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Reductive dechlorination of 1,2-dichloroethane and chloroethane by cell suspensions of methanogenic bacteria

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Abstract

Concentrated cell suspensions of methanogenic bacteria reductively dechlorinated 1,2-dichloroethane *via* two reaction-mechanisms: a dihalo-elimination yielding ethylene and two hydrogenolysis reactions yielding chloroethane and ethane, consecutively. The transformation of chloroethane to ethane was inhibited by 1,2-dichloroethane. Stimulation of methanogenesis caused an increase in the amount of dechlorination products formed, whereas the opposite was found when methane formation was inhibited. Cells of *Methanosarcina barkeri* grown on H_2/CO_2 converted 1,2-dichloroethane and chloroethane at higher rates than acetate or methanol grown cells.

Abbreviations: BrES – 2-bromoethanesulfonic acid, CA – chloroethane, 1,2-DCA – 1,2-dichloroethane, F_{430} – Ni(II)tetrahydro-(12β, 13α)-corphin with an uroporphinoid (III) ligand skeleton

Introduction

Chlorinated aliphatic hydrocarbons are found to be biotransformed under methanogenic conditions *via* reductive dechlorination in sewage sludge, aquifers, and sediments (Bouwer et al. 1981; Bouwer & McCarty 1983; Kloepfer et al. 1985; Parsons & Lage 1985; Vogel & McCarty 1985). Reductive dechlorination by pure cultures of anaerobic bacteria was first reported for the degradation of hexachlorocyclohexane isomers by different *Clostridia* strains (MacRae et al. 1969; Jagnow et al. 1977). Recently, it was shown that sulfate reducing, methanogenic, and fermenting bacteria have the ability to dechlorinate 1- and 2-carbon halogenated aliphatic compounds (Egli et al. 1987; Belay & Daniels 1987; Egli et al. 1988; Gälli & McCarty 1989). The products were lower chlorinated compounds or even non-toxic products like carbon dioxide, ethylene or ethane.

We report here the production of ethylene and chloroethane (CA) from 1,2-dichloroethane (DCA) and ethane from CA by four strains of methanogens. Evidence is provided that the rate of dechlorination is dependent on the metabolic activity of the cells.

Materials and methods

Organisms

Methanosarcina barkeri (DSM 2948) and *Methanococcus mazei* (DSM 2053) were obtained from the Deutsche Sammlung von Mikroorganismen

(Braunschweig, FRG). *Methanobacterium thermoautotrophicum* strain Marburg (DSM 2133) was obtained from Prof. Thauer, Marburg, FRG. *Methanothrix soehngenii* (DSM 2139) was the Opfikon strain isolated by Huser et al. (1982).

Growth conditions

Cultivation of *M. barkeri* and *M. mazei* on acetate (100 mM) or methanol (250 mM) was performed at 37° C in 120 ml serum bottles with 60 ml of imidazol buffered medium as described by Scherer & Sahm (1981), supplemented with 0.02% yeast extract. The gas phase was N_2 and the initial pH of the medium, 6.4. Subcultures on H_2/CO_2 (80%/20%) of *M. barkeri* and *M. thermoautotrophicum* strain Marburg (incubated at 55° C) were made in 120 ml serum bottles with 20 ml of a phosphate/bicarbonate buffered medium described by Huser et al. (1982). *M. soehngenii* was subcultured at 37° C on the phosphate/bicarbonate buffered medium in 500 ml portions in 1-L bottles with 80 mM sodium acetate as energy substrate and a gas phase of N_2/CO_2 (80%/20%).

Dechlorination experiments

Where necessary, handlings were carried out in an anaerobic glovebox (Coy Laboratories Products, Toepffer GmbH, Göppingen, FRG). The oxygen concentration was kept low with R-20 palladium catalyst provided by BASF (Arnhem, NL).

Cells at the late log phase were harvested aseptically and anaerobically by centrifugation in sterile 300 ml stainless steel centrifugation tubes (Sorvall Instruments, Meyvis, Bergen op Zoom, NL) at 27,500 × g for 45 min at 4° C. Cells were washed twice in sterile medium without substrate, NH_4Cl or yeast extract and resuspended in the same medium. Protein concentration was measured by the method of Lowry et al. (1951) with bovine serum albumin as standard. 5 ml portions of cell suspension were transferred to sterile 35 ml serum bottles. The bottles were sealed with sterile 10 mm thick viton stoppers and kept on ice. The gas phase was changed with N_2, N_2/CO_2, or H_2/CO_2. Where necessary, the methanogenic substrate (methanol or acetate) and 1,2-DCA or CA dissolved in ethanol (10 µl) was added by syringe (Kloehn, Inacom Instruments B.V., Veenendaal, NL). The final ethanol concentration was 34.8 mM or 0.2%. Control experiments showed that ethanol did not lead to appreciable formation of ethylene or ethane. The mesophilic cultures were incubated in a water bath at 37° C, *M. thermoautotrophicum* at 55° C. For each data point two or three cultures were sacrificed for analysis. The gas phase was analyzed for ethylene, ethane, and methane. Subsequently, 5 ml hexane was added by syringe and the bottles were vigorously shaken for 1 min to extract 1,2-DCA and possible chlorinated products. The bottles were stored at 4° C until analysis of the hexane extract.

Analyses

C-2 gases were determined by two different methods. In the first method 200 µl headspace was injected into a 417 Packard gas chromatograph equipped with an FID connected to a Porapak T column (3 m by 1/8″). Operating temperatures of the detector and the column were 120 and 60° C, respectively. Carrier gas was nitrogen at a flow rate of 30 ml/min. In the second method 500 µl headspace was injected into a 438A Chrompack Packard gas chromatograph equipped with an FID connected to a capillary column (25 m by 0.32 mm [inner diameter], Poraplot Q, 10 µm, Chrompack, NL) and a splitter injector (ratio 1 : 80). Operating temperatures of the injector, column, and detector were 250, 60, and 300° C, respectively. Carrier gas was nitrogen with an inlet pressure of 30 kPa. In most of the experiments the first method was used because it was faster. When small amounts of ethane had to be separated from large amounts of ethylene the second method was applied. Methane was measured with a 417 Packard gas chromatograph equipped with TCD at 100 mA connected to a molecular sieve column (13X, 180 cm by 1/4″, 60–80 mesh). Operating temperature of the detector and the column was 100° C. Carrier gas was argon at a flow rate of 30 ml/min. For 1,2-DCA and

CA analysis 1 μl hexane sample was injected into a 436 Chrompack Packard gas chromatograph equipped with a ^{63}Ni-ECD connected to a capillary column (25 m by 0.32 mm [inner diameter], Sil 5CB, 1.22 μm, Chrompack, NL) and a splitter injector (ratio 1 : 50). Operating temperatures of the injector, column, and detector were 250, 90, and 300° C, respectively. Carrier gas was nitrogen with an inlet pressure of 30 kPa. Trichloroethylene served as internal standard. The retention times and peak areas were determined with a Shimadzu C-3A computing integrator. CA was positively identified with GC-MS.

Chemicals

1,2-Dichloroethane (DCA) was purchased from Aldrich, Brussels, Belgium, 1-iodopropane and chloroethane (CA) from E. Merck, Darmstadt, FRG. All other chemicals were of analytical grade and used without further purification. Gases were purchased from Hoekloos, Schiedam, NL.

Results

1,2-DCA was reductively dechlorinated to ethylene and CA by concentrated cell suspensions of four strains of methanogenic bacteria (Table 1). Transformation of 1,2-DCA was not only catalyzed by methanogens which solely grow on H_2/CO_2 (Egli et al. 1987; Belay & Daniels 1987), but also by the acetoclastic organism *M. soehngenii* and by methanogens with a broader substrate range such as *M. barkeri* and *M. mazei* grown on methanol. This shows that this dechlorination activity may be characteristic for methanogens and is not restricted to one primary substrate. Because a complete mass balance of the chlorinated substrate and products was seldom achieved (Table 1), it is possible that other chlorinated products were formed. If the initial step in the reductive dechlorination of 1,2-DCA is a single electron transfer, and therefore takes place *via* a radical, dimerization could occur and C-4 compounds could be formed. Peaks were never observed, however, at the positions of the GC runs where such compounds should appear. Non-volatile and non-hexane-extractable products could not be analyzed by our methods. Although abiotic dehalogenation by the medium, which could lead to the formation of alcohols and thiols (Barbash & Reinhard 1989), is not likely since the rates of such nucleophilic substitution reactions are too slow to be significant during the time-frame involved here. A decrease in 1,2-DCA concentration of 0.17% per day could be expected at 37° C with the concentrations of the nucleophilic agents OH^-, HS^-, and HPO_4^{2-} used in our experiments. This decrease is calculated with the rate constants reported by Barbash & Reinhard (1989). However, biological formation of such polar compounds cannot be excluded. Besides other chlorinated products inaccuracies in the GC measure-

Table 1. Dechlorination of 1,2-DCA by cell suspensions of four strains of methanogenic bacteria.

Strain[a]	Substrate[b]	Incubation-time	(nmole culture^{-1})			
			0 h	After incubation		
		(h)	1,2-DCA	1,2-DCA	Ethylene	CA
M. barkeri	Methanol	24	329 ± 9	271 ± 3	4 ± 0.2	34 ± 2
M. mazei	Methanol	24	356 ± 5	205 ± 17	3 ± 0.2	107 ± 3
M. thermoautotrophicum[c]	H_2/CO_2	48	333 ± 5	260 ± 11	21 ± 2	54 ± 4
M. soehngenii	Acetate	96	246 ± 15	168 ± 12	9 ± 0.3	29 ± 3

[a] Protein content per culture was initially: *M. barkeri* 7.4 mg; *M. mazei* 5.2 mg; *M. thermoautotrophicum* 5.3 mg; *M. soehngenii* 2.3 mg.
[b] Substrate for growth and incubation of cell suspensions.
[c] Incubated at 55° C.

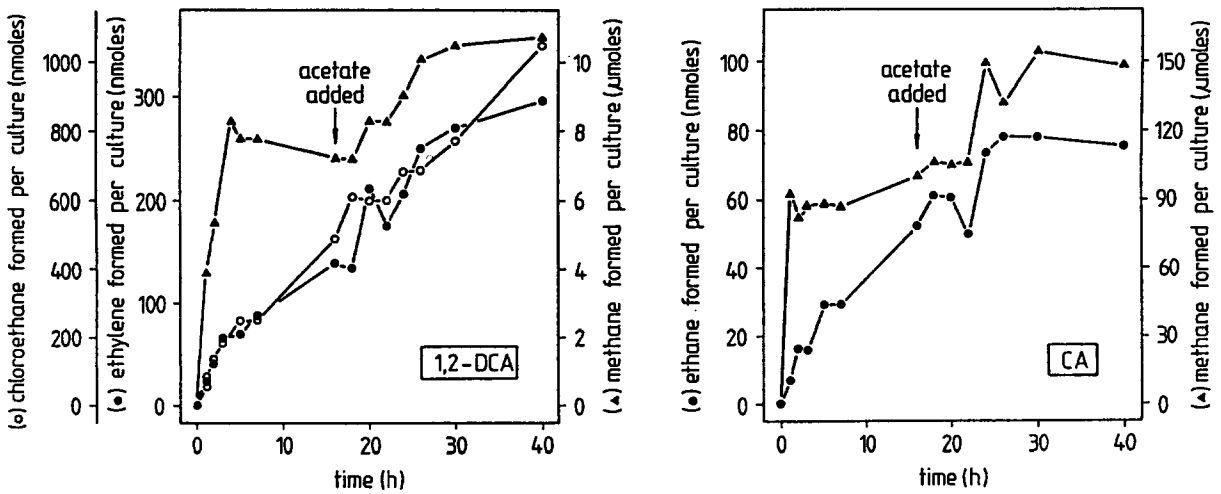

Fig. 1. Dechlorination pattern of 1,2-DCA and CA by concentrated cell suspensions of *M. barkeri* grown and incubated on acetate. Protein content per culture was initially 18.5 mg. 20 mM of acetate was added after 0 and 18 h of incubation. The initial concentration of 1,2-DCA or CA was approx. 1 mM or 5 μmoles per culture, respectively.

ments and sorption to biomass are possible additional factors which could account for the incomplete mass balance. Comparing cells grown on different substrates could give some indication what enzymes are involved in the dechlorination. Therefore, *M. barkeri* was chosen as model-organism for further study.

CA was transformed to ethane by cell suspensions of *M. barkeri.* This dechlorination reaction was inhibited 37–59% in the presence of 1,2-DCA (Table 2). The reductive dechlorination of 1,2-DCA and CA by cell suspensions of *M. barkeri* did not strictly follow methane formation (Fig. 1). Formation of dechlorination products decreased slightly when methane production stopped, and increased with methane formation after a second addition of methanogenic substrate. Interestingly, dechlorination went on even after cease of methane production.

The dependence of dechlorination on biological activity was exhibited by stimulating or inhibiting methanogenesis. Stimulation done by adding different concentrations of methanogenic substrate, resulted in increased amounts of dechlorination products (Fig. 2). The amount of ethylene and CA formed per mole of methane was not constant. In the presence of e.g. 2.5 mM methanol, 1.14 mmoles ethylene and 1.42 mmoles CA were formed per mole of methane; whereas 250 mM methanol resulted only in 0.20 mmoles ethylene and 0.42 mmoles CA per mole of methane. Inhibition of methanogenesis by 2-bromoethanesulfonic acid (BrES) or 1-iodopropane caused a decrease in dechlorination products (Fig. 3). BrES, as an analogue of methyl coenzyme M, is a specific inhibitor of the last step of methane formation. While it completely inhibited formation

Table 2. Ethane production from CA in the presence and absence of 1,2-DCA by cell suspensions of *Methanosarcina barkeri.* [a]

CA[b]	1,2-DCA[b]	Ethylene[c]	Ethane[c]
(nmole culture^{-1})		(nmole culture^{-1})	
500	0	0.0	1.35 ± 0.07
500	5000	65.2 ± 3.3	0.85 ± 0.07
2500	0	0.0	5.55 ± 0.07
2500	5000	74.8 ± 0.8	2.55 ± 0.49
5000	0	0.0	10.40 ± 0.85
5000	5000	64.4 ± 7.1	4.25 ± 2.05
0	5000	67.3 ± 1.8	0.25 ± 0.07

[a] The experiment was carried out with cells grown on methanol. Protein content per culture was initially 8.6 mg. Ethylene and ethane were analyzed by the second method described in 'Materials and methods'.
[b] Added at time 0.
[c] Measured after 24 h of incubation.

of CA, ethylene was still produced even at high BrES concentrations (Fig. 3A). Methanogens can also form ethylene from BrES (Belay & Daniels 1987). To account for this, ethylene formed by cultures containing only BrES was subtracted from ethylene formed on 1,2-DCA and BrES together. Ethylene production on BrES alone was maximum 18% of ethylene formed on 1,2-DCA and BrES. 1-Iodopropane is an inhibitor of corrinoid enzyme catalyzed methyl transfer reactions (Brot & Weissbach 1965) and was used to study the role of a corrinoid enzyme in methane formation (Eikmanns & Thauer 1985). This compound inhibited both ethylene and CA production (Fig. 3B).

An increase in 1,2-DCA or CA concentration resulted in increasing amounts of dechlorination products formed per mole of methane (Fig. 4). Methane production was not affected in the concentration range used in these experiments (data not shown). Cells grown on H_2/CO_2 produced the highest amounts of ethylene, ethane, and CA (Fig. 4). To gain insight into the enzymes responsible for

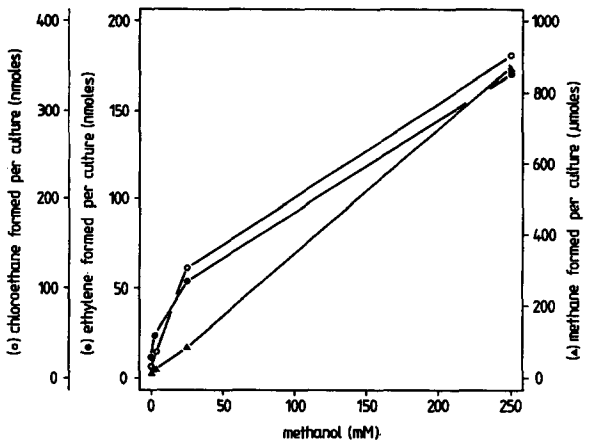

Fig. 2. Effect of stimulation of methanogenesis on dechlorination of 1,2-DCA. Different concentrations of methanol (0, 2.5, 25, and 250 mM) were added to cell suspensions of *M. barkeri* (initially 18.3 mg of protein per culture) and the products were measured after 24 h of incubation. The initial concentration of 1,2-DCA was approx. 1 mM or 5 μmoles per culture, respectively.

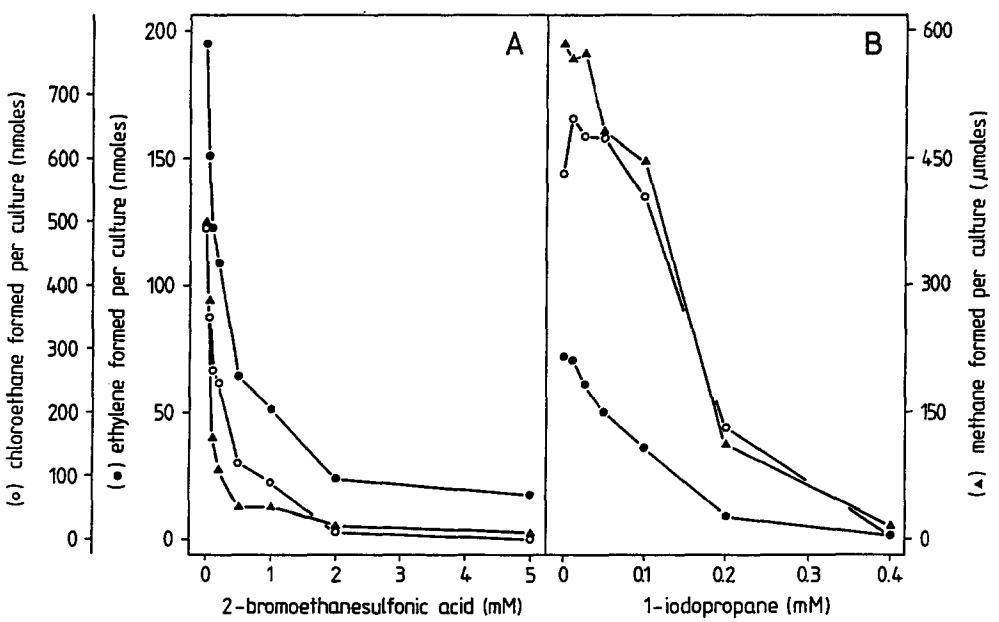

Fig. 3. Effect of inhibition of methanogenesis by (*A*) 2-bromoethanesulfonic acid or (*B*) 1-iodopropane on dechlorination of 1,2-DCA by cell suspensions of *M. barkeri.* The methanogenic substrate was methanol (250 mM) and protein content of the cultures for the experiments with 2-bromoethanesulfonic acid and 1-iodopropane was initially 16.2 mg and 12.5 mg, respectively. 2-Bromoethanesulfonic acid was added by syringe from an aqueous solution, 1-iodopropane from a stock solution in ethanol. The products were measured after 24 h of incubation and the initial concentration of 1,2-DCA was approx. 1 mM or 5 μmoles per culture, respectively.

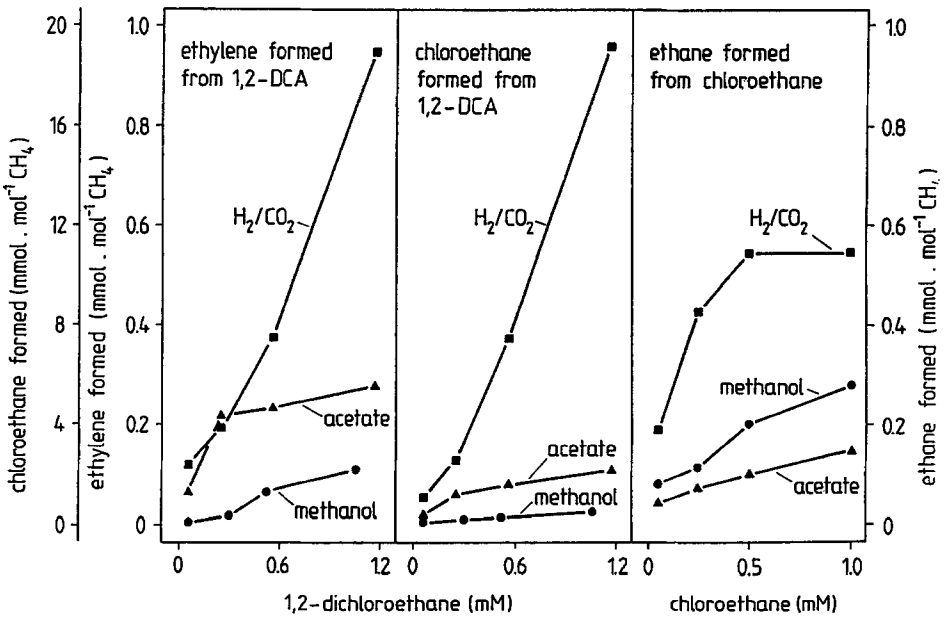

Fig. 4. Effects of different concentrations of 1,2-DCA or CA on product formation rates by cell suspensions of *M. barkeri* grown and incubated on different methanogenic substrates. The cultures on methanol, acetate, and H_2/CO_2 contained initially 7.9 mg, 0.9 mg, and 5.1 mg of protein, respectively. Methanol (250 mM) and acetate (100 mM) were added by syringe from stock solutions, H_2/CO_2 (80%/20%) was present in the gasphase with a pressure of 1.8 bar. Dechlorination products and methane was measured after 24 h of incubation.

the dechlorination reaction, initital production rates by cell suspensions grown and incubated with different substrates were measured (Table 3). The rates, in nmol per mg of protein per h, did not differ significantly among cell suspensions grown and incubated on methanol, acetate, or H_2/CO_2. Approximately the same amounts of dechlorination products were formed per mg of protein per h when CO was provided as electron donor. However, when rates were expressed in mmoles of dechlorination products per mole of methane, cells grown in H_2/CO_2 showed the highest activities. Methane formation on CO could not be quantified because the CO used was contaminated with methane.

Discussion

The transformation of 1,2-DCA to ethylene, catalyzed by methanogens, was shown previously (Egli et al. 1987; Belay & Daniels 1987). We demonstrate here that methanogens also form CA from 1,2-DCA and that CA is dechlorinated to ethane. In addition, we show that the ability to reductively dechlorinate 1,2-DCA is a property of both hydrogenotrophic and acetoclastic methanogenic bacteria and that 1,2-DCA can be dechlorinated by two different reductive reaction-mechanisms (Fig. 5). The transformation of 1,2-DCA to ethylene is a dihalo-elimination and transformation to CA a hydrogenolysis (Vogel et al. 1987). CA is dechlorinated *via* hydrogenolysis.

The inhibition of ethane production by 1,2-DCA is probably due to competition of the two chlorinated compounds for the same electrons. Such a dechlorination pattern, where the dechlorination of a lower chlorinated compound is inhibited by the higher chlorinated compound, has also been observed in mixed microbial systems like sewage sludge or soil for as well aromatic compounds (Suflita et al. 1982; Boyd & Shelton 1984; Mikesell & Boyd 1986; Fathepure et al. 1988; Bosma et al. 1988) as aliphatic compounds (Barrio-Lage et al. 1986; Parsons & Lage 1985; Vogel & McCarty

1985). These sequential dechlorination reactions in pure cultures and in mixed microbial systems indicate that the dechlorination pattern observed in complex communities could also be due to a competition for the same electrons in one kind of organism.

Dechlorination of 1,2-DCA and CA by *M. barkeri* in relation to methane formation differed from the dechlorination pattern reported for tetrachloroethylene by *Methanosarcina* sp. strain DCM (Fathepure & Boyd 1988). The transformation of tetrachloroethylene to trichloroethylene by this organism strongly depended on the amount of methanogenic substrate consumed. In our experiments, however, dechlorination of 1,2-DCA or CA did not strictly follow methanogenesis. This difference may be due to the different systems used. Dechlorination experiments with tetrachloroethylene were performed in growing cultures whereas our experiments were carried out in concentrated cell suspensions. Reducing power could still be present in these cells and may have influenced the rates at low substrate concentrations.

Although reductive dechlorination of 1,2-DCA is catalyzed by pure cultures of methanogens, it is

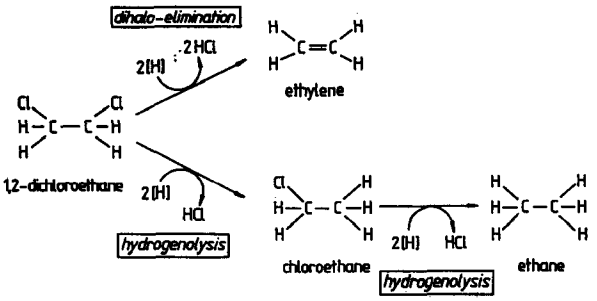

Fig. 5. Degradation pathway of 1,2-DCA by cell suspensions of methanogenic bacteria.

not certain whether these reactions account for significant transformation in mixed cultures. Bouwer & McCarty (1983) found ^{14}C-labeled carbon dioxide from [^{14}C]-1,2-DCA in methanogenic batch cultures seeded with a mixed culture from sewage sludge. Ethylene and ethane, formed in the reductive dechlorination of 1,2-DCA by methanogens, are thought to be stable in methanogenic mixed cultures (Schink 1985, 1988). It is unlikely then, that the mineralization found by Bouwer &

Table 3. Ethylene and CA production rates from 1,2-DCA and ethane production rates from CA by cell suspensions of *Methanosarcina barkeri.*

Substrate		Product formation rates[a]		
		from 1,2-DCA		from CA
Growth	Cell suspension	Ethylene	CA	Ethane
Methanol	Methanol	1.64 ± 0.20 (0.27 ± 0.07)	3.47 ± 0.70 (0.56 ± 0.10)	0.40 ± 0.10 (0.07 ± 0.01)
	Acetate	1.08 ± 0.15 (1.72 ± 0.46)	2.38 ± 0.22 (3.61 ± 0.50)	0.45 ± 0.09 (0.58 ± 0.10)
	H_2/CO_2	2.07 ± 0.18 (0.83 ± 0.16)	5.13 ± 0.33 (2.07 ± 0.24)	1.06 ± 0.15 (0.44 ± 0.10)
	CO	0.95 ± 0.04 n.a.[b]	3.25 ± 0.22 n.a.	0.46 ± 0.04 n.a.
Acetate	Methanol	1.39 ± 0.15 (3.28 ± 0.42)	1.65 ± 0.45 (3.74 ± 0.73)	0.35 ± 0.08 (0.57 ± 0.12)
	Acetate	1.30 ± 0.06 (0.47 ± 0.02)	2.15 ± 0.10 (0.77 ± 0.06)	0.55 ± 0.12 (1.01 ± 0.26)
	H_2/CO_2	1.44 ± 0.27 (0.95 ± 0.24)	2.25 ± 0.30 (1.52 ± 0.52)	0.87 ± 0.19 (0.49 ± 0.08)
	CO	1.33 ± 0.18 n.a.	2.05 ± 0.30 n.a.	0.39 ± 0.05 n.a.
H_2/CO_2	Methanol	0.97 ± 0.38 (4.72 ± 1.50)	4.00 ± 0.55 (16.74 ± 4.19)	0.47 ± 0.08 (1.59 ± 0.28)
	Acetate	1.23 ± 0.14 (15.62 ± 1.86)	3.95 ± 0.40 (47.75 ± 5.85)	0.18 ± 0.04 (1.28 ± 0.26)
	H_2/CO_2	1.32 ± 0.15 (5.52 ± 0.68)	6.25 ± 0.60 (26.09 ± 3.20)	0.41 ± 0.08 (0.83 ± 0.39)
	CO	1.03 ± 0.11 n.a.	4.85 ± 0.85 n.a.	0.15 ± 0.03 n.a.

[a] Rates are expressed in nmole.mg of protein^{-1} · h^{-1}, data in parentheses represent rates in mmole · mole of CH_4^{-1}. After 2 and 4 hours of incubation two cultures were sacrificed for analysis. Product formation was linear within this time-frame. Cultures from cells grown on methanol, acetate, and H2/CO_2 contained initially 26.4 mg, 10.4 mg, and 18.9 mg of protein, respectively. 250 mM of methanol, 100 mM of acetate, 1.8 bar H_2/CO_2 (80%/20%), or 1.2 bar CO/N_2 (80%/20%) were added to cell suspensions at time 0.

[b] n.a. = not applicable because CH_4 formation on CO could not be quantified.

McCarty (1983) resulted from the two C-2 gases formed by the total dechlorination of 1,2-DCA. This indicates that 1,2-DCA could also be degraded *via* reactions other than reductive dechlorination under anaerobic conditions.

Specific rates of ethylene, CA, and ethane production showed that cells grown on H_2/CO_2 had the highest activity relative to methane formation. Studies of the degradation of chloromethanes by anaerobic bacteria suggest that corrinoids (Krone et al. 1989a; Egli et al. 1988. Wood et al. 1968) or, in methanogens, factor F_{430} (Krone et al. 1989b) are involved in the reductive dechlorination of these halocompounds. However, since *M. barkeri* grown on H_2/CO_2 did not contain the highest amounts of corrinoids or factor F_{430} (Krzycki & Zeikus 1980; Gorris & Van den Drift 1986; Dangel et al. 1987; Diekert et al. 1981), this does not explain the highest dechlorination activities for these cells. Studies which compared the activity of oxidoreductases, hydrogenase or methyl-coenzyme M reductase in cell extracts of *M. barkeri* grown on different substrates (Baresi & Wolfe 1981; Krzycki et al. 1982) do not give any indications as to which enzymes could be involved in the cometabolic transformation of 1,2-DCA by methanogenic bacteria. Inhibition experiments with 1-iodopropane do not prove that only corrinoids are involved in the dechlorination reactions because methane formation was inhibited as well. The dihalo-elimination of 1,2-dibromoethane to ethylene by iron(II)porphyrins (Wade & Castro 1973) or by low valent transition metal ions like Cr^{2+} (Castro & Kray 1963) and the hydrogenolysis of 1,1,1-trichloroethane by iron(II) porphyrins (Klecka & Gonisor 1984) yielding 1,1-dichloroethane suggest that cofactors with a tetrapyrrole structure and a transition metal are involved in the cometabolic dechlorination reactions catalyzed by anaerobic bacteria. Research is underway to investigate whether this might be the case for the reductive dechlorination of 1,2-DCA and CA by methanogenic bacteria.

Conclusions

Hydrogenotrophic and acetoclastic methanogenic bacteria dechlorinate 1,2-dichloroethane (1,2-DCA) reductively by two reaction-mechanisms. Ethylene is formed *via* a dihalo-elimination and chloroethane (CA) *via* a hydrogenolysis. CA is transformed to ethane *via* hydrogenolysis. This reaction is inhibited in the presence of 1,2-DCA. The rate of dechlorination is dependent on metabolic activity of the cells; *Methanosarcina barkeri* cells grown on H_2/CO_2 show higher dechlorinating activities than cells grown on methanol or acetate.

Acknowledgements

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