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Anaerobic dechlorination of tetrachloromethane and 1,2-dichloroethane to degradable products by pure cultures of *Desulfobacterium* sp. and *Methanobacterium* sp.

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1. SUMMARY

Methanobacterium thermoautotrophicum grew autotrophically and *Desulfobacterium autotrophicum* grew heterotrophically in the presence of one of several highly chlorinated aliphatic hydrocarbons in the low μM range. *M. thermoautotrophicum* transformed a portion of 1,2-dichloroethane, and ethene was identified as the product. *D. autotrophicum* also displayed substrate specificity; it reduced tetrachloromethane quantitatively to trichloromethane and dichloromethane, and converted 1,1,1-trichloroethane to 1,1-dichloroethane.

2. INTRODUCTION

Chlorinated solvents comprise a quarter of the priority pollutants listed by the U.S. EPA [1]. A few of these compounds, generally with a low level of chlorination, are subject to aerobic degradation [2–7] or transformation [8–10; see also 11], and some of these degradative capacities can be applied to waste or water treatment [5,12,13]. But for the most part, the highly chlorinated solvents are subject only to anaerobic transformation [14–22].

Anaerobic soil samples and mixed cultures from activated sludge catalyze reductive dehalogenation of tetrachloromethane to putative trichloromethane under several conditions [15,16,19,20] and the product has been conclusively identified [19]. Similarly, 1,1,1-trichloroethane is reduced to 1,1-dichloroethane and chloroethane [19], tetrachloroethene is reduced via trichloroethene and the dichloroethenes to chloroethene [16,19–21] and sometimes chloroethane [21], and dehalogenation of 1,2-dibromoethane to putative ethene is know [17; cf. 22]. Conversion of haloaliphatics to unknown products [19] and to CO_2 [14–16] illustrates that other reaction types also occur.

A major problem in studying these anaerobic reactions is the fact that they occur in undefined mixed cultures, and the organisms involved are unknown [cf. 22]. It is easier to contemplate understanding and possibly optimizing these reactions if they are available in pure culture. We now report the release of ethene from e.g., 1,2-dichloro-

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ethane in pure cultures of *Methanobacterium thermoautotrophicum* and of methylene chloride and 1,1-dichloroethane from tetrachloromethane and 1,1,1-trichloroethane, respectively, in pure cultures of *Desulfobacterium autotrophicum* and, to a lesser extent, in *M. thermoautotrophicum*.

3. MATERIALS AND METHODS

3.1. Materials

The chlorinated hydrocarbons ($\geqslant 99\%$) were from Fluka (Buchs, Switzerland), except 1,1-dichloroethane (Alfa, Danvers, MA), chloromethane and chloroethane (Matheson, East Rutherford, NJ). Standard alkanes and ethene were from Matheson. Other chemicals were the highest quality from Fluka or Merck-Schuchardt (Munich, F.R.G.). Gas-tight syringes (Precision Sampling, Baton Rouge, LA) were used. Viton septa were from Gummi Maag, Zürich, Switzerland.

3.2. Analyses

Chlorinated hydrocarbons, methane and ethene were determined after separation on packed columns in a GC equipped with FID [23]. A Porapak P column was used routinely [7] and Durapak [24], Chromosorb 101 [24] and Tenax [7] columns were used to confirm the identity of each product by co-chromatography with authentic material. Sample size was 0.3 ml of headspace, and syringes were flushed in a stream of nitrogen between injections. Standards were prepared in culture vessels containing the appropriate sterile growth medium. Growth was estimated as optical density [7].

3.3. Culture vessel

Thick-walled, 130-ml, screw-cap bottles with metal septum screwcaps were used. The main seal was viton. It was 11 mm thick, fitted tightly in the opening, and was flanged (4 mm thick) to fit snugly in the cap. A back-up septum (2 mm) of natural rubber lay between the viton septum and the cap.

3.4. Organisms and growth conditions

M. thermoautotrophicum Marburg (DSM 2133) was grown at 63°C in 20-ml portions of salts medium [25]. The medium was inoculated through the septum with a fresh culture and incubated till the OD_{546} reached 0.1 (about 2 h), when the chlorinated solvent (200 mM in glacial acetic acid) was added by syringe. *D. autotrophicum* DSM 3382 was grown at 27°C in 25-ml portions of 5 mM lactate-minimal medium with a H_2/CO_2 (4 : 1) gas phase [26]. Chlorinated compounds (20 mM in water, and sonicated immediately prior to use) were added by syringe.

4. RESULTS

We needed to follow kinetically the behaviour of chlorinated solvents in individual cultures, so we required an anaerobic closure that could withstand repeated removal of gas samples. The use of Mininert valves [cf. 7] led to frequent leaks; septa of natural, silicone or butyl rubber were either permeable to or sorptive agents for the solvents, and teflon-coated closures [18,19] permitted only single sampling. We found that viton was effectively non-sorptive and was impermeable to oxygen and to chlorinated solvents. Pierced septa closed only slowly on withdrawal of a needle, so a permanently gas-tight back-up septum was used, in addition to retaining the viton septum very tightly in the bottle opening with a screw cap. The growth of *M. thermoautotrophicum* was unaffected by regular sampling of a control culture, so negligible oxygen was introduced on sampling. The loss of chlorinated solvent over 3 week periods was $< 5\%$.

M. thermoautotrophicum reached the stationary state in about 36 h and released a normal amount of methane [27], the only hydrocarbon observed. The presence of 7 (of 10) halohydrocarbons (Table 1) had no effect on growth; three compounds partially inhibited growth. None of the halogenated compounds was subject to extensive transformation ($\leqslant 11\%$, Table 1) and half of the compounds were not transformed in significant amounts.

The disappearance of limited amounts of 1,2-dichloroethane was accompanied by the release of putative ethene, and this was seen to be a

Table 1

Transformations of halogenated alkanes and alkenes during and after autotrophic growth of *Methanobacterium thermoautotrophicum*

Halogenated compound [a] (1000 nmol/culture)	Growth [b]	Product observed	Product formed (nmol) after incubation for		
			1 day	4 days	8 days
None [c]	Normal	None [d]			
CH_3-CH_2Cl	Normal	None			
CH_2Cl-CH_2Cl	Normal	$CH_2 = CH_2$	None	30	70
CH_3-$CHCl_2$	Normal	None			
CH_3-CCl_3	Poor	CH_3-$CHCl_2$	None	50	110
cis-CHCl=CHCl	Normal	None			
trans-CHCl=CHCl	Normal	None			
$CHCl=CCl_2$	Normal	None			
$CCl_2=CCl_2$	Normal	$CHCl = CCl_2$	None	None	20
$CHCl_3$	Poor	CH_2Cl_2	None	Trace	10
CCl_4	Poor	$CHCl_3$	None	110	No assay

[a] The halogenated compound was stable in sterile medium (loss < 5%) and gave rise to no measurable product; the presence of autoclaved cultures did not alter this. In growing cultures, disappearance of the halogenated compound was at most 11%. The concentration of the solvent in the growth medium is uncertain, because the portion in the gas phase at 63° C is unknown.

[b] Growth was scored as turbidity and methane formation.

[c] Liquid solvents were dissolved in acetic acid to allow accurate dosage. Acetic acid was added to the control and where the chlorinated compound was added as a gas. The acetic acid had no effect on growth.

[d] The limit of detection represented about 10 nmol/culture or about 1% transformation of the halogenated compound. No alkane (except methane) was observed in the absence of halogenated hydrocarbons.

biotransformation (Table 1). The identification of the product was confirmed by co-chromatography with authentic material on three different GC columns. The ethene (7% of the educt) was formed continuously during the incubation, whereas the growth period was short.

Table 2

Transformation of chlorinated alkanes and alkenes during heterotrophic growth of *Desulfobacterium autotrophicum*

Halogenated compound [a] (2000 nmol/culture)	Growth [b]	Products formed	Solvents present (nmol) after incubation for			
			1 day	6 days	10 days	19 days
None	Normal	None [c]				
CCl_4	Poor		1520	0	0	0
		$CHCl_3$	520	1640	1360	1220
		CH_2Cl_2	0	480	920	1080
$CHCl_3$	Normal		2000	1700	1000	1060
		CH_2Cl_2	0	620	840	1100
CH_2Cl_2	Normal	None	2000		2020	1860
CH_3-CCl_3	Normal		1900	1500	1020	1040
		CH_3-$CHCl_2$	40	820	940	1060
CH_3-$CHCl_2$	Normal	None	1800	2000	1900	1720
$CCl_2=CCl_2$	Normal	None	1960	1940	1980	1840
$CHCl=CCl_2$	Normal	None	2000	1620	1940	2000

[a] The halogenated compound was stable and gave rise to no measurable product in sterile medium and in sterile medium containing 20 mM sulfide (loss < 5%). The presence of autoclaved cultures did not alter this. The concentration of solvent in the medium was probably about 20 μM, allowing for the gas : liquid partition coefficient [29] and the large volume of the gas phase.

[b] Growth was scored as turbidity.

[c] The limit of detection represented about 10 nmol/culture or about 0.5% transformation of the halogenated compound. The error associated with each assay was about 5–10%. No product was observed in the absence of halogenated hydrocarbons.

In contrast to the complete removal of the chlorine substituents from 1,2-dichloroethane, four other solvents were subject to partial dechlorination (Table 1). Recovery of solvent carbon was quantitative. It is thus clear that a pure culture can transform a halohydrocarbon and that the system is relatively specific in the substrates converted.

The heterotrophic growth of *D. autotrophicum* was little affected by the presence of chlorinated solvents (Table 2), but autotrophic growth was completely inhibited by tetra- and trichloromethane. The most rapid transformation observed was the dechlorination of tetrachloromethane, which was complete in six days, yielding trichloromethane and small amounts of dichloromethane, whose concentration increased as the chloroform in turn was reduced (Table 2). There was no significant transformation of dichloromethane and the recovery of carbon from tetrachloromethane was quantitative. The homologue of trichloromethane, 1,1,1-trichloroethane, was reduced to 1,1-dichloroethane, which, like several other solvents, was not subject to significant transformation. The recovery of carbon from the educt was quantitative.

During heterotrophic growth, *D. autotrophicum* released small amounts of methane [cf. 26]. This reaction was inhibited by about 45% in the presence of tetra- and trichloromethane. This, coupled with the selective inhibition of autotrophic growth by these compounds, leads us to suspect that, in *D. autotrophicum*, one (or more) enzyme(s) of C1 metabolism is involved in reductive dechlorination.

5. DISCUSSION

This work confirms the assumption [14,16,21] that a methanogen can transform halogenated compounds. It also confirms the supposition [15] that other strict anaerobes, here a sulfate reducer, can dehalogenate highly chlorinated aliphatics. We used laboratory strains that had no previous exposure to chlorinated solvents. It thus appears that a limited range of halohydrocarbon transformation is inherent in these bacteria.

The reactions we observe have been observed in mixed cultures [17,19], but, in contrast to the mixed cultures, each organism transforms only a limited number of substrates. This specificity strongly implies enzymic catalysis and not non-specific reactions with cell components [22,28; cf. 21]. We seem to have observed only reductive dehalogenations, and not other reactions (see Introduction), because we have high recoveries of the halohydrocarbon carbon. The mechanism(s) of the reductions is unknown. But the fact that one can now examine the reaction in pure culture should allow the mechanism to be explored.

The products from two of these reactions, ethene and dichloromethane, are subject to rapid and complete aerobic degradation [e.g., 2, 5] so one can postulate complete mineralization of tetrachloromethane and trichloromethane. Aerobic transformations of 1,2-dichloroethane [3,4] are more rapid than this anaerobic conversion. The product from 1,1,1-trichloroethane (Table 2) is subject to biotransformation to chloroethane [19], which is subject to complete aerobic degradation [7], so here too one could contemplate complete mineralization for waste disposal.

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