/MS) analysis. The metabolites of TCE were identified using a Shimadzu Model GCMS 9000C GC/MS instrument (Kyoto, Japan) equipped with a Shimadzu Model GC-MSPAC 500 computer system. A silicon DC500 glass column (2 m by 1 mm) was used, and the injector and the oven temperatures were 250°C and 100°C, respectively. The flow rate of the carrier gas (He) was 10 ml/min.

TCE metabolites were identified by comparison of their mass spectra with those of authentic standards.

* Corresponding author.
Chemicals. Trichloroethylene was purchased from Wako Pure Chem.
Co. (Osaka, Japan). The purity was checked by gas chromatography and
exceeded 99.5%.

Results

During the degradation of TCE, a high concentration of
cells was used to achieve complete degradation of TCE at
a high concentration. In fact, more than 95% of the TCE
was degraded, based on GC analysis, suggesting that only
low levels of metabolites could be detected. In the ether
extract, two strong peaks (peaks 1 and 3) and a weak peak
(Peak 2) were detected by GC analysis, as shown in Fig. 1.

No other peak was observed. These three peaks were not
found in the absence of cells. Various methyl esters of
organic acids considered to be TCE metabolites were assay-
ed by gas chromatography as standards. As a result, the
retention time of peaks 1 and 3 corresponded to those of
authentic dichloroacetic acid and trichloroacetic acid,
respectively. Moreover, a more rigorous analysis of both
peaks was done by GC/MS. Figure 2A shows the mass
spectrum of peak 1. Characteristic fragment peaks were
observed at m/z 111 (-OCH3), 83 (-COOCH3), and 59
(-CHCl3). In Fig. 3A, major spectral features of peak 3
were also observed at m/z 141 (-Cl), 117 (-COOCH3), 82
(-Cl-COOCH3), and 59 (-CCl3). These mass spectra were
identical with those of the authentic methyl esters (Fig. 2B,
3B), indicating that peaks 1 and 3 corresponded to di-
cloroacetic acid and trichloroacetic acid, respectively.

Since no methyl esters of standard organic acids surveyed
corresponded to peak 2 by GC analysis, investigations by
GC/MS were also done (Fig. 4A). A weak molecular ion
peak was observed at m/z 148. Typical isotopic abundance
patterns at m/z 117, 119, and 121, in addition to 131, 133,
and 135, were indicative of a trichloro-compound. Other
abundances were also observed at m/z 113, 82, which were
indicative of a dichloro-compound, and 77, with additional
ions at m/z 31, which was indicative of a -CH2OH group.
Based on these data, peak 2 was assumed to correspond to
2,2,2-trichloroethanol. The mass spectrum of authentic
2,2,2-trichloroethanol is shown in Fig. 4B. By comparison
of the peak 2 spectrum with that of an authentic standard,
peak 2 was subsequently found to correspond to 2,2,2-
trichloroethanol.

Discussion

In this report we described the production of trichloro-
acetic acid during TCE degradation by a type II meth-
annotrophic bacterium, strain M, with additional produc-
tion of dichloroacetic acid and 2,2,2-trichloroethanol. In
our preliminary experiment with [14C] TCE,22 we also
confirmed by HPLC and GC, respectively, that glyoxylic
acid and CO were degradation products. It is known that
glyoxylic acid, formic acid, chloral, CO, etc. are formed as
degradation products of TCE by methanotrophs.6,7,19 In
contrast, in the urine of rats and mice dosed with TCE,
Fig. 5. A Hypothetical Pathway of TCE Degradation by Strain M.

MMO, methane monoxygenase; (1), chloral; (2), trichloroacetic acid; (3), 2,2,2-trichloroethanol; (4), TCE oxide; (5), carbon monoxide; (6), formic acid; (7), glyoxylic acid; (8), dichloroacetic acid. Proposed pathways are indicated by dashed lines.

several other metabolites including oxalic acid have been identified and a metabolic pathway of TCE in animals was proposed by Dekant et al.23 On the basis of the studies reported here and the results of the former studies, a hypothetical pathway of TCE degradation in strain M is presented in Fig. 5. This pathway is partially similar to that proposed by Little et al.111 and Miller and Guengerich,24 but differs in that trichloroacetic acid and 2,2,2-trichloroethanol, which are formed via chloral as indicated below, are considered to be substantial metabolites of TCE degradation.

It is well known that one of the first steps of TCE degradation, the epoxidation of TCE to TCE oxide, is catalyzed by methane monoxygenase in the case of methanotrophs.6,20 On the other hand, in animals, microsomal cytochrome-P450 is involved in this step. It has been reported that cytochrome P-450 is distributed among procaryotes as well as eucaryotes,25,26 which indicates that the first epoxidation step of TCE could be catalyzed by cytochrome P-450 even in methanotrophs. Our preliminary experiments showed that TCE degradation by a cell-free extract of strain M did not occur in the presence of acetylene, which is a specific inhibitor of methane monoxygenase. On the other hand, when the cell-free extract was incubated with CO, which is an inhibitor of cytochrome P-450, TCE degradation was not inhibited (data not shown). These results suggested that strain M may not contain cytochrome P-450, and that the epoxidation of TCE is not catalyzed by cytochrome P-450, but by methane monoxygenase.

Miller and Guengerich24 demonstrated that TCE oxide was converted to glyoxylic acid and dichloroacetic acid under acidic aqueous conditions, spontaneously, while under basic conditions, CO and formic acid were formed, due to the instability of TCE oxide. At pH 7.7, these four products were formed, with formic acid predominating. In the case of strain M, the formation of dichloroacetic and glyoxylic acids, and CO, could also be due to the same non-enzymatic reaction. Formic acid was expected to be formed in sufficient amounts to be detected, because the pH for degradation was 7.4. Since formic acid was not detected in this study, it is likely that strain M may convert it rapidly, probably to CO₂.

Recently, Fox et al.20 have reported that through the catalysis of soluble MMO, TCE could be converted to chloral as well as TCE oxide. In this novel pathway, TCE oxide is no obligate intermediate, instead, a chlorine shift occurred for TCE, followed by the formation of chloral. This type of conversion is known to be mediated by cytochrome P-450 in animals, in which chloral is a major product of TCE conversion, indicating that the degradation pathway via chloral predominates. Chloral could then be converted to trichloroacetic acid or 2,2,2-trichloroethanol, and the formation of both products was confirmed in this study. In animals, the reductive step from chloral to 2,2,2-trichloroethanol, and the oxidative step of chloral to trichloroacetic acid were reported to be catalyzed by liver alcohol dehydrogenase [EC 1.1.1.1] and NAD-dependent liver cytosol enzyme, respectively.23 In strain M, although we have not identified such enzyme activities, similar enzymatic systems may operate for further decomposition of chloral.

It remains to be determined whether the pathway of TCE degradation by strain M proceeds via TCE oxide or via chloral. Since both trichloroacetic acid and dichloroacetic acid were detected in our study, both pathways could be used. The metabolite profile shown in Fig. 1 demonstrated that the amount of trichloroacetic acid formed was about
Trichloroacetic Acid as a Metabolite of TCE

Two times as large as that of dichloroacetic acid during TCE degradation, implying the possibility that the degradation pathway of TCE via chloral by methane monoxygenase may be significant in strain M, but trichloroacetic acid is a predominant dead-end product of TCE degradation via the chloral pathway, while dichloroacetic acid is one of the TCE degradation products as well as glyoxal acid, formic acid, and CO. Therefore, additional studies with [14C] TCE are necessary to find which pathway is more responsible for TCE degradation. Since degradation products of chloral were not reported in type I methanotrophs, such a characteristic methane monoxygenase may be specific to type II methanotrophs.

Trichloroacetic and dichloroacetic acids are known to be major by-products formed during the process of water chlorination. Although only a high dose of trichloroacetic acid in drinking water causes a significant toxic effect in an animal model, these chlorinated acetic acids were reported to be carcinogens for mouse liver. At present, since the information about the exact amounts of the acids formed by TCE degradation is limited, further detailed investigations are required. These results also indicate the need to consider the use of heterotrophic bacteria in addition to methanotrophs for further decomposition of these products to non-toxic compounds, in TCE bioremediation processes. Furthermore, as described above, metabolite profiles during the TCE degradation varied with the bacteria used and the experimental conditions, implying the necessity to identify and measure the metabolites before field application.

Acknowledgment. We wish to thank Professor T. Tabuchi for his helpful advice.

References