Journal of Industrial Microbiology, 4 (1989) 29–35
Elsevier

SIM 00155

Metabolism of chlorinated methanes, ethanes, and ethylenes by a mixed bacterial culture growing on methane

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Received 21 March 1988
Revised 1 June 1988
Accepted 6 June 1988

Key words: Methane-utilizing bacteria; Halogenated hydrocarbons; Trichloroethylene; Chloroform; Dichloromethane

SUMMARY

Soil was taken from the top 10 cm of a soil column that removed halogenated aliphatic hydrocarbons in the presence of natural gas. This soil was used as an enrichment inoculum to determine that the removals seen in the soil column were in fact of a microbiological nature. Methane served as the source of carbon and energy and was consumed immediately by the enrichments. After several transfers of the enrichments, a stable consortium of at least three bacterial types was obtained. The predominant bacterium was a non-motile, gram-negative coccus. This stable consortium was able to remove chlorinated methanes, ethanes, and ethylenes when grown with methane and oxygen in the headspace. Methane was required for the removals to be observed. Acetylene inhibited the removals, which further suggests the involvement of methanotrophs. Benzene and toluene were removed by the mixed culture with or without methane in the headspace. Fatty acid analysis of the mixed culture resulted in a profile that indicated that the predominant organism was a type II methanotroph. This study provides further evidence that methanotrophic bacteria are capable of cometabolizing a wide range of chlorinated methanes, ethanes, and ethylenes.

INTRODUCTION

The intentional or accidental introduction of pollutant compounds into the terrestrial subsurface results in environmental problems that are often difficult to solve. Microbiological degradation of these pollutant compounds may be an effective mechanism for restoration of contaminated subsoils and ground water. One particular class of pollutant compounds is the halogenated aliphatic compounds (halocarbons) which may be substantial components of leachate from municipal and industrial

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landfills, as well as other sources, and contribute to the contamination of ground water [18,19]. The halocarbons most often detected in contaminated ground water contain one to three carbons with chlorine and bromine substitution [18].

In aerobic environments, halocarbons are not removed from aqueous solution as the result of microbial activity in nonamended soils [20,21] or in the presence of primary sewage effluent [1,2]. An approach that stimulates the growth of gaseous hydrocarbon-utilizing bacteria by adding natural gas to the headspace of a soil column, however, results in the removal of halocarbons from aqueous solution ([22]; J.M. Henson, J.W. Cochran, and J.T. Wilson, Abstr. Soc. Environ. Toxicol. Chem. 1985, p. 100). Additional reports have shown the removal of chlorinated ethylenes by a mixed culture of methane-utilizing bacteria [6] as well as the removal of chloroform from soil amended with natural gas [17]. A unique biochemical mechanism for the removal of trichloroethylene was reported by Nelson et al. [10,11].

This report describes the removal of chlorinated methanes, ethanes, and ethylenes by a mixed culture of bacteria enriched on methane isolated from the soil column described previously ([22]; Henson et al., Abstr. Soc. Environ. Toxicol. Chem. 1985, p. 100).

MATERIALS AND METHODS

Enrichment of methane-utilizing bacteria. Soil was removed from the top 10 cm of a soil column used previously for investigating the removals of various halocarbons ([22]; Henson et al., Abstr. Soc. Environ. Toxicol. Chem. 1985, p. 100). Five-gram amounts of the soil were placed into 50 ml of a sterile salts medium [16] contained in 160-ml serum bottles (Wheaton Scientific, Millville, NJ) and shaken at room temperature (25°C). Black rubber stoppers and aluminum crimps were used to close the serum bottles. The headspace of the closed serum bottles was composed of air and methane in a 4 to 1 ratio. Control cultures were prepared in the same manner except methane was omitted from the headspace. The methane enrichments were transferred after examination by phase-contrast microscopy. The cultures were transferred several times until the observation of similar morphological types after each transfer indicated a stable community.

Preparation of an aqueous halocarbon solution. The halocarbons were obtained from Aldrich Chemical Co. (99+% quality, Milwaukee, WI) or Chem Service, Inc. (West Chester, PA). The stock solution of halocarbons was prepared by adding an aliquot of the neat halocarbon to sterile deionized water that completely filled a 160-ml serum bottle, preventing a headspace. The serum bottle was closed with a Teflon-lined silicone septum (20 mm, No. 23244, Supelco, Inc., Bellefonte, PA) and sealed with an aluminum crimp (Wheaton Scientific, Millville, NJ). The sealed serum bottle was covered with aluminum foil and stirred overnight at room temperature.

Determination of halocarbon removal. The ability of the methane-utilizing mixed culture to metabolize various halocarbons was determined by transferring aseptically 0.1 ml of the mixed culture grown overnight into 10 ml of the sterile salts medium contained in a 27-ml serum tube (Belco Glass, Inc., Vineland, NJ). An aliquot of an aqueous stock solution of the halocarbons was added to each serum tube to achieve a final concentration of between 200 and 300 μg/l for each halocarbon. The serum tube was sealed with a Teflon-lined silicone septum and aluminum crimp as described above. Methane was added aseptically to the headspace of the experimental tubes ($n = 3$) while nitrogen was added aseptically to one set of control tubes ($n = 2$). Additional controls were prepared by adding an aliquot of the halocarbon stock solution to the medium without the mixed culture ($n = 2$). All septa received an equal number of punctures. The second set of controls resulted in data very similar to the first set of controls, therefore the percent remaining was determined as follows:

$$[1 + ((A - B)/B)] \times 100$$

where A = concentration of halocarbon in the experimental tubes and B = concentration of halocarbon in the control tubes with nitrogen.

Chemical analysis. The concentrations of halocarbons were determined by purging the halocarbons from each serum tube into Tenax resin followed by thermal desorption onto a fused silica capillary column (30 m × 0.25 mm i.d., 1 μm DB-5 phase, J & W Scientific, Folsom, CA). This was a modification of the purge-and-trap gas chromatography method of Pankow and Rosen [15] and has been described in detail in a separate communication (Cochran, J.W., M.V. Yates and J.M. Henson, J. Microbiol. Methods, in press). The headspace of the serum tubes was analyzed for methane and oxygen by removing samples with a gas-tight syringe. Oxygen was quantified after detection by thermal conductivity. Methane concentration was determined after flame ionization detection.

Analysis of fatty acids. Phospholipid fatty acids of the mixed culture were extracted and identified by previously described techniques [12,13]. Nomenclature of the fatty acids is as follows: number of carbon atoms:unsaturation, position of unsaturation, geometry of unsaturation. The following abbreviations are used: 't' and 'c' for *trans* and *cis*, respectively.

RESULTS

Microbial enrichment

The soil was initially placed into enrichments with either methane or propane as the carbon and energy source. The enrichments with methane were more stable and resulted in better and more reproducible growth. After several transfers, a mixed culture grown on methane was obtained that consisted of at least three morphological types with the predominant bacterium being a non-motile gram-negative coccobacillus (Fig. 1). This bacterium was about 1.5 μm in diameter. Attempts to obtain a pure culture resulted in colonies on solid medium that were pinpoint in size and required about 2 weeks for visible growth to occur. The colonies were opaque and white. The culture obtained was erratic in growth and in reactions to the halocarbons, therefore only results from the more stable mixed culture are reported here.

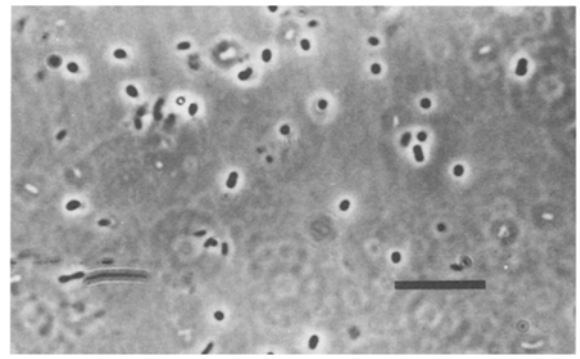

Fig. 1. Phase-contrast photomicrograph of the mixed culture grown on methane. The predominant microorganism is a coccus while occasionally a rod-shaped cell is seen. The bar represents 10 μm.

Removal of halogenated compounds

Removal of the halocarbons by the methane-utilizing mixed culture are shown in Figs. 2–4. The halocarbons were added to the culture as a mixture but are shown in groups of methanes, ethanes, and ethylenes for ease of comparison.

Of the halogenated methanes examined, dichloromethane was removed most rapidly and to the greatest extent, while carbon tetrachloride was the most resistant to microbial removal (Fig. 2). The removal of chloroform was almost as rapid and extensive as that of dichloromethane. Only dichloromethane was completely removed within the 30-day incubation period.

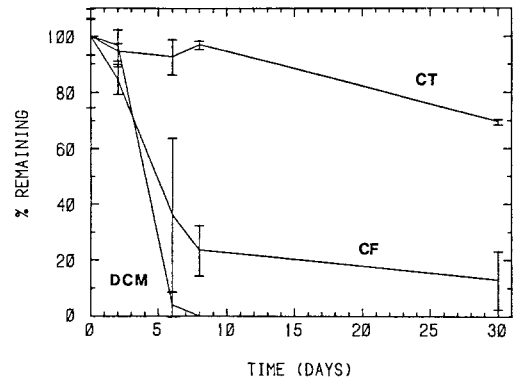

Fig. 2. Removal of the chlorinated methanes dichloromethane (DCM), chloroform (CF), and carbon tetrachloride (CT) by a mixed microbial culture growing on methane. Error bars are ± 1 S.D.

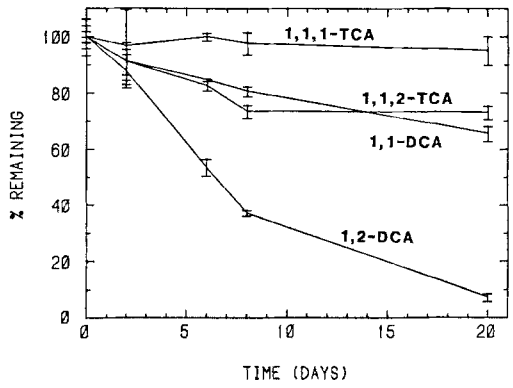

Fig. 3. Removal of the chlorinated ethanes 1,2-dichloroethane (1,2-DCA), 1,1-dichloroethane (1,1-DCA), 1,1,2-trichloroethane (1,1,2-TCA), and 1,1,1-trichloroethane (1,1,1-TCA) by a mixed microbial culture growing on methane. Error bars are ±1 S.D.

Fig. 3 shows the removal of the halogenated ethanes examined. No compound was completely removed during the incubation period used. The compound most amenable to removal, with respect to rate and extent, was 1,2-dichloroethane. The compound 1,1,1-trichloroethane was not removed, while the removals of 1,1-dichloroethane and 1,1,2-trichloroethane were intermediate and similar to each other.

The results for the removal of the chlorinated ethylenes are presented in Fig. 4. The rates of removal of trichloroethylene and *cis*- and *trans*-1,2-dichloroethylene were rapid, although a considerably longer period of time was required for the complete removal of trichloroethylene as compared with the other two compounds. Tetrachloroethylene (PCE) was removed to a much lesser extent in comparison to the other chlorinated ethylenes.

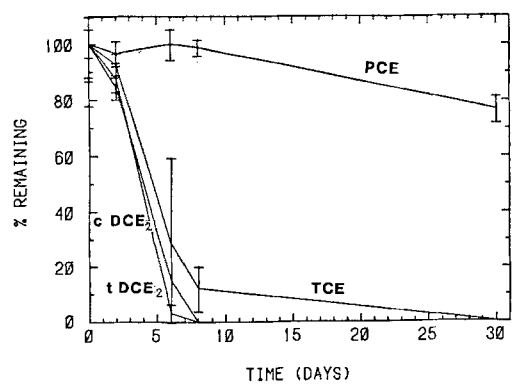

Fig. 4. Removal of chlorinated ethylenes *trans*-1,2-dichloroethylene (tDCE), *cis*-1,2-dichloroethylene (cDCE), 1,1,2-trichloroethylene (TCE), and 1,1,2,2-tetrachloroethylene (PCE) by a mixed microbial culture growing on methane. Error bars are ±1 S.D.

Removal of non-halogenated compounds

This mixed microbial culture was capable of removing benzene and toluene when these compounds were added as a separate mixture. The removal of benzene and toluene was similar whether methane was present or not.

Utilization of oxygen and methane

The utilization of gases by the mixed culture is presented in Fig. 5. Both oxygen and methane were rapidly removed until the availability of methane became the rate-restricting substrate between day 8 and day 20. The increase in oxygen observed on day 20 was probably because of the influx around the septum from the atmosphere. These removals were not corrected to standard temperature and pressure and, therefore, do not represent absolute measurements but a measurement of a general trend.

Acetylene addition

Acetylene, an inhibitor of methane monooxygenase [4], was added to the headspace of serum tubes to determine the effect that this inhibitor might have on the removal of the halocarbons (Table 1). The removal of the halocarbons was prevented by the addition of the acetylene.

Fatty-acid profile

The analysis of the phospholipid fatty acids (PLFA) of the mixed culture indicated that fatty acids 18 carbons in length were the predominant component (80%) of the membrane phospholipids. The predominant fatty acid found in the PLFA profile was 18:1ω8 that comprised 45.5 mol% of the total PLFAs. In addition, diunsaturated 18-carbon fatty acids (14.5 mol%) were detected but the positions of unsaturation were not determined for all these PLFAs. Other PLFAs found in excess of 1 mol% were (mol%): 15:0 (1.2), 16:1ω7c (4.6), 16:1ω7t (1.3), 16:0 (6.1), *anteiso*-17:1ω8 (1.0), 17:0 (1.2), and 18:1ω7c (20.6). An analysis of the mem-

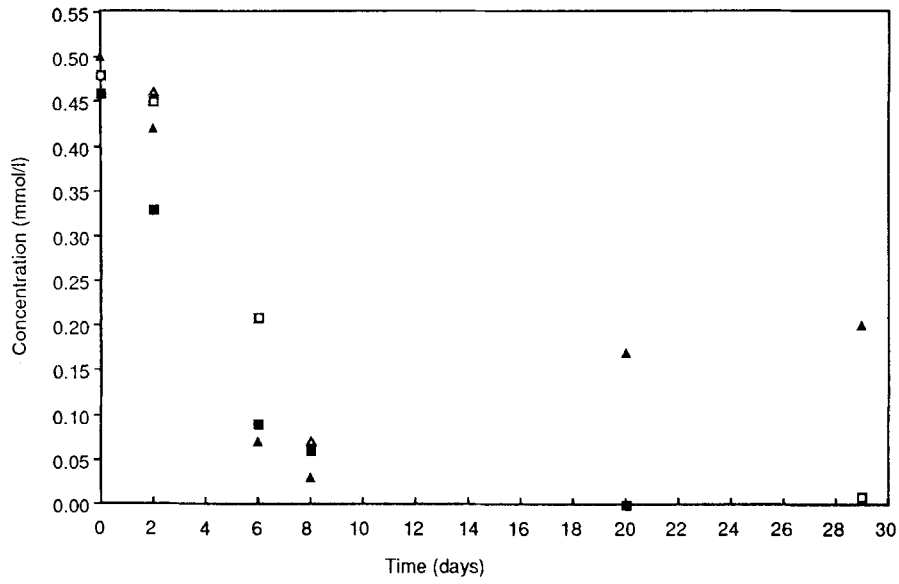

Fig. 5. Consumption of oxygen (triangles) and methane (squares) by a mixed microbial culture. These results are from two experiments with solid and open figures representing the mean (duplicates) of each experiment. Analysis of the headspace of tubes without microorganisms showed that at day 30, the concentration of methane would be about 50% of that at day 0.

brane phospholipids from the pure culture capable of growth on methane resulted in PLFAs in excess of 1 mol% as follows (mol%): 16:1ω7c (5.0), 16:0 (3.8), 18:1ω8 (21.5), 18:1ω7c (65.0), and 10-methyl-18:0 (1.0).

DISCUSSION

Previous studies have shown that when Lincoln fine sand was amended with natural gas, several halocarbons were removed to greater extents than was

Table 1

The effect of acetylene on the removal of halocarbons by a methane-utilizing mixed microbial culture

The results are means of triplicate samples ± 1 S.D. DCM = dichloromethane; t-DCE = *trans*-1,2-dichloroethylene; TCE = 1,1,2-trichloroethylene; PCE = 1,1,2,2-tetrachloroethylene. The concentrations are μg/l.

Compound	Day 0		Day 3		Day 5	
	+ bact	− bact	+ bact	− bact	+ bact	− bact
DCM	843 ± 33.4[a]	852 ± 41.7	18.6 ± 4.38	660 ± 29.0	5.89 ± 2.5	629.5 ± 27.6
t-DCE	264 ± 9.2	284 ± 26.2	1.90 ± 0.014	164 ± 2.1	0.42 ± 0.19	143 ± 0.0
TCE	348 ± 14.1	374 ± 35.4	138 ± 33.2	229 ± 5.6	160.5 ± 2.1	226 ± 7.1
PCE	248 ± 7.8	284 ± 19.1	137 ± 19.8	154 ± 2.8	154.5 ± 9.2	168.5 ± 6.4
DCM	884 ± 70.0[b]	869 ± 72.1	594 ± 137.9	728 ± 10.6	611.5 ± 146.4	624.5 ± 64.3
t-DCE	260 ± 11.3	270 ± 29.7	146 ± 37.5	188 ± 6.4	134.5 ± 33.2	156 ± 5.7
TCE	326 ± 18.4	338 ± 48.8	176 ± 32.5	224 ± 1.4	194 ± 35.4	217.5 ± 3.5
PCE	221 ± 11.3	230 ± 33.9	119 ± 27.7	160 ± 12.0	135.5 ± 34.6	147 ± 9.9

[a] Methane.
[b] Methane + acetylene.

observed in a non-amended soil column ([22]; Henson et al., Abstr. Soc. Environ. Toxicol. Chem. 1985, p. 100). This study shows that methane (a major component of the natural gas used in the previous studies) enrichment of soil taken from the top 10 cm of the above soil column resulted in a mixed microbial culture capable of achieving similar trends of removals to those observed for the amended soil column. In a separate study, the use of methane to enrich a microbial community in Lincoln fine sand incubated under conditions other than in a column was also capable of removing several halocarbons [7]. Thus, amendment of soils with methane stimulates naturally occurring bacteria that are capable of removing a variety of halocarbons.

Other authors reported the stimulation of naturally occurring bacteria that resulted in the removal of several halocarbons. A mixed culture of methane-enriched bacteria from pond sediment was capable of removing chlorinated ethylenes [6]. The removal of trichloroethylene was also observed when an aerobic bacterium, isolated from a water sample from a holding pond at an industrial waste treatment facility, degraded trichloroethylene as the result of induction of enzymes responsible for metabolism of aromatic compounds [10,11]. A chlorinated form of methane, chloroform, was removed from soil when incubated with methane in the headspace [17].

These studies show that stimulation of naturally occurring mixed bacterial cultures results in the removal of a variety of halocarbons in addition to trichloroethylene as originally reported by Wilson and Wilson [22]. Methane monooxygenase, the enzyme that oxidizes methane to methanol in methanotrophs, is capable of reacting with a variety of hydrocarbon substrates including alkenes and halogenated methanes [5]. The product of the reaction of methane monooxygenase with ethene is the corresponding epoxide [5]. Epoxide intermediates were reported in the degradation of the *cis* and *trans* isomers of dichloroethylene by a mixed culture growing on methane (Leahy, M.C., M. Dooley-Danna, M. Young and M. Fogel. 1987. Abstr. Annu. Meet. Am. Soc. Microbiol., K-178, p. 232). Several reports have shown that pure cultures of bacteria are capable of transforming halocarbons. Dichloromethane is used as a growth substrate by a *Pseudomonas* sp. [3]. Other reports show that cometabolism of 1,2-dichloroethane [9] and *trans*-1,2-dichloroethylene [8] occurs with pure cultures of methanotrophic bacteria. The results of the last two studies strongly suggest that methane monooxygenase was responsible for the initial event in the metabolism of the halocarbons. The epoxide of *trans*-1,2-dichloroethylene was formed by pure cultures of *Methylomonas methanica* and *Methylosinus trichosporium* [8].

A different type of cometabolism was observed by Nelson et al. [10] for a pure culture capable of removing trichloroethylene in the presence of several aromatic compounds. The metabolism of trichloroethylene by this culture involved the degradation pathway that uses the *meta* fission pathway [10] although the enzyme responsible is as yet unknown. This bacterium is not a methanotroph [10].

Addition of methane to Lincoln fine sand in the column stimulated a microbial community composed of a high proportion of methanotrophs as indicated by the profile of phospholipid fatty acids [13]. The fatty acid profile of the mixed culture in this study has a large percentage of 18:1 fatty acids and was therefore similar to the profile reported for the soil column studied previously [13]. Methanotrophic bacteria that are classified as type II have predominantly fatty acids that are 18 carbons in length [14]. This result suggested that the predominant members of the type II methane-utilizing bacteria within the soil column were enriched in this study.

Although biochemical studies have yet to confirm the reactions that resulted in the removal of the halocarbons in this study, it seems possible that methane monooxygenase induced by enhancing the population of methane-utilizing bacteria may have reacted with the compounds studied to produce intermediates that are subsequently degraded by the enhanced microbial community. Stimulation of naturally occurring bacteria with methane appears to be a feasible approach to remove pollutant compounds from the environment and requires further study.

ACKNOWLEDGEMENTS

Although the research in this article has been funded by the United States Environmental Protection Agency as an in-house research project at the Robert S. Kerr Environmental Research Laboratory, it has not been subjected to the Agency's required peer and policy review and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred. We thank Janet Nichols, Dave Ringleburg, and David White for the analysis of the phospholipid fatty acids. We also thank Bill Dunlap, John Wilson, and Steve Hutchins for critical review of the manuscript and Kathy Clinton for preparation of the figures.

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