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Sulfide-Dependent Methane Production and Growth of a Thermophilic Methanogenic Bacterium

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Two strains of a thermophilic methanogenic bacterium, designated MT1 a and b, were isolated from a coastal surface sediment in the northern part of the Swedish West Coast. The two strains were identical in appearance and nutritional requirements and resembled *Methanobacterium thermoautotrophicum*. MT1 had an extraordinary requirement for sulfide. At sulfide levels below 0.1 mM, growth was poor and the methane production rate decreased. Other sources of sulfur, such as cysteine, sulfate, or thiosulfate, could not replace sulfide. The results indicate that a fast turnover rate of reduced sulfur compounds is involved in the energy metabolism of this organism.

A great deal of recent literature has dealt with various aspects of methanogenesis and methanogenic bacteria, but only a few papers have considered their relation to sulfide.

Methanogenic bacteria are known to require reduced sulfur, e.g., sulfide or cysteine, as a source of a sulfur for growth (6, 19, 20), but it has also been reported that 0.1 mM hydrogen sulfide inhibited the growth of a *Methanobacterium* sp. (7). In recent reports, there are indications that methane formation is highly dependent on the availability of sulfide, much more so than was previously anticipated (12, 17). One reason for this being overlooked is the common practice of reducing media with a mixture of cysteine and sodium sulfide when culturing fastidious anaerobes, thereby supplying ample amounts of sulfide. Frequently, sulfide is added in a concentration of 2 mM (0.5 g of $Na_2S \cdot 9H_2O$ per liter of medium) (1, 5, 14).

The present study describes a thermophilic methanogenic bacterium isolated from a coastal surface sediment and reveals its requirement of large amounts of sulfide for growth and methane formation.

MATERIALS AND METHODS

Culturing technique and growth conditions. Mixtures of hydrogen and carbon dioxide, 80 and 20%, respectively (vol/vol), unless otherwise stated, were made from pure gases in proportion to the flow of each gas. Flow regulators (Variflow Corp.; SC 440 XF) with upstream reference were fitted with digital dials (Amphenol-Tuchel; D 121). The gas flow for each gas was calibrated against its dial with a bubble-flow meter, so that any desired gas mixture could be composed by setting the dials. Accurate mixing is achieved by this procedure when a stable continuous flow is maintained through the regulators. The type of gassing manifold described by Balch and Wolfe (2) was modified to meet this requirement. A four-way ball valve (Whitey; B-43Y) was installed to switch the gas mixture to a bypass line at the same time that the vacuum was connected to the gassing needles. The two lines were connected and led to an open flame, where the outflowing gas was burnt. All gases used were passed over copper filings heated to 320°C to remove traces of oxygen.

The standard Hungate technique and modifications thereof (4, 10, 11) were used for isolation and maintenance of the methanogenic organisms. A 300-ml, triple-baffled nephelo flask (Bellco; 2581-19133) with side arm (19 by 130 mm) was used for growth studies. A rubber stopper was fitted tightly into the large opening. A Bellco aluminum seal stopper (2048-11800) was aseptically fitted in the clean-out port, and a bored-through screw cap held the rubber stopper in position. Methanogenic basal medium (50 ml) was transferred aseptically and anaerobically from a pressurized serum bottle through a 20-gauge Venoject needle to a nephelo flask. The nephelo flasks were then pressurized with the gas mixture to a total pressure of 200 kPa and, after inoculation, were kept in an incubator (New Brunswick Scientific Co.; G-25) at 60°C and 170 rpm.

A glass vessel containing up to 1 liter of medium was also used for growth studies (13), with the standard gas mixture flowing through the medium at 120 ml per min. The redox potential was monitored during each growth experiment. The outgoing gas was led through a sampling valve connected to the gas chromatograph and then through an open flame where it was burnt.

The composition of methanogenic basal medium (MBM) is shown in Table 1. The solid medium used for growth in roll tubes was supplied with 3% agar (MBA). All media used were reduced with titanium citrate (18) to a final concentration of 0.1 mM Ti^{3+}, and sulfide was added as indicated. The pH of the medium was 7.2 when the CO_2 pressure was 40 kPa in

TABLE 1. *Composition of methanogenic basal medium*[a]

Compound	Element concentration
Macro mineral	
$NaHCO_3$	3.20 g/liter; 38.1 mM
NH_4HCO_3	0.70 g/liter; 8.9 mM
NaCl	0.90 g/liter; 15 mM
KH_2PO_4	0.96 g/liter; 7.05 mM
Na_2HPO_4	1.00 g/liter; 7.05 mM
Trace element	
$MgCl_2 \cdot 6H_2O$	20.0 mg/liter; Mg, 97 μM
$CaCO_3$	11.0 mg/liter; Ca, 110 μM
$MnCl_2 \cdot 4H_2O$	13.0 mg/liter; Mn, 66 μM
$FeCl_3 \cdot 6H_2O$	17.8 mg/liter; Fe, 66 μM
$CoCl_2 \cdot 6H_2O$	3.60 mg/liter; Co, 15 μM
$ZnSO_4 \cdot 7H_2O$	3.50 mg/liter; Zn, 12 μM
$NiCl_2 \cdot 2H_2O$	1.00 mg/liter; Ni, 4.2 μM
$CuCl_2 \cdot 2H_2O$	0.20 mg/liter; Cu, 1.2 μM
$AlK(SO_4)_2 \cdot 12H_2O$	0.33 mg/liter; Al, 0.7 μM
$Na_2MoO_4 \cdot 2H_2O$	0.20 mg/liter; Mo, 0.9 μM
H_3BO_3	0.20 mg/liter; B, 3.2 μM
Nitrilotriacetic acid	100.0 mg/liter; 52.3 μM
Vitamin	
Thiamin	0.12 mg/liter
Riboflavin	0.12 mg/liter
Pyridoxin-hydrochloride	0.23 mg/liter
Cyanocobalamin	0.01 mg/liter
Niacin	0.12 mg/liter
Para amino benzoic acid	0.12 mg/liter
Folic acid	0.05 mg/liter
Lipoic acid	0.12 mg/liter
Biotin	0.05 mg/liter

[a] In addition to the compounds listed above, the medium contained 10 ml of 0.2% Tween 80 and 2.0 ml of 0.1% resazurin per liter. The pH of the medium was 7.2 at 60°C and at a total pressure of 200 kPa, with a CO_2 pressure of 40 kPa.

the pressurized tubes and bottles (total pressure, 200 kPa). In cultures in which the total pressure was close to atmospheric (100 kPa) the amount of $NaHCO_3$ was reduced to 1.4 g/liter so that pH remained at 7.2

The optical density of the cultures was determined at 610 nm with a Beckman B spectrophotometer either in a 1-cm cuvette or in the side arm of the nephelo flasks. Samples (10 to 50 ml) for dry-weight determinations were centrifuged at 3000 × *g* for 20 min, washed once with distilled water, and dried overnight at 105°C.

Enrichment and isolation. The sediment sampling was carried out in Idefjorden, which is in the most northern part of the Swedish West Coast. The sampling site was off the mouth of the Tista River. The river has been the waste recipient of pulp and paper industries, and huge deposits of fibrous material were found in the fjord sediment. The sediment was collected by an Ekman dredge and was poured into a 5-liter plastic container, which was completely filled and tightly stoppered. The sediment was pitch black and consisted mainly of fibrous cellulose-like material. Approximately 150 ml of sediment was pressure filtered under N_2 directly into a stoppered serum bottle through a 20-gauge needle. One milliliter of this fiber-free but turbid solution was used as an inoculum to 50 ml of medium. After 1 day of incubation under the standard gas mixture at 60°C, the bottles were checked for gas consumption and then were repressurized. After several transfers with a 2% inoculum to the mineral medium, a serial dilution was made, and roll tubes with MBA were incubated. From a roll tube with about 20 colonies, a single colony was picked and subsequently cultured in MBM. Two strains, a and b, isolated from the organism designated MT1, were checked for purity by serial dilution and by repeated single colony picking.

Test of heterotrophic ability. The strains were transferred to serum bottles containing 10 ml of methanogenic basal medium with a limited amount of H_2, where the remaining gas phase consisted of N_2 plus CO_2. Except for the control culture, 0.5% of different organic compounds were added, and methane was analyzed after incubation for 1 month.

Methane and sulfide analyses. Methane was analyzed on a steel column (3.17 mm by 2 m) packed with Porapak-R in a Varian 3700 gas chromatograph equipped with a flame ionization detector. Gas samples were injected either with a gas sampling valve or with a 100-μl gas- and pressure-tight syringe (Pressure-Lok series A-2). Strict anaerobic technique was used when standard Na_2S solutions were prepared, and sulfide was analyzed as methylene blue, as described previously (16).

RESULTS

Characterization of MT1 strains a and b. Microscopic examination of wet mounts by phase-contrast microscopy and of Gram stains from a liquid culture showed all cells to be slender, irregularly crooked rods with rounded ends; sometimes they formed short chains of three to four cells. They were 0.75 μm wide and 4.5 to 8.0 μm long, nonmotile, and gram positive to gram variable. The optimum temperature for growth was between 60 to 65°C, but the cells also grew well at 45 and 75°C but not at 40 and 80°C. The pH optimum was between 6.8 and 7.1, and they also grew at 5.3 and 7.8. The pH range was obtained by adding different amounts of HCO_3^- to the medium while the Na^+ concentration was constant at 165 mM.

The strains used H_2 as their only source of energy. None of the organic compounds tested were utilized, viz.; formate, acetate, butyrate, methanol, glutamate, aspartate, cellobiose, maltose, xylose, and arabinose. With these compounds, no more methane was generated than in the control cultures without organic compounds.

The isolates grew in methanogenic basal medium with 0, 1, or 2% NaCl but not in medium containing 3% NaCl. They did not require vitamins or yeast extract and could be grown autotrophically on a mineral medium. Cysteine stimulated growth in the presence of sulfide.

Sulfide dependence. All experiments were

run with both strains except for the experiment presented in Fig. 2, in which only one strain was used. The responses of the two strains were almost identical.

The isolated strains required sulfide for growth and methane formation. The response to sulfide can be seen in Fig. 1 through 4. If less than 0.10 mM sulfide was present in the medium, growth was poor and quickly ceased. Addition of sulfide to sulfide-starved cells resulted in a specific growth rate (μ) of 0.16 h^{-1} until sulfide seemed to be depleted (Fig. 1). After further addition of sulfide, growth started again at the same rate as before. The growth yield for the cultures with ample amounts of sulfide (growth curves A and B in Fig. 1) was 1.20 g (dry weight) of cells per mol of methane produced, and for the cultures with limited amounts of sulfide, growth yield was 1.13 g (dry weight) of cells per mol (curves C and D in Fig. 1). Prolonged sulfide starvation did not affect the subsequent growth rate after sulfide addition. The linear relationship among growth, methane formation, and available sulfide is shown in Fig. 2. The sulfur requirement corresponds to 2.6% of the dry weight. Other sources of sulfur, such as sulfate or thiosulfate, could not restore growth. Growth sustained with only cysteine was slow owing to the slow hydrolysis of the thiol group which released sulfide. Slow growth resulting from a small addition of sulfide remained unaffected by the addition of thiosulfate.

When the MT1 strains were cultured in the glass vessel and the medium was reduced with cysteine and sodium sulfide, only frequent injection of sulfide sustained rapid growth and continuous methane production (Fig. 3). By giving only a few injections of sulfide, calculated to give a final concentration of 0.5 mM, to sulfide-starved cells, we obtained a quite different methane production pattern (Fig. 4). The sulfide concentration in the medium rapidly decreased until it was depleted. This could be explained by the decreased solubility of H_2S at 60°C and by the gas flow stripping the medium of H_2S. The solubility of H_2S ranges from 5.068 g/liter at 10°C

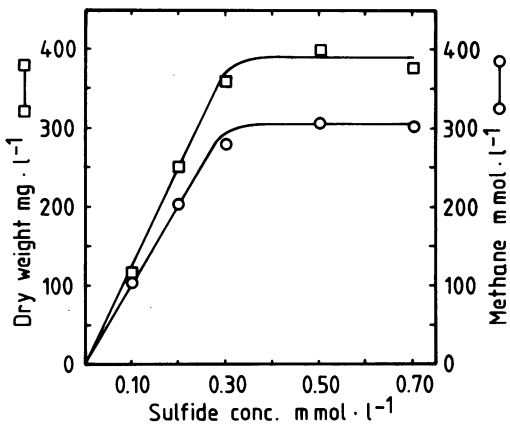

FIG. 2. *Dependence of growth and methane production on available amounts of sulfide in nephelo flasks, each containing 50 ml of medium, at 60°C. The values presented are the means of duplicates. All the media were reduced with titanium citrate (final concentration, 0.1 mM).*

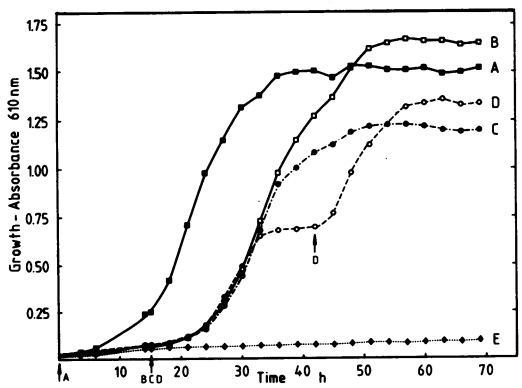

FIG. 1. *Growth of sulfide-starved MT1 cells in nephelo flasks in relation to different amounts of sulfide added to the media at 60°C. All the media were reduced with titanium citrate (final concentration, 0.1 mM). Lines A and B, 0.6 mM Na_2S; line C, 0.32 mM Na_2S; line D, 2 × 0.2 mM Na_2S; line E, no sulfide added. The arrows show when sulfide was added. The growth curves represent the means of duplicates.*

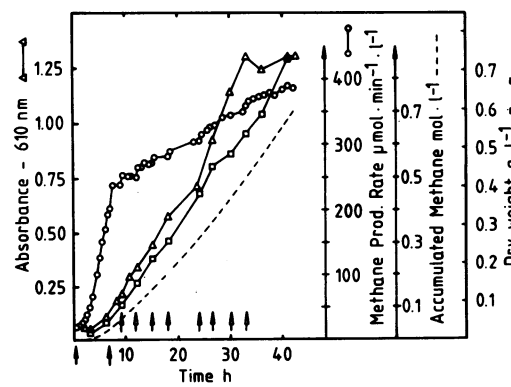

FIG. 3. *Growth of MT1 cells at 60°C in a 1-liter glass vessel with a gas flow of 120 ml per min. Growth was measured as absorbance at 610 nm in a 1-cm cuvette and as dry weight. The methane production rate was calculated, per liter of culture fluid, from the methane concentration measured in the outgoing gas. The arrows indicate additions of Na_2S to final concentrations of 1.00 mM.*

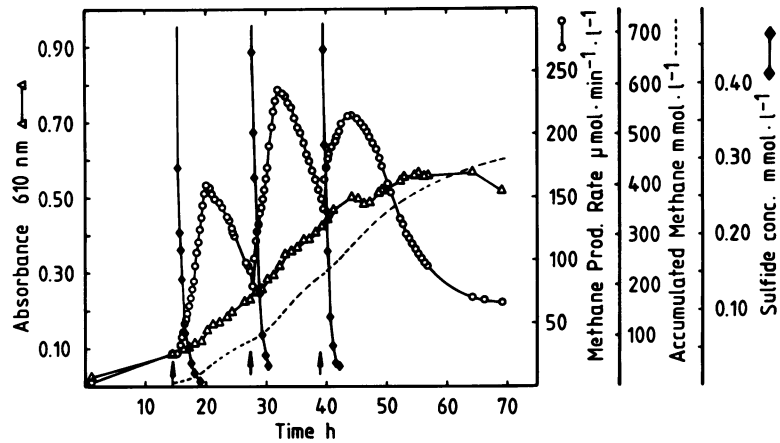

FIG. 4. *Growth of sulfide-starved cells of MT1 at 60°C after three injections of Na_2S. Growth was measured as absorbance at 610 nm in a 1-cm cuvette. The methane concentration was measured in the outgoing gas, and the methane production rates and the accumulated amounts of methane were calculated per liter of culture fluid. The gas flow was 120 ml per min. The arrows indicate the additions of Na_2S to final concentrations of 0.50 mM.*

to 1.478 g/liter at 60°C at a total pressure of 101 kPa (8). After injection of sulfide, there was a large increase in the methane production rate within 30 min after an initial small decrease. When sulfide was depleted, the methane production rate peaked and then decreased rapidly. The same pattern was repeated with further injections. During the experiment, growth increased steadily after the first sulfide addition. The accumulated amounts of methane, calculated from the methane production rate, paralleled growth.

DISCUSSION

The methanogenic strains, isolated from a marine sediment, resemble *Methanobacterium thermoautotrophicum,* which was isolated from digestor sludge (9, 21).

It has been shown that methanogenic bacteria require a fully reduced sulfur source (6, 20). Strain MT1 requires sulfide for growth, and the sulfide can not be replaced by cysteine; this is also true for *M. thermoautotrophicum, Methanobrevibacter ruminantium,* and *Methanobrevibacter arboriphilus,* but not for *Methanobacterium byrantii* or *M. arboriphilus* strain AZ (6, 17, 20). Strain MT1 is evidently capable of supplying its reduced sulfur compounds from sulfide, in contrast to *M. ruminantium,* which requires coenzyme M even if sulfide is available (2).

The response of the methane production rate to the sulfide injections was dramatic (Fig. 4). After restoration of the sulfide concentration, methane production increased as expected, but the methane production rate decreased rapidly and did not level off when sulfide is depleted. This observation suggests a fast turnover of a reduced sulfur compound that takes part in methane formation and then changes into a sulfur compound which cannot be used for methane formation. It is well known that methanogens possess a unique sulfur-rich cofactor, coenzyme M (2-mercaptoethanesulfonic acid), used in methane formation. Balch and Wolfe reported coenzyme M concentrations of between 0.3 and 16 µmol/g of dry weight for various methanogenic species (3). However, there are no reports of a turnover of coenzyme M. The reported amounts of coenzyme M correspond to a maximum of 4% of the sulfur consumed by strain MT1. Furthermore, methanogenic bacteria contain, in addition to coenzyme M, a large number of iron-sulfur proteins (15), which presumably take part in the electron transport.

The suggested uncoupling of growth and methane production at elevated sulfide concentrations (7.5 mM [12]) was not evident in the studied concentration range when the amounts of methane and growth were compared. Furthermore, the linear relationship among growth, methane production, and available amounts of sulfide (Fig. 2) do not suggest an uncoupling.

The observations made above indicate that the consumed sulfide is used for two main purposes: primarily to produce sulfur compounds taking part in the energy production and also to form sulfur-containing amino acids, proteins, etc. (i.e., as a general sulfur source for new building blocks required for growth).

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