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Methanogenic Conversion of 3-*S*-Methylmercaptopropionate to 3-Mercaptopropionate

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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*-methylmercaptopropionate (MMPA) and subsequently to 3-mercaptopropionate. The methanogenic archaea *Methanosarcina* sp. strain MTP4 (DSM 6636), *Methanosarcina acetivorans* DSM 2834, and *Methanosarcina (Methanobolus) sicilicae* 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 μ M) resulted in the production of methanethiol which was subsequently converted to methane (417 μ M). In the presence of the antibiotics ampicillin, vancomycin, and kanamycin (20 μ g/ml each), 275 μ M methane was formed from 380 μ M 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.

Dimethylsulfoniopropionate (DMSP), an osmolyte found in many marine algae and certain plants (8, 11, 24), is the major source of dimethylsulfide (DMS) in the marine environment. In the atmosphere, DMS contributes through its oxidation products methanesulfonic acid and sulfuric acid to cloud formation and acid precipitation (2). DMSP and DMS are biologically converted in oxic as well as anoxic environments (12, 13, 15, 17, 18, 27). The production of DMSP by benthic algae and the settling of dead algae cause the presence of DMSP in marine sediments. Relatively high concentrations of DMSP (from approximately 1 up to 70 to 110 μ mol liter of sediment⁻¹) have been measured in the surface sediment of a salt marsh and other types of intertidal sediments (12, 30). In estuarine carbonate sediments, DMSP is degraded by cleavage to DMS and acrylate or by a demethylation to 3-*S*-methylmercaptopropionate (MMPA) and subsequently to 3-mercaptopropionate (MPA [16, 17]). MMPA is also degraded to methanethiol (MT) and presumably acrylate (17); acrylic acid and hydrogen sulfide can slowly react to form MPA in a chemical process (28). Kiene and Taylor (17) suggested that a *Eubacterium limosum*-like organism might be responsible for the sequential demethylation of DMSP to MPA. Thus far, no anaerobic microorganisms that are able to demethylate DMSP all the way to MPA have been isolated. Anoxic most-probable-number incubations (in the presence of 5 mM NO₃⁻) of mud from the upper 5 mm of a cyanobacterial mat indicated the presence of considerable populations of MPA-producing microorganisms (30), but the characteristics of the organisms are unknown. Recently, it was shown that the marine sulfate-reducing bacterium *Desulfobacterium* strain PM4 demethylates DMSP to MMPA (29). This organism is not able to demethylate MMPA to MPA. In this communication, we present data that show that some methanogenic archaea are able to demethylate MMPA to MPA.

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MATERIALS AND METHODS

Sediment sampling, preparation, and incubation. Anoxic intertidal sediment was collected from the Wadden Sea near Westermierland, 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 μ M DMSP measured as DMS by headspace analysis after alkalization 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 μ g/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 MMPA (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.

Methanosarcina 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: *Methanosarcina acetivorans* MS (DSM 2834) precultured on methanol (10 mM); *Methanosarcina mazei* C16 (DSM 3318; also known as "*Methanosarcina frisia*" C16) precultured on methanol (25 mM); *Methanosarcina sicilicae* T4/M (DSM 3028; this strain was formerly designated *Methanobolus sicilicae*; cf. reference 20) precultured on methanol (25 mM); *Methanobacterium* sp. strain C8 (DSM 3821) precultured on H₂-CO₂ (80:20); *Methanococcoides methylutens* TMA-10 (DSM 2657) precultured on trimethylamine (25 mM); *Methanohalophilus zhilinaeae* WeN5 (DSM 4017) precultured on trimethylamine (10 mM); *Methanospirillum hungatei* JF1 (DSM 864) precultured on H₂-CO₂ (80:20) and acetate (2.5 mM); *Methanosarcina 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

Nijmegen, The Netherlands. All of these strains were cultivated in the media as described in the Deutsche Sammlung von Mikroorganismen und Zellkulturen catalog of strains (3). *Methanococcoides* sp. strain PM2 (culture collection of the Department of Microbiology, University of Groningen), precultured on methanol (10 mM), was cultivated in the medium of Heijthuisen and Hansen (9). The strains were grown at 37°C except for the *Methanococcoides* strains (30°C), *Methanohalophilus zhilinaeae* (45°C) and *Methanobacterium* sp. strain C8 (30°C).

Analyses. Headspace analyses of methane and MT were performed by a slightly modified method of Visscher and van Gernerden (31), in which a Supelpak S instead of a Porapak column was used. The concentration of MT in the liquid phase was calculated by use of a distribution coefficient of 7.9 (13). In pure culture studies, methane was measured by gas chromatography on a Porapak Q column with thermal conductivity detection (9).

MMPA and MPA were measured after esterification of 0.5 ml of sample (after centrifugation to remove cells or sediment) with methanol in sulfuric acid (50%) by the gas chromatographic method of Laanbroek et al. (19) for analysis of lactate. Succinate was used as an internal standard. MMPA and MPA were also analyzed by a high-performance liquid chromatography (HPLC) method as described previously (29).

Chemicals. MMPA was made by alkaline hydrolysis of its methyl ester (Aldrich, Steinheim, Federal Republic of Germany) as described by Wackett et al. (32). The identity and purity of the product were checked by ¹H nuclear magnetic resonance; the MMPA content was estimated by organic carbon analysis. MPA was obtained from Aldrich.

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 mM methane from 20 mM MMPA. No MT or acrylate could be detected. Epifluorescence microscopy showed large numbers of irregular coccoid cells which had a characteristic fluorescence at 420 nm. After treatment with the antibiotics ampicillin, vancomycin, and kanamycin (20 μg ml⁻¹ final concentration each), which all act against bacteria but not against archaea, MMPA was still converted to methane and MPA. When 2-bromoethanesulfonic acid (25 mM), a specific inhibitor of methanogenesis (26), was added, no MMPA was converted to MPA and methane. 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 MMPA to MPA and methane.

Conversion of MMPA by pure cultures of methanogenic archaea. *Methanosarcina* sp. strain MTP4 was found to be able to grow with MMPA 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 MMPA 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 μmol of methane per ml of medium were formed. The conversion corresponds to the following reaction: 4MMPA + 2H₂O → 4MPA + CO₂ + 3CH₄. The identity of the organic compound formed after growth of strain MTP4 on MMPA was established to be MPA by ³H nuclear magnetic resonance and

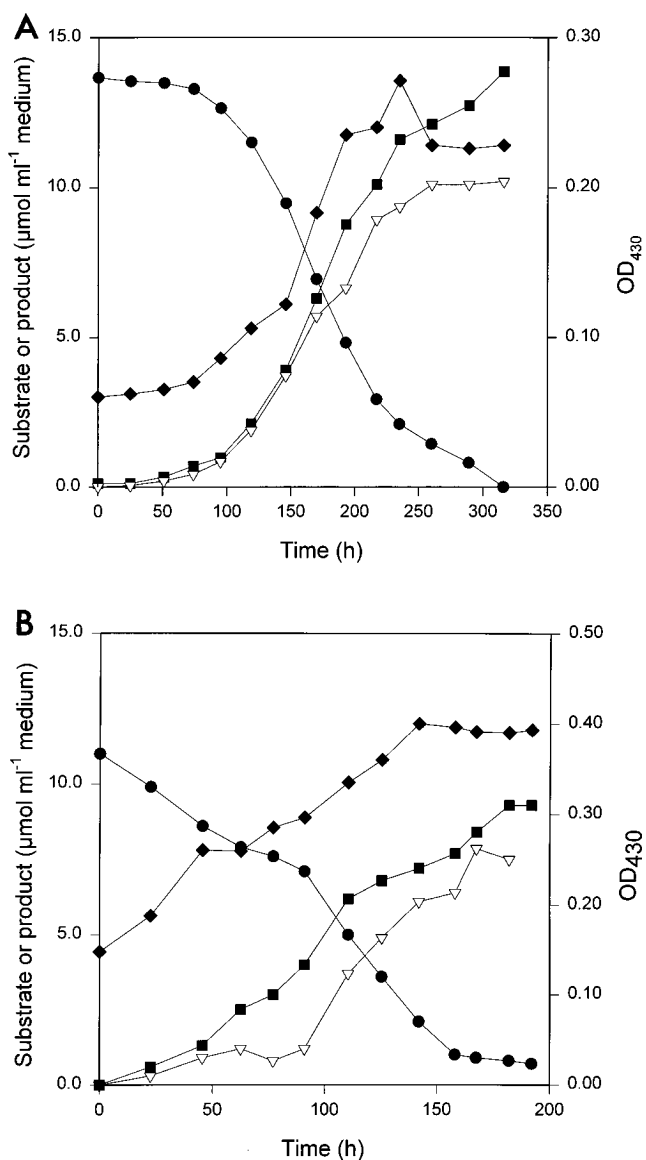


FIG. 1. (A) Growth of *Methanosarcina* sp. strain MTP4 on MMPA (13.5 mM). (B) Growth of *M. acetivorans* DSM 2834 on MMPA (10 mM). Symbols: ◆, optical density at 430 nm (OD₄₃₀); ▽, methane; ●, MMPA; ■, MPA. Cultures were grown in 120-ml crimp-seal bottles with 50 ml of medium under an atmosphere of N₂-CO₂ (80:20 [vol/vol]). The methane line indicates the total amounts present in both the gas and the liquid.

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. mazeii* DSM 3318, *Methanobacterium* sp. strain C8 DSM 3821, *Methanococcoides methylutens* DSM 2657, *Methanohalophilus zhilinaeae* DSM 4017, *Methanospirillum hungateii* DSM 864, *M. barkeri* Fusaro, *M. barkeri* MS, and *Methanococcoides* 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.

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: $\text{H}_2\text{-CO}_2$, 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 $\text{H}_2\text{-CO}_2$ 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 C_1 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 (5), but in the marine environment DMSP is most probably the major source of DMS. DMSP 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 $\text{H}_2\text{-CO}_2$ -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

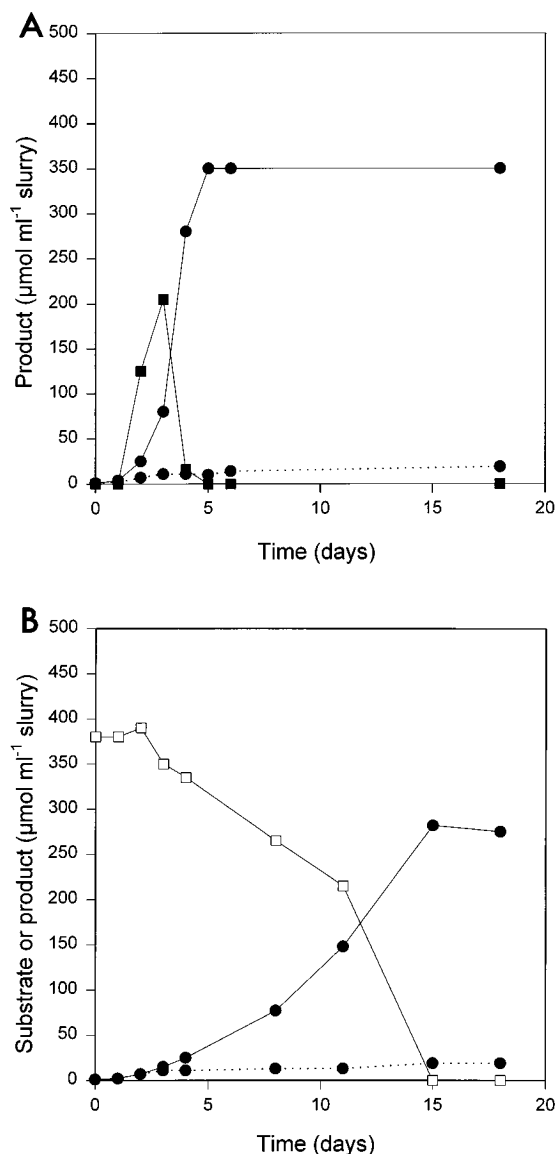


FIG. 2. (A) Accumulation of MT (■) and methane (●) in a sediment suspension after the addition of MMPA (500 μM). (B) Accumulation of methane (●) in a sediment suspension after the addition of MMPA (□) and antibiotics; no MT was detected in the presence of antibiotics. Dotted lines are controls without MMPA addition. Sediment slurries (40 ml) were incubated in 70-ml crimp-seal bottles under a nitrogen atmosphere. The methane and MT lines indicate the total amounts present in both the gas and liquid.

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 DMSP. Thus far, acetogenic bacteria that can demethylate DMSP 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 DMSP 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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