Methanogenic Conversion of 3-S-Methylmercaptocaptopropionate to 3-Mercaptocaptopropionate

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Anaerobic metabolism of dimethylsulfoniopropionate, an osmolyte of marine algae, in anoxic intertidal sediments involves either cleavage to dimethylsulfide or demethylation to 3-S-methylmercaptocaptopropionate (MMPA) and subsequently to 3-mercaptocaptopropionate. The methanogenic archaea Methanosarcina sp. strain MTP4 (DSM 6636), Methanosarcina acetivorans DSM 2834, and Methanosarcina (Methanoblobus) sicilae DSM 3028 were found to use MMPA as a growth substrate and to convert it stoichiometrically to 3-mercaptopropionate. Approximately 0.75 mol of methane was formed per mol of MMPA degraded; methanethiol was not detected as an intermediate. Eight other methanogenic strains did not carry out this conversion. We also studied the conversion of MMPA in anoxic marine sediment slurries. Addition of MMPA (500 μM) resulted in the production of methanethiol which was subsequently converted to methane (417 μM). In the presence of the antibiotics ampicillin, vancomycin, and kanamycin (20 μg/ml each), 275 μM methane was formed from 380 μM MMPA; no methanethiol was formed during these incubations. Only methanethiol was formed from MMPA when 2-bromoethanesulfonate (25 mM) was added to a sediment suspension. These results indicate that in natural environments MMPA could be directly or indirectly a substrate for methanogenic archaea.

Materials and Methods

Sediment sampling, preparation, and incubation. Anoxic intertidal sediment was collected from the Wadden Sea near Westerierland, The Netherlands. The sediment consisted of a black sulfide-rich layer covered by a 0- to 2-cm-thick oxic sandy layer. Sediment cores were taken with perspex cores (10-cm length, 2.5-cm diameter). After sampling, the cores were sealed with butyl rubber stoppers. The samples were transported in an N₂-flushed anaerobic jar at ambient temperature, and suspensions were made in an anaerobic glove box (equipped with catalyst R0-20 from BASF Aktiengesellschaft, Ludwigshafen, Federal Republic of Germany) within a few hours. Fresh sediments contained 40 to 60 μM DMSP measured as DM by headspace analysis after alkanilization of the sample with NaOH (final concentration, 5 M).

The sediment was suspended in degassed seawater (approximately 4 ml/g of sediment [wet weight]). The suspension was thoroughly mixed with a blender for 1 min, and 40-ml aliquots were poured into 70-ml bottles while the sediment was kept in suspension. The bottles were sealed with a screw cap containing a butyl rubber stopper through the central hole; a Viton disc, which is impermeable to volatile sulfur compounds, was placed beneath the rubber stopper (7). The headspace was flushed with oxygen-free N₂ for 2 min. Then, if required, the inhibitor 2-bromoethanesulfonic acid (final concentration, 25 mM) or the antibiotics ampicillin, vancomycin, and kanamycin (20 μg/ml final concentration each) were added from aqueous stocks, and the suspension was incubated at 25°C overnight to remove remaining oxygen; then the incubations were started by addition of the substrate.

Microorganisms and growth conditions. An enrichment culture of MMPA-degrading microorganisms was obtained by inoculation of anoxic sediment (2.5-ml suspension) in bicarbonate-buffered (50 mM) mineral medium (9) with sulfate (20 mM), yeast extract (50 mg liter⁻¹), and MMA (10 to 20 mM). Sulfate was omitted after several transfers into fresh medium. Incubations were done in 120-ml bottles filled with 50 ml of medium at 28°C.

Methanosarina sp. strain MTP4 (DSM 6636) was grown in 120-ml bottles filled with 50 ml of medium as described elsewhere (6) under an atmosphere of N₂-CO₂ (80/20) at 30°C; inoculation (5%) was from late-log-phase cultures. Strain MTP4 was isolated with MT as a substrate by Finster et al. (6) from sediment of a salt marsh near Bordeaux, France. Growth was monitored by measurement of the optical density at 430 nm. The following strains were also used: Methanosarina acetivorans MS (DSM 2834) precultured on methanol (10 mM); Methanosarina mazei C16 (DSM 3318; also known as Methanosarcina frisia) (C16) precultured on methanol (25 mM); Methanosarina sicilae T4M (DSM 3028; this strain was formerly designated Methanolobus sicilae; cf. reference 20) precultured on methanol (25 mM); Methanobacterium sp. strain C8 (DSM 3821) precultured on H₂-CO₂ (80/20); Methanoococcosidex methylatis TMA-10 (DSM 2657) precultured on trimethylamine (25 mM); Methanohalophilus zhiliareae WeN5 (DSM 4017) precultured on trimethylamine (10 mM); Methanospiillum hungatei JF1 (DSM 864) precultured on H₂-CO₂ (80/20) and acetate (2.5 mM); Methanosarina barkeri Fusaro precultured on acetate (5 mM); and Methanosarcina barkeri MS precultured on methanol (10 mM). M. barkeri strains Fusaro and MS were kindly provided by J. T. Keltjens, University of
RESULTS

Enrichment culture. After inoculation of mineral medium containing MMPA (20 mM) and yeast extract (50 mg liter\(^{-1}\)) with anoxic marine sediment, MMPA was converted to methane, with MT as an intermediate. After several transfers into fresh medium, MMPA was still converted to methane; this also took place when sulfate was omitted from the medium. The enrichment culture that was obtained in this way produced 14 mmol of methane per ml of medium. The conversion correlated with an increase in the proportion of MT. These observations made us speculate that methanogenic archaea present in the enrichment culture might have directly converted MMPA to MPA and methane. Because of the morphological similarity of the methanogens present in the enrichment culture to coccoid Methanosarcina strains and the ability of Methanosarcina sp. strain MTP4 to metabolize MT (6), a possible intermediate of MMPA degradation, we tested strain MTP4 for the ability to convert MPA to MTPA and methane.

Conversion of MMPA by pure cultures of methanogenic archaea. Methanosarcina sp. strain MTP4 was found to be able to grow with MPA as a substrate. A lag phase of approximately 7 days was observed when a methanol-grown culture was transferred to medium containing MMPA as a substrate. Transfer of an MMPA-grown culture to fresh medium with methanol or MPA as a substrate gave no significant lag phase. During growth, MMPA was converted to MPA and methane (Fig. 1A). The specific growth rate was 0.033 h\(^{-1}\) (doubling time \(t_d\), 21 h), on the basis of exponential production of methane between hours 51 and 119. In a separate experiment, the conversion stoichiometry was determined; from 13.5 mM MMPA, 13.5 mM MPA and 10.2 \(\mu\)mol of methane per ml of medium were formed. The conversion corresponded to the following reaction: 4MMPA + 2\(\text{H}_2\text{O} \rightarrow 4\text{MPA} + \text{CO}_2 + 3\text{CH}_4\). The identity of the organic compound formed after growth of strain MTP4 on MMPA was established to be MPA by \(^3\)H nuclear magnetic resonance and cochromatography (HPLC and gas chromatography) with authentic MPA as a reference (data not shown).

Ten other methanogenic strains were tested for the ability to grow on MMPA. Only M. acetivorans DSM 2834 (see Fig. 1B) and M. siciliae DSM 3028 (data not shown) were found to be able to grow on MMPA; the latter strain grew more slowly than strain MTP4 and M. acetivorans. M. mazei DSM 3318, Methanobacterium sp. strain C8 DSM 3821, Methanococciodes methylutens DSM 2657, Methanohalophilus zhilinaeae DSM 4017, Methanospirillum hungatei DSM 864, M. barkeri Fusaro, M. barkeri MS, and Methanococciodes sp. strain PM2 did not grow with MMPA as a single substrate and did not convert MMPA during growth on their regular substrate (see Materials and Methods). The MMPA-utilizing methanogenic strains were unable to convert DMSP.

FIG. 1. (A) Growth of Methanosarcina sp. strain MTP4 on MMPA (13.5 mM). (B) Growth of M. acetivorans DSM 2834 on MMPA (10 mM). Symbols: •, optical density at 430 nm (OD\(_{430}\)); ◦, methane; ◇, MPA; ■, MPA. Cultures were grown in 120-ml crimp-seal bottles with 50 ml of medium under an atmosphere of \(\text{N}_2\)-\(\text{CO}_2\) (80:20 [vol/vol]). The methane line indicates the total amounts present in both the gas and the liquid.
Conversion of MMPA in sediment suspensions. MT was formed within 1 day after addition of MMPA (500 μM) to a sediment suspension (Fig. 2A). The maximum concentration of MT was approximately 200 μM; MT started to decrease after 2 days. No MT could be detected after 5 days. Methane formation started in the same period and reached its maximum after 5 days (417 μM). No MT was formed in the presence of the antibiotics ampicillin, vancomycin, and kanamycin (Fig. 2B). Methane formation in these incubations was much slower than in the incubations without additions. The concentration of MMPA decreased at a rate similar to the increase in the concentration of methane. MT but not methane was formed when 2-bromoethanesulfonic acid (25 mM) was added to the suspension.

DISCUSSION

This is the first report in which it is shown that a pure culture of a methanogenic archaeon can utilize MMPA as a substrate for growth. It adds to the limited number of compounds that are known as methanogenic substrates or electron donors for methanogenesis: H₂-CO₂, formate, CO, methanol, acetate, tri-, di-, and monomethylamine, dimethylsulfide, MT, 1-propanol, 2-propanol, ethanol, 1-butanol, 2-butanol, 1,3-butanediol, cyclopentanol, and pyruvate (1, 6, 21–23, 33). The most important methanogenic substrates usually are H₂-CO₂ and acetate, but in marine environments methylated compounds such as trimethylamine and DMS are thought to predominate (33). *Methanosarcina* strain MTP4 utilizes MMPA as a typical C₁ substrate and demethylates it to MPA. *Methanosarcina* strain MTP4 was isolated from a salt marsh with MT as the enrichment substrate (6); it can also grow on DMS. DMS and MT can be formed from methoxylated aromatics (3), but in the marine environment DMS is most probably the major source of DMS. DMS is also a precursor of MMPA, as suggested by sediment slurry experiments (16, 17) and shown in pure culture studies with *Desulfobacterium* sp. strain PM4 (29). Thus, strain MTP4 originates from an environment in which both DMS and MMPA are present. Similarly, *M. acetivorans* DSM 2834 was isolated from marine sediment and is now known to metabolize both DMS (20) and MMPA (this study). *M. siciliae* DSM 3028 was shown to be closely related to *M. acetivorans* (20).

The biochemical mechanism of MMPA demethylation by methanogens is still obscure. Wackett et al. (32) showed that in crude cell extracts of H₂-CO₂-grown *Methanobacterium thermoautotrophicum* ΔH, MMPA, which is a structural analog of methyl-S-coenzyme M, can serve as a substrate for the methyl-S-coenzyme M reductase, an enzyme involved in the last step of methanogenesis (4). It is therefore possible that strain MTP4 is able to take up MMPA and use the methyl-S-coenzyme M reductase to convert MMPA. However, of the 11 methanogenic strains tested, only *Methanosarcina* sp. strain MTP4, *M. acetivorans* DSM 2834, and *M. siciliae* DSM 3028 were able to convert MMPA. It is therefore not very likely that MMPA utilization by strain MTP4 and strain DSM 2834 is due to a general lack of specificity of the methyl-S-coenzyme M reductase. Alternatively, an MMPA-coenzyme M methyltransferase system might be used in the conversion of MMPA. Specific methyltransferases are known to be involved in the metabolism of methanol and methylamines, respectively (4).

The methanogenic conversion of MMPA to MPA has also been found to occur in slurries prepared from anoxic marine sediments, but only when antibiotics were added. Under normal conditions, MMPA was readily converted to MT and presumably acrylate. MT is further converted to methane by methanogenic archaea. These results suggest that in situ MMPA can serve as a substrate for methanogenic archaea but that the major pathway for conversion might be demethylation; it should be kept in mind, however, that at the low natural concentrations of MMPA the ratio between demethylation and demethiolation may be very different. Kiene and coworkers (14, 17) concluded that demethylation is a major transformation pathway for MMPA in intertidal sediments. They suggested that *Eubacterium limosum*-like bacteria might be responsible for the sequential demethylation of DMS. Thus far, acetogenic bacteria that can demethylate DMS have not been isolated. The combined activities of DMSP-demethylating, sulfate-reducing bacteria (29) and MMPA-demethylating methanogenic archaea may also be responsible for the observed conversion of DMSP to MPA.
Cleavage of DMSP results in the formation of DMS. Part of the DMS escapes to the atmosphere, where it is oxidized to sulfuric acid and methanesulfonic acid (2). These compounds may act as cloud condensation nuclei, and thus DMS may exert a negative effect on global warming. Anaerobic metabolism of DMS results in the formation of methane (6,15), which can act as a greenhouse gas (10), although part of the DMS might be oxidized to CO₂ by sulfate-reducing bacteria (15). Demethylation as well as demethiolation of MMPA can also result in the direct or indirect formation of methane. Part of the methane that is formed in the anoxic sediment can be oxidized in the upper oxic layer by methane-oxidizing bacteria, but methane fluxes from salt marshes into the atmosphere have been found to exist (25). Therefore, we conclude that anaerobic demethylation of DMS results in the production of a positive effector (methane) of global warming, whereas the cleavage into DMS and acrylate leads to both a positive (methane) and a negative (DMS) effector.

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