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Investigation of Mercaptans, Organic Sulfides, and Inorganic Sulfur Compounds as Sulfur Sources for the Growth of Methanogenic Bacteria

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Abstract. A variety of compounds were investigated for use as sulfur sources for the growth of methanogenic bacteria. *Methanococcus* (*Mc.*) *deltae*, *Mc. maripaludis*, *Methanobacterium* (*Mb.*) species *GC-2B*, *GC-3B*, and *MMY*, *Methanobrevibacter* (*Mbr.*) *ruminantium*, and *Methanosarcina* (*Ms.*) *barkeri* strain 227 grew well with sulfide, S°, thiosulfate, or cysteine as sole sulfur source. *Mbr. ruminantium* was able to grow on $SO_4^=$ or $SO_3^=$, and *Ms. barkeri* strain 227 was able to grow on $SO_3^=$, but not on $SO_4^=$ as a sole sulfur source. *Mc. jannaschii* grew with sulfide, S°, thiosulfate or $SO_3^=$, but not on cysteine or $SO_4^=$ as sole surface source. *Mc. thermolithotrophicus*, *Mc. jannaschii*, *Mc. deltae*, and *Mb. thermoautotrophicum* strains Marburg and ΔH were able to grow with methanethiol, ethanethiol, *n*-propanethiol, *n*-butanethiol, methyl sulfide, dimethyl sulfoxide, ethyl sulfide, or CS_2 as a sulfur source, when very low levels (20–30 μM) of sulfide were present; no growth occurred on 5–100 μM sulfide alone. Methanethiol, ethanethiol, and methyl sulfide-using cultures produced sulfide during growth.

Methanogenic bacteria have been studied for many years, but recent technical advances with tubes or bottles pressurized with the substrates H_2–CO_2 (80 : 20 vol/vol) have facilitated small-scale growth of these bacteria [1, 2]. This system involves using O_2-free medium with Na_2S or cysteine or both to assure strict anaerobic conditions and provide a sulfur source. The present understanding in regard to sulfur nutrition and metabolism in these bacteria is limited [36].

It has been thought that methanogenic bacteria require fully reduced sulfur (i.e., sulfide) as a sulfur source [5, 6, 13, 16, 22, 26–28, 35, 37], but recently Stetter and Gaag [30] and Daniels et al. [9] have shown that many methanogens can reduce elemental sulfur to sulfide. Most recently, Daniels et al. [10] have demonstrated the effects of a wide range of inorganic sulfur-containing compounds (especially sulfate, sulfite, and thiosulfate) on the growth of four methanogens. In continuation of these studies we report here the effects of sulfate, sulfite, thiosulfate, and cysteine on the growth of several other methanogenic bacteria, especially from the marine environment. Also, since fermentor cultures of *Mb. thermoautotrophicum* strain Marburg produce mercaptans (B.S. Rajagopal, unpublished data), we investigated the use of mercaptans (methanethiol, ethanethiol, *n*-propanethiol and *n*-butanethiol) as sulfur sources for growth, as well as methyl sulfide (diemthyl sulfide) and its oxidized product dimethyl sulfoxide (DMSO), ethyl sulfide (diethyl sulfide), carbon disulfide (CS_2), and carbonyl sulfide (COS). We report that a wide variety of sulfur compounds, including all the mercaptans and organic sulfides examined, serve as sulfur sources for the methanogens studied.

Materials and Methods

Organisms. *Methanococcus (Mc.)*[1] *deltae* strains ΔRC and ΔLH, *Mc. maripaludis*, *Methanobacterium* (*Mb.*) species *MMY*, *GC-2B*, and *GC-3B* were obtained from Dr. J. Reeve [7, 14, 18]. *Mc. jannaschii* [17] and *Methanobrevibacter* (*Mbr.*) *ruminantium* [1] were gifts of Dr. R.S. Wolfe. *Mc. thermolithotrophicus* was obtained from Dr. K.O. Stetter [15], and *Mb. thermoautotrophicum* strain ΔH (DSM 1053 = ATCC 29096) was a gift of Dr. J. Winter [39]. *Mb. thermoautotrophicum* strain Marburg (DSM 2133) was provided by Dr. Georg Fuchs [12]. *Methanosarcina* (*Ms.*) *barkeri* strain 227 was obtained from Dr. S.H. Zinder [21].

[1] Since all methanogen genus names begin with M, for clarity we use the nomenclature for abbreviation suggested by Daniels et al. [11].

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Medium and growth conditions. Cells were grown by a modification of the method of Balch and Wolfe [1]. *Mc. deltae* strains ΔRC and ΔLH were grown at 37°C in a medium modified from that previously described [7]; it consisted of the following components (mM) in distilled and deionized water: K_2HPO_4 (1.72), KH_2PO_4 (2.2), NaCl (513.3), sodium acetate (12.2), NH_4Cl (50.5), $MgCl_2 \cdot 6H_2O$ (20), Na_2CO_3 (1.9), resazurin (0.003). $MgCl_2$ was added separately after autoclaving; 10 ml/liter each of 100 × concentrated solution of trace elements [10] and vitamin mix [38] was also added. The pH of the medium was adjusted to 6.9 by addition of Na_2CO_3 while the medium was flushed with H_2–CO_2 (80:20 vol/vol).

Mc. maripaludis was grown at 37°C in a medium modified from that previously described [18], which consisted of the following components (m*M*): K_2HPO_4 (0.8), KCl (4.43), NH_4Cl (4.67), $CaCl_2 \cdot 2H_2O$ (0.34), NaCl (342.23), $MgCl_2 \cdot 6H_2O$ (15), Na_2CO_3 (1.9), resazurin (0.003); the mineral elixir and vitamin mix were the same as for *Mc. deltae*. $MgCl_2$ was added separately after autoclaving. The pH was adjusted to 6.8 in the same manner as for *Mc. deltae*. *Mc. jannaschii* and *Mc. thermolithotrophicus* were grown at 62°C in a medium described previously [10].

Mbr. ruminantium was grown at 37°C in a medium modified from that previously described [1]; it consisted of the following components (m*M*): K_2HPO_4 (5.74), NH_4Cl (3.36), NaCl (15.4), $MgCl_2 \cdot 6H_2O$ (0.58), sodium acetate (30.5), $CaCl_2 \cdot 2H_2O$ (0.11), $NaHCO_3$ (29.8), Coenzyme M (0.76×10^{-3}), resazurin (0.003); the mineral elixir and vitamin mix were the same as for *Mc. deltae*; yeast extract and trypticase were added at 0.5 g/liter each; isobutyric, α-methylbutyric, isovaleric, and valeric acids were each added at a final concentration of 0.05% (vol/vol); Tween 80 was added at a final concentration of 0.002% (vol/vol); pH was adjusted to 7.0 with Na_2CO_3 while the medium was gassed with H_2–CO_2 (80:20 vol/vol).

Methanobacterium species *GC-2B* and *GC-3B* were grown at 37°C in a medium that consisted of the following components (m*M*): K_2HPO_4 (1.72), KH_2PO_4 (2.2), NaCl (10.26), sodium acetate (12.2), NH_4Cl (50.5), $CaCl_2 \cdot 2H_2O$ (0.05), $MgCl_2 \cdot 6H_2O$ (0.49), Na_2CO_3 (1.9), resazurin (0.003); the mineral elixir and vitamin mix were the same as for *Mc. deltae*. The pH was adjusted to 6.5 in the same manner as described for *Mc. deltae*. *Methanobacterium* species *MMY* was grown in the same medium, but was supplemented with 428 m*M* NaCl and 38 m*M* $MgCl_2 \cdot 6H_2O$. *Mb. thermoautotrophicum* strain ΔH and Marburg were grown at 62° as described previously [10]. *Ms. barkeri* strain 227 was grown at 37°C in a medium described previously [24].

The techniques for the preparation of media were as described previously [10]. Elemental sulfur was added as a solid prior to making the media anaerobic. When sulfide was the sulfur source, $Na_2S \cdot 9H_2O$ (1.8 m*M*) was added by syringe from an anaerobic stock solution after the media were made anaerobic. The tubes with about 70 kPa overpressure of H_2–CO_2 were then autoclaved. Breakage of the tubes during this procedure owing to vacuum or pressure is very rare. After sterilization, sodium salts of sulfate, sulfite, thiosulfate or cysteine, or 2-mercaptoethanol (2-ME) were added to the tubes without sulfide or S° by sterile syringe from filter-sterilized stock solutions prepared under a gas phase of argon. Before inoculation with cultures, the tubes (except those containing sulfide as a sulfur source, which were pressurized after inoculation) were re-evacuated, flushed, and pressurized with 140 kPa H_2–CO_2 (80:20 vol/vol) by use of sterile hoses.

The experiments with mercaptans and organic sulfides as sulfur source were done in 250-ml stoppered bottles (Wheaton Scientific no. 223950 sealed with cutoff no. 1 black stoppers and 30-mm OD aluminum seals, Wheaton Scientific no. 224187), as we were unable to obtain appreciable growth when the experiments were conducted in tubes. Medium (50 ml) was dispensed into these bottles and made anaerobic, as described previously [10]; due to larger volume, evacuation and gassing require considerably more time than with tubes. (Unlike the safety record of tubes, bottles present an explosion hazard when removing them from the autoclave; we have developed a protective cover from a large plastic bottle for use during autoclaving [8].) Solutions of mercaptans (methanethiol, ethanethiol, *n*-propanethiol, and *n*-butanethiol) and organic sulfides (methyl sulfide and its oxidized product, DMSO [dimethylsulfoxide], ethyl sulfide, and CS_2) were made in sterile anaerobic distilled and deionized water, and these solutions were resterilized using Millex-GV syringe filters (Millipore Corporation, Bedford, Massachusetts; prior to filtering, the filters were made anaerobic with sterile anaerobic water). These solutions were not evacuated, as some of the compounds might be lost under vacuum. Required quantities of these solutions (kept cold at 4°C in an ice bucket) were added to the bottles with a sterile syringe before the cultures were inoculated. Carbonyl sulfide (COS) was transferred from the cylinder into a sterile anaerobic bottle through a rubber hose fitted with cutoff glass–metal Luer lock 3-ml syringe and Millex-GV filter unit, and the required quantity of the gas was added from this bottle with a sterile syringe fitted with gas-tight Mininert syringe valve (Alltech Associates, Deerfield, Illinois). After inoculation with cultures, all the bottles were pressurized with 140 kPa H_2–CO_2 (80:20 vol/vol) through sterile hoses.

Inocula for sulfur source experiments were prepared in 250-ml bottles containing 50 ml medium and 0.7 m*M* sulfide, thus giving a maximum of about 70 μ*M* carryover sulfide in the inoculum since inocula were typically 10%.

Incubation was carried out with the tubes and bottles horizontal in a gyratory shaker at 150 rpm unless indicated otherwise. Growth was measured by absorbance at 600 nm with a Spectronic 20 spectrophotometer. All data points were averages of triplicate tubes. All the cultures were routinely checked for contaminants by microscopic (phase contrast) examination, by inoculation into medium under N_2–CO_2 (80:20 vol/vol) atmosphere supplemented with 0.2% each of yeast extract and peptone, and 0.05% each of glucose, sucrose, xylose, and glycerol, and by growth in sulfide or other sulfur sources ($SO_4^=$, $SO_3^=$, or thiosulfate) in the presence of antibiotics (53.6 μg/ml each of streptomycin and kanamycin for *Mc. jannaschii*, and 53.6 μg/ml each of streptomycin and vanomycin for other organisms). None of the cultures showed any contaminants.

Analysis of sulfur-containing compounds. The gas phase (by direct injection [3]) and liquid phase (by benzene extraction [33]) of uninoculated and inoculated tubes containing different sulfur compounds was analyzed for volatile sulfur compounds with a Shimadzu GC 9A chromatograph equipped with a flame photometric detector (394 nm) and a 3 m × 3.2 mm (ID) Teflon (FEP) column packed with chromosil 330. The gas chromatograph was operated at 70°C with H_2 flow rate of 105 ml/min, He flow rate of 50 ml/min, and air flow rate of 55 ml/min. Also, dissolved sulfide was measured spectrophotometrically by a chemical assay [32].

Results

Examination of uninoculated media for the abiological production of reduced sulfur compounds. As an aid to the interpretation of the sulfur source experi-

Table 1. Use of various sulfur sources for growth by methanogens

Sulfur source	Substrate concentration (m*M*)	A_{600}[a] *Methanococcus deltae* Strain ΔRC		*Methanococcus deltae* Strain ΔLH		*Methanococcus maripaludis*		*Methanococcus jannaschii*	
		A	B	A	B	A	B	A	B
Sulfide[b]	0.07	0.04	0.08	0.04	0.05	0.05	0.06	0.09	0.08
Sulfide	1.90	0.85	0.45	0.88	0.40	0.85	0.42	0.85	0.55
Sulfate	3.65	0.05	0.10	0.05	0.06	0.05	0.06	0.05	0.08
Sulfite	0.50	0.06	0.08	0.04	0.05	0.04	0.05	0.80	0.15
Thiosulfate	4.00	0.88	0.48	0.92	0.52	0.75	0.50	0.70	0.45
Sulfur	3.6 mg/ml	0.92	0.54	0.80	0.45	0.90	0.48	0.85	0.50
Cysteine	3.90	0.90	0.80	0.90	0.85	0.80	0.85	0.05	0.06
		Methano-brevibacter raminantium		*MMY*		*Methanobacterium* *GC-2B*		*Methanobacterium* *GC-3B*	
		A	B	A	B	A	B	A	B
Sulfide	0.07	0.12	0.15	0.09	0.08	0.08	0.09	0.10	0.12
Sulfide	1.90	0.82	0.45	0.95	0.40	0.90	0.54	0.95	0.40
Sulfate	3.65	0.80	0.14	0.15	0.12	0.14	0.15	0.12	0.15
Sulfite	0.50	0.50	0.12	0.12	0.14	0.12	0.14	0.14	0.15
Thiosulfate	4.00	0.80	0.45	0.85	0.45	0.80	0.45	0.80	0.50
Sulfur	3.6 mg/ml	0.78	0.40	0.95	0.40	0.95	0.52	0.90	0.48
Cysteine	3.90	0.88	0.90	0.95	0.80	0.90	0.82	0.95	0.90

A, sulfur source without 2-mercaptoethanol.
B, sulfur source with 10 m*M* 2-mercaptoethanol.
[a] Maximum A_{600} was recorded after incubation for 20–120 h for *Mc. deltae* and *Mc. maripaludis*, 15–30 h for *Mc. jannaschii*, and 3–12 days for *Mbr. ruminantium* and *Methanobacterium* species. An absorbance of <0.2 is taken as no growth.
[b] Residual from inoculum.

ments described below, the headspace and liquid of media containing different sulfur compounds were examined by gas chromatography to detect the abiological production of volatile sulfur compounds during incubation for 30 days at both 37° and 62°C. Thiosulfate, sulfate, sulfite, and S° yielded no detectable volatile compounds during abiological incubation, but when 2-mercaptoethanol (2-ME) (2.5 m*M*) was present with thiosulfate (4 m*M*) or S° (4 mg/ml), sulfide was produced at a level of 0.5–0.9 m*M*. None of the mercaptans or organic sulfides yielded detectable sulfide or other volatile compounds. However, COS was chemically unstable at both 37° and 62°C and yielded large quantities of sulfide.

Sulfur sources used for growth. Tables 1 and 2 describe the ability of several sulfur compounds (inorganic and organic) to serve as sulfur sources for growth of several methanogenic bacteria. Because of previous workers' use of 2-ME as a medium reductant [4, 5], the data describe experiments done either in the presence or absence of 2-ME. In all cases, carryover sulfide (0.07 m*M*) or 2-ME did not result in growth, while added sulfide (1.8 m*M*) allowed full growth.

The initial growth (i.e., cells transferred from sulfide medium into medium containing other sulfur sources) of all the cultures on thiosulfate, sulfate, sulfite, cysteine, mercaptans, or other sulfur sources under normal tube-shaking conditions was preceded by a lag of 12–20 h, growth was often inconsistent, and the medium in some of the cultures turned pink owing to oxidation during this lag period. However, when cells were incubated initially without shaking (with the tubes or bottles in upright position) for 12–20 h, followed by normal shaking, repeatable growth could be observed. Thus, for the first transfer it was decided to incubate all the cultures without shaking for the first 12–20 h of growth irrespective of sulfur source. After the initial adaptation to these sulfur sources, all the cultures were repeatedly transferred without a lag. None of the cultures showed a lag with sulfide or S° as sulfur source.

Sulfide. All methanogens used in this study ex-

Table 2. Use of mercaptans and organic sulfides as sulfur source for growth of methanogenic bacteria

Sulfur source	Substrate concentration (m*M*)	A_{600}[a] *Methanococcus thermolithotrophicus*	*Methanobacterium thermoautotrophicum* strain Marburg	*Methanobacterium thermoautotrophicum* strain ΔH	*Methanococcus jannaschii*	*Methanococcus deltae* strain ΔRC	*Methanococcus deltae* strain ΔLH
Sulfide[b]	0.07	0.03	0.05	0.05	0.06	0.09	0.08
Methanethiol	1.0	0.72	0.75	0.75	0.45	0.68	0.65
Ethanethiol	1.0	0.58	0.80	0.80	0.34	0.45	0.40
n-Propanethiol	1.0	0.62	0.70	0.70	0.35	0.40	0.35
n-Butanethiol	1.0	0.60	0.75	0.75	0.38	0.39	0.33
Methyl sulfide	1.0	0.54	0.70	0.70	0.40	0.35	0.30
DMSO	1.0	0.50	0.60	0.65	0.35	0.38	0.34
Ethyl sulfide	1.0	0.62	0.68	0.68	0.35	0.34	0.30
CS_2	1.0	0.55	0.60	0.60	0.40	0.40	0.35

[a] Maximum A_{600} was recorded after incubation for 12–15 h for *Mc. thermolithotrphicus*, 15–30 h for *Mc. jannaschii*, and 20–120 h for *Mc. deltae* and *Mb. thermoautotrophicum* strains Marburg and ΔH. An absorbance of <0.2 is taken as no growth.
[b] Residual from inoculum.

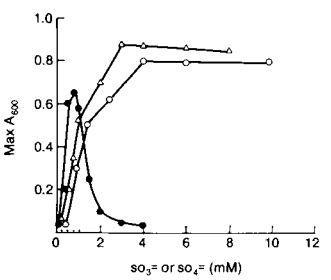

Fig. 1. Effect of sulfate and sulfite concentrations on growth of *Methanobrevibacter ruminantium* and sulfite concentrations on growth of *Methanococcus jannaschii*: ○, sulfate with *Mbr. ruminantium*; ●, sulfite concentration with *Mbr. ruminantium*; and △, sulfite concentration with *Mc. jannaschii*.

hibited an optimum level of 1–2 m*M* sulfide. *Mc. deltae* strains ΔRC and ΔLH, *Mc. marpaludis*, and *Mbr. ruminantium* were inhibited at levels beyond 4 m*M*, while *Mc. jannaschii* and *Methanobacterium* species *MMY*, *GC-2B*, and *GC-3B* grew well even at 10 m*M* sulfide. 2-ME at 10 m*M* and above was inhibitory to growth of all the organisms on sulfide.

Sulfate. None of the cultures except *Mbr. ruminantium* were able to use $SO_4^=$ as sole sulfur source (Table 1); this ability was confirmed by repeated transfer. The level of $SO_4^=$ required for optimal growth was 4–6 m*M*, and higher concentrations had no further effect (Fig. 1). 2-ME at >5 m*M* was inhibitory to growth of *Mbr. ruminantium* on $SO_4^=$. *Ms. barkeri* strain 227 was unable to grow with 4–5 m*M* $SO_4^=$ in the presence or absence of 2.5–5.0 m*M* 2-ME (data not shown).

Sulfite. Of the eight organisms examined that are shown in Table 1, only *Mc. jannaschii* and *Mbr. ruminantium* were able to grow on $SO_3^=$ as a sole sulfur source. 2-ME at >5 m*M* was inhibitory to growth of both the organisms on $SO_3^=$. After 4–5 repeated transfers, the effect of $SO_3^=$ concentration on growth was studied. Optimal growth of *Mbr. ruminantium* occurred at 0.5–1 m*M*, and concentrations >1 m*M* were greatly inhibitory (Fig. 1). However, optimal growth of *Mc. jannaschii* occurred at 2.5–3.0 m*M*, and concentrations up to 6 m*M* had no further effect (Fig. 1). *Ms. barkeri* strain 227 was also able to grow with 0.25–0.50 m*M* $SO_3^=$ (data not shown).

Thiosulfate. Interestingly, all the eight organisms examined were able to grow on thiosulfate as a sole sulfur source (Table 1). 2-ME at >10 m*M* was inhibitory to growth of all the organisms on thiosulfate. All the cultures growing on thiosulfate were transferred 4–5 times, and the effect of thiosulfate concentration on growth was studied. Optimal growth of all the organisms occurred at 4–5 m*M*, and higher concentrations had no further effect (data not shown). *Ms. barkeri* strain 227 was also able to grow with 3–4 m*M* of thiosulfate as sole sulfur source (data not shown).

Elemental sulfur. All the eight organisms examined were able to grow on elemental sulfur as a sole sulfur source (Table 1). 2-ME at >10 m*M* was inhib-

itory to growth of all the organisms on elemental sulfur.

Cysteine. Of the eight organisms examined, all except *Mc. jannaschii* were able to grow on cysteine as a sole sulfur source (Table 1). After 4–5 repeated transfers, effect of cysteine concentration on growth was determined. The level of cysteine required for optimal growth of all the organisms was about 5–6 m*M*, and higher concentrations had no further effect (data not shown). *Ms. barkeri* strain 227 was also able to grow with 3–4 m*M* cysteine as sole sulfur source (data not shown).

Mercaptans and organic sulfides. In this series of experiments, the ability of methanethiol, ethanethiol, *n*-propanethiol, *n*-butanethiol, methyl sulfide, dimethyl sulfoxide (DMSO), ethyl sulfide, and carbon disulfide (CS_2) to serve as sulfur source for the growth of *Mc. thermolithotrophicus, Mc. jannaschii, Mc. deltae* strains Δ*RC* and Δ*LH*, and *Mb. thermoautotrophicum* strains Marburg and ΔH was studied. Interestingly, all the organisms tested were able to grow with any of these sulfur compounds as a sulfur source (Table 2) when the medium was supplemented with very low levels of sulfide. Initially, we were unable to obtain growth on these compounds when the experiment was conducted in tubes, but when the experiment was repeated in bottles (250 ml), growth occurred and all the cultures were transferred repeatedly. Probably owing to decreased surface area exposed to air and thicker rubber stoppers, the bottles provide a more reduced environment than with tubes. (We have observed this bottle-preference phenomenon with our N_2-fixing cultures of *Mc. thermolithotrophicus, Mb. bryantii,* and *Methanospirillum (Msp.) hungatei* [N. Belay, unpublished observations].)

All the cultures needed about 20–30 μ*M* sulfide along with the mercaptans or organic sulfides. The reasons for this requirement of sulfide is not clear, but it may help to keep the medium at a more reduced state, thereby enabling the organisms to grow on mercaptans or organic sulfides. None of the organisms grew on 25–100 μ*M* sulfide alone. Methanethiol, ethanethiol, and methyl sulfide-using cultures produced 50–100 μ*M* sulfide during growth; uninoculated medium produced no sulfide. In a separate experiment, influence of 5–100 μ*M* sulfide alone or in combination with methanethiol or ethanethiol (1 m*M*) on *Mc. thermolithotrophicus* was studied. *Mc. thermolithotrophicus* grew on methenethiol or ethanethiol (1m*M*) in the presence of sulfide at concentrations >20 μ*M*, but not on 5–100 μ*M* sulfide alone (Fig. 2).

Out of several mercaptans and organic sulfides examined, methanethiol was the best sulfur source

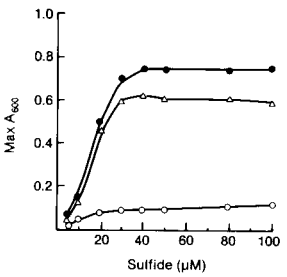

Fig. 2. Effect of sulfide concentration on growth of *Methanococcus thermolithotrophicus* in the presence of methanethiol or ethanethiol: ○, sulfide alone; △, 1 m*M* ethanethiol; and ●, 1 m*M* methanethiol.

(giving growth as good as with sulfide) for all the methanogenic bacteria tested (Table 2). Ethanethiol, *n*-propanethiol, *n*-butanethiol, methyl sulfide, DMSO, ethyl sulfide, and CS_2 provided for maximal growth densities (approaching that with sulfide) of *Mc. thermolithotrophicus* and *Mb. thermoautotrophicum* strains Marburg and ΔH, while growth of *Mc. jannaschii* and *Mc. deltae* strains ΔRC and ΔLH on these sulfur sources was comparatively lower. After four repeated transfers, the effects of concentrations of methanethiol, ethanethiol, and methyl sulfide on the growth of *Mc. thermolithotrophicus* were studied; the level of all the three compounds required for optimal growth was 1–2 m*M*, and higher concentrations had no further effect; 50%–80% of maximal growth occurred at 0.5 m*M* (data not shown).

Discussion

Virtually all media described for the growth of methanogenic bacteria contain sulfide added to make an initial concentration of 1–2 m*M*; cysteine has also been used in conjunction with sulfide [1, 2, 6, 12, 13, 15, 16, 22, 25–28, 35, 37–39]. Several workers have studied other potential sources of sulfur for media reduction or growth requirements, but the implication has been that fully reduced sulfur is required by all methanogens [13, 27, 28, 35]. Recently, Daniels et al. [9, 10] found that *Mc. thermolithotrophicus* and *Mb. thermoautotrophicum* strains Marburg and ΔH can grow on $SO_3^=$, thiosulfate, or elemental sulfur as a sole sulfur source; *Mc. thermolithotrophicus* was also able to grow with $SO_4^=$. Thus it seems clear from our work [9, 10] (this study) that a variety of sulfur-containing compounds, with the sulfur at several different oxidation states (in both inorganic and organic forms), serve as sole sources of sulfur for growth of methanogenic bacteria.

When methanogens are tested for their ability to grow on sulfur sources other than sulfide, growth may be poor or may not occur at all when cultures are shaken from the start of the incubation period. Cultures may be subjected to more oxidation under shaking conditions until they adapt to the new substrate. Thus, we recommend that when methanogens are grown on sulfur sources other than sulfide or S°, a period, of standing incubation should be allowed.

Gas chromatographic and chemical analysis of tubes or bottles containing the different sulfur sources has demonstrated that chemical-mediated production of sulfide or other reduced sulfur compounds from most of the compounds used in this study is negligible. COS was chemically unstable and yielded significant quantities of sulfide, however, and thus the growth on COS as sole sulfur source is probably due to sulfide and not COS. Likewise, when 2-ME is included in the medium along with thiosulfate or S°, chemical-mediated sulfide production occurs. A thorough discussion on nonbiological dismutation of oxidized sulfur compounds used in this paper has been presented in our earlier study [10], and all our data indicate that chemical-mediated reduction of sulfur compounds used in this study is negligible.

2-ME did not serve as sulfur source for the growth of methanogens tested in this and our earlier study [10]. Bhatnagar et al. [4] have also demonstrated that 2-ME was not metabolized by two strains of *Methanobacterium*. However, growth of many of the methanogens we have studied is inhibited by 2-ME. The use of 2-ME may lead to a false conclusion that a sulfur compound is not used by a methanogen when in fact its utilization may be inhibited by 2-ME; thus, its use is not recommended.

The sulfide and especially sulfite concentration curves suggest that it is very important to determine the levels for optimal growth of each organism (as shown here, and by other workers) [10, 16, 22, 26, 28, 37]. Bhatnagar et al. [4] used 5 m*M* $SO_3^=$ in testing methanogens for their growth on this compound; however, we have found that more than 1–2 m*M* is greatly inhibitory to all strains we have tested so far except *Mc. jannaschii* (this study) [10]. Care should be taken with elemental sulfur, since it could be metabolized rapidly to form toxic levels of hydrogen sulfide.

The data suggest that the methanogens used in this and an earlier study [10] have a variety of enzymes capable of using diverse sulfur sources like S°, $SO_4^=$, or $SO_3^=$. Sulfate can not be used as a significant sulfur source by most of a variety of methanogens examined (this work) [5, 6, 10, 16, 22, 27, 28, 37]; this suggests that the methanogens do not have enzymes for sulfate reduction. *Ms. barkeri* contains P_{590}, a sulfite reductase [23], although growth on sulfite had not been demonstrated prior to our work. Of the organisms examined, only *Mc. thermolithotrophicus* and *Mbr. ruminantium* seem to have a full set of enzymes for sulfate reduction, but most methanogens have a "sulfur reductase."

Several microorganisms produce volatile sulfur compounds, including alkyl thiols (mercaptans) and alkyl sulfides [19], but little is known about their biological decomposition. Some of these products occur naturally and are also produced by paper mills using the Kraft pulp process [29] and cause an industrial odor problem because of their low odor threshold. Lovelock et al. [20] detected methyl sulfide in ocean waters and in the atmosphere over soils and have suggested that methyl sulfide plays a major role in the atmospheric sulfur cycle. We have shown here that many methanogens can use several mercaptans and organic sulfides as sulfur sources for growth; although the cultures need a supplement of 20–30 μM sulfide to initiate growth, several mercaptans and organic sulfides produced sulfide during growth. Given lack of growth on the very low sulfide levels alone and the conversion of mercaptans to sulfide, we conclude that these compounds are serving as sulfur sources. Anaerobic metabolism of methionine by mud samples from Lake Mendota (Wisconsin) yielded methanethiol, which was further metabolized to methane, carbon dioxide, and hydrogen sulfide [43, 44]. Likewise, microbial populations present in anaerobic fresh water sediments and in anaerobic sewage digester sludge metabolized the carbon in methanethiol, methyl sulfide, and dimethyl disulfide to methane and carbon dioxide [43, 44]; however, no production of methane or carbon dioxide from either methanethiol or methyl sulfide by pure cultures of *Mbr. ruminantium, Mb. thermoautotrophicum*, or *Ms. barkeri* was detected. We have verified this work with *Ms. barkeri* (B.S. Rajagopal, unpublished data). It will be of interest to study whether these volatile organic sulfur compounds can serve as both a carbon and sulfur source for growth of any methanogens. The methanogens possibly play an important role in anaerobic habitats, which serve as sinks in the biogeochemical cycling of these compounds.

The reduction of the sulfur-containing compounds with hydrogen as the reductant is thermodynamically favorable: $SO_4^=$, $SO_3^=$, and S° reduction

to $S^=$ yields free energies ($\Delta G^{\circ\prime}$) of −152, −173, and −28 kJ per reaction. The free energy for the fermentation of methanethiol is −35 kJ per reaction, whereas the free energy for the reduction by hydrogen of methanethiol to methane and hydrogen sulfide is −69 kJ per reaction [31, 34]. The free energies for methyl sulfide metabolism are similar. Several anaerobic archaebacteria, including *Thermoproteus, Desulfurococcus*, and *Thermococcus* species obtain all their energy from S° reduction [40–42]; *Desulfovibrio* species obtain their energy by $SO_4^=$ reduction. However, in many bacteria, assimilatory sulfate reduction supplies cells with a sulfur source, but no energy. It is likely that in methanogens the assimilatory route occurs, but the possibility exists that they can produce ATP by hydrogen sulfide production: the distinction between these two possibilities is a relevant topic for future investigation.

The marine environment is high in sulfate, and it was expected that some of the marine isolates would be able to reduce $SO_4^=$ to use it as a sulfur source. However, the only methanogen in this study capable of this was *Mbr. ruminantium*, which is a nonmarine organism isolated from the rumen of a cow [6]. The only other methanogen known to use $SO_4^=$ is *Mc. thermolithotropicus* [10], and it is a marine isolate. Of the five marine organisms examined here, none used $SO_4^=$; this suggests it is not a common attribute with methanogens in this environment.

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Literature Cited

1. Balch WE, Wolfe RS (1976) New approach to the cultivation of methanogenic bacteria: 2-mercaptoethane sulfonic acid (HS-COM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl Environ Microbiol 32:781–791
2. Balch WE, Fox GE, Magrum LJ, Woese CR, Wolfe RS (1979) Methanogens: reevaluation of a unique biological group. Microbiol Rev 43:260–296
3. Banwart WL, Bremner JM (1974) Gas chromatographic identification of sulphur gasses in soil atmospheres. Soil Biol Biochem 6:113–115
4. Bhatnagar L, Henriquet M, Zeikus JG, Aubert JP (1984) Utilization of mercapto-2-ethanol as medium reductant for determination of the metabolic response of methanogens towards inorganic sulfur compounds. FEMS Microbiol Lett 22:155–158
5. Bhatnagar L, Jain MK, Aubert JP, Zeikus JG (1984) Comparison of assimilatory organic nitrogen, sulfur and carbon sources for growth of *Methanobacterium* species. Appl Environ Microbiol 48:785–790
6. Bryant MP, Tzeng SF, Robinson IM, Joyner AE (1971) Nutrient requirements of methanogenic bacteria. Adv Chem Ser 105:23–40
7. Corder RE, Hook LA, Larkin JM, Frea JI (1983) Isolation and characterization of two new methane-producing cocci: *Methanogenium olentangyi*, sp. nov., and *Methanococcus deltae*, sp. nov. Arch Microbiol 134:28–32
8. Daniels L (1985) A protective device for use during autoclaving bottles of methanogen medium. ASM News 51:60–61
9. Daniels L, Belay N, Mukhopadahyay B (1984) Considerations for the use and large-scale growth of methanogenic bacteria. In: Scott CD (ed) Proceedings of the 6th symposium on biotechnology in fuels and chemicals. Biotechnology and bioengineering symposium no. 14, New York: John Wiley and Sons, pp 199–213
10. Daniels L, Belay N, Rajagopal BS (1986) Assimilatory reduction of sulfate and sulfite by methanogenic bacteria. Appl Environ Microbiol 51:703–709
11. Daniels, L, Sparling R, Sprott GD (1984) The bioenergetics of methanogenesis. Biochim Biophys Acta 768:113–163
12. Fuchs G, Stupperich E, Thauer RK (1978) Acetate assimilation and the synthesis of alanine, aspartate and glutamate in *Methanobacterium thermoautotrophicum*. Arch Microbiol 117:61–66
13. Gunnarsson LAH, Rönnow PH (1982) Variation of the ATP-pool in thermophilic methanogenic bacteria during nitrogen or sulfur starvation. FEMS Microbiol Lett 14:317–320
14. Hook LA, Corder RE, Hamilton PT, Frea JI, Reeve JN (1984) Development of a plating system for genetic exchange studies using a modified low oxygen chamber. In: Strohl I, Tuovinen OH (eds) Microbial chemoautotrophy. Columbus: Ohio State Press, pp 275–289
15. Huber J, Thomm M, Konig H, Thies G, Stetter KO (1982) *Methanococcus thermolithotrophicus*, a novel thermophilic lithotrophic methanogen. Arch Microbiol 132:47–50
16. Jones WJ, Whitman WB, Fields RD, Wolfe RS (1983) Growth and plating efficiency of methanococci on agar media. Appl Environ Microbiol 46:220–226
17. Jones WJ, Leigh JA, Mayer F, Woese CR, Wolfe RS (1983) *Methanococcus jannaschii* sp. nov., an extremely thermophilic methanogen from a submarine hydrothermal vent. Arch Microbiol 136:254–261
18. Jones WJ, Paynter MJB, Gupta R (1983) Characterization of *Methanococcus maripaludis* sp. nov., a new methanogen isolated from salt marsh sediment. Arch Microbiol 135:91–97
19. Kadota H, Ishida Y (1972) Production of volatile sulfur compounds by microorganisms. Ann Rev Microbiol 26:127–138
20. Lovelock JE, Maggs RJ, Rasmussen RA (1972) Atmospheric dimethyl sulfide and the natural sulfur cycle. Nature 237:452–453
21. Mah R, Smith MR, Baresi L (1978) Studies on an acetate-fermenting strain of *Methanosarcina*. Appl Environ Microbiol 35:1174–1184
22. Mountfort DO, Asher RA (1979) Effect of inorganic sulfide on the growth and metabolism of *Methanosarcina barkeri* strain DM. Appl Environ Microbiol 37:670–675
23. Moura JJG, Moura I, Santos H, Xavier AV, Scandellari M, LeGall J (1982) Isolation of P_{590} from *Methanosarcina barkeri*: evidence for the presence of sulfite reductase activity. Biochem Biophys Res Commun 108:1002–1009

24. Murray PA, Zinder SH (1984) Nitrogen fixation by a methanogenic archaebacterium. Nature 312:284–286
25. Patel GB, Roth LA, Van den Berg L, Clark DS (1976) Characterization of a strain of *Methanospirillum hungatei*. Can J Microbiol 22:1404–1410
26. Ronnow PH, Gunnarsson LAH (1981) Sulfide-dependent methane production and growth of a thermophilic methanogenic bacterium. Appl Environ Microbiol 42:580–584
27. Rönnow PH, Gunnarsson LAH (1982) Response of growth and methane production to limiting amounts of sulfide and ammonia in two thermophilic methanogenic bacteria. FEMS Microbiol Lett 14:311–315
28. Scherer P, Sahm H (1981) Influence of sulphur-containing compounds on the growth of *Methanosarcina barkeri* in a defined medium. Eur J Appl Microbiol Biotechnol 12:28–35
29. Sivelä S, Sundman V (1975) Demonstration of *Thiobacillus*-type bacteria which utilize methyl sulphides. Arch Microbiol 103:303–304
30. Stetter KO, Gaag G (1983) Reduction of molecular sulphur by methanogenic bacteria. Nature 305:309–311
31. Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. Bact Rev 41:100–180
32. Trüper HG, Schlegel HG (1964) Sulphur metabolism in Thiorhodaceae. 1. Quantitative measurements on growing cells of *Chromatium okenii*. Antonie Van Leeuwenhoek J Microbiol Serol 30:225–238
33. Tsibul'skii VV, Vitenberg AG, Khripun IA (1978) Application of equilibrium concentrating in the gas-chromatographic determination of trace impurities of unstable sulfur-containing compounds in gases. J Anal Chem (USSR) 33:921–925
34. Weast RC (ed) (1968) Handbook of chemistry and physics, 49th edn. Cleveland: CRC Press
35. Wellinger A, Wuhrmann K (1977) Influence of sulfide compounds on the metabolism of *Methanobacterium* strain A_2. Arch Microbiol 115:13–17
36. Whitman WB (1985) Methanogenic bacteria: In: Woese CR, Wolfe RS (eds) The bacteria, vol 8: archaebacteria. Orlando FL: Academic Press, pp 3–84
37. Whitman WB, Ankwanda E, Wolfe RS (1982) Nutrition and carbon metabolism of *Methanococcus voltae*. J Bacteriol 149:852–863
38. Wolin EA, Wolin MJ, Wolfe RS (1963) Formation of methane by bacterial extracts. J Biol Chem 238:2882–2886
39. Zeikus JG, Wolfe RS (1972) *Methanobacterium thermoautotrophicum*, sp. nov., an anaerobic, autotrophic, extreme thermophile. J Bacteriol 109:707–713
40. Zillig W, Holz I, Janekovic D, Schäfer W, Reiter WD (1983) The archaebacterium *Thermococcus celer* represents a novel genus within the thermophilic branch of the archaebacteria. Syst Appl Microbiol 4:88–94
41. Zillig W, Stetter KO, Prangishvilli D, Schäfer W, Wunderl S, Janekovic D, Holz I, Palm P (1982) *Desulfurococcaceae*, the second family of the extremely thermophilic, anaerobic, sulfur-respiring Thermoproteales. Zbl Bakteriol Hyg I [Abt Orig C] 3:304–317
42. Zillig W, Stetter KO, Schäfer W, Janekovic D, Wunderl S, Holz I, Palm P (1981) *Thermoproteales*: a novel type of extremely thermoacidophilic anaerobic archaebacteria isolated from icelandic solfataras. Zbl Bakteriol Hyg I [Abt Orig C] 2:205–227
43. Zinder SH, Brock TD (1978) Methane, carbon dioxide, and hydrogen sulfide production from the terminal methiol group of methionine by anaerobic lake sediments. Appl Environ Microbiol 35:344–352
44. Zinder SH, Brock TD (1978) Production of methane and carbon dioxide from methanethiol and dimethyl sulfide by anaerobic lake sediments. Nature 273:226–228