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Effect of Inorganic Sulfide on the Growth and Metabolism of *Methanosarcina barkeri* Strain DM

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Minimal growth of *Methanosarcina barkeri* strain DM occurred when sulfide was omitted from the growth medium, and addition of either sodium sulfate or coenzyme M to sulfide-depleted media failed to restore growth. Optimal growth occurred in the presence of 1.25 mM added sulfide, giving a molar growth yield (Y_{CH_4}) of 4.4 mg (dry weight) of cells per mmol of CH_4 produced. Increasing sulfide to 12.5 mM led to a decrease in Y_{CH_4} (1.9 mg [dry weight]/mmol of CH_4), in the specific growth rate and in the intracellular levels of adenosine triphosphate. However, the specific rate of methane production increased. The data suggested that at elevated sulfide levels (12.5 mM) the decrease in Y_{CH_4} might be a result of an increase in the relative energy needed for maintenance and of uncoupling of growth from energy production.

In recent years there have been numerous studies on the relationship between sulfide-producing and methane-producing bacteria. In anaerobic microbial ecosystems with sulfate present, there is increasing evidence that methanogenesis is inhibited by sulfate-reducing bacteria which compete for methanogenic substrates such as hydrogen or acetate or both (1, 2, 5, 28). In some ecosystems inhibition of methanogenesis has been attributed to elevated levels of sulfide (7, 14, 28). Some methanogens are dependent on the production of sulfide since they may require it as the sulfur source, whereas others may utilize it in addition to cysteine (6, 27, 30, 31). Thus, there appears to be a critical relationship between the activity of the sulfate-reducing bacteria and the survival of the methanogenic population.

Little detailed attention has been given to the study of the effects of sulfide on the growth and metabolism of methanogens. In many methanogens at least part of the requirement for sulfide is as a precursor of coenzyme M, which in its methylated form functions as the terminal electron acceptor during methanogenesis (23).

The aim of this communication is to describe how sulfide affects the production and utilization of energy during the growth of *Methanosarcina barkeri* on methanol.

MATERIALS AND METHODS

Bacterial strain. *M. barkeri* strain DM (ATCC 29894) was isolated from an anaerobic digestor supplemented with bovine waste. During enrichment and isolation methanol was the growth substrate. Purity of the culture was frequently checked by inoculation into a medium for sulfate reducers (18) and into AC medium (Difco).

The strain grew well in methanol or under an atmosphere of 70% H_2-30% CO_2. Acetate was degraded slowly in the absence of other methanogenic substrates. The strain formed large aggregates of cells resembling morphotype I of *Methanosarcina* described by Zhilina (32).

Growth medium. The medium was prepared and sterilized under a strictly anaerobic atmosphere of 70% nitrogen and 30% CO_2 by a modification of the Hungate technique (12, 13). The growth medium contained, per liter: K_2HPO_4, 0.75 g; KH_2PO_4, 0.37 g; NH_4Cl, 0.5 g; $MgCl_2 \cdot 6H_2O$, 0.2 g; trace mineral solution (29), 10 ml; trace vitamin solution (29), 10 ml; yeast extract (Baltimore Biological Laboratory [BBL]), 2 g; Trypticase (BBL), 2 g; Na_2CO_3, 2 g; methanol, 10 ml; phenosafranine, 2 mg; $Na_2S \cdot 9H_2O$, 0.3 g; dithiothreitol, 0.5 g; and cysteine hydrochloride, 0.5 g. The final pH of the medium was 7.0. For liquid cultures, media were dispensed anaerobically in 7.5-ml quantities into Bellco anaerobic culture tubes (16 by 125 mm), which were then sealed with butyl septum stoppers, or in 30- or 100-ml quantities into 50- or 150-ml culture flasks, which were then sealed with butyl rubber stoppers.

Culture techniques. The techniques described by Hungate (12, 13) were adapted for the maintenance and subculturing of the organism. Liquid cultures were routinely inoculated by transferring 1/10 to 1/20 the amount of a 3- to 7-day-old culture under strictly anaerobic conditions. Cultures were incubated at 37°C without shaking.

Growth measurements. Absorbances were measured at 600 nm using a Pye Unicam SP6-400 spectrophotometer. Growth curves obtained by this method approximated those obtained by measuring methane production (data not shown). However, the absorbance method frequently gave spurious results presumably because of interference by large aggregates of

Methanosarcina and was therefore not routinely used in growth measurements. Methane production was routinely used for the measurement of growth.

Measurement of methanol and methane. For the determination of methane the gas composition of the atmosphere above the culture medium was monitored by gas chromatography as previously described (16). Total gas production was determined daily by measuring the volume of gas produced by displacement of the plunger of a glass piston syringe. The calculation of methane from the gas composition and measured volumes of the gas phase of the vessel and syringe was by the same procedure as described by Mah et al. (15). Methanol was determined by gas chromatography at 150°C, using a Porapak Q column connected to a flame ionization detector.

Measurement of sulfide. Values given in the text are for added sulfide and are higher than the true sulfide levels in the growth medium because of loss of sulfide to the gas phase. This is a result of equilibration between the S^{2-}, HS^-, and H_2S species. At pH 7 dissolved sulfide exists mainly as the HS^- and H_2S forms (pK = 7, for $H_2S \leftrightharpoons HS^- + H^+$ [3]), the latter of which readily escapes into the gas phase. This was verified by the methylene blue method of Truper and Schlegel (25), which showed that the true sulfide levels in the growth medium were 0.87 and 7.5 mM, respectively, for 1.25 and 12.5 mM added sulfide.

Cell yields. Cells were harvested by centrifugation at 7,000 × *g* for 20 min at 0 to 2°C, washed twice with distilled water (no lysis occurred during washing), and added to preweighed planchettes. Planchettes and cells were then dried to a constant weight by incubating at 60°C in a vacuum oven and were reweighed after drying. The increase in weight was taken as the cell dry weight. For determination of the molar growth yield (Y_{CH_4}) the dry weight of cells produced (milligrams) was divided by the quantity of methane produced (millimoles). The dry weight of cells was corrected for the dry weight of the inoculum, using a plot similar to that of Fig. 4 to estimate the latter. For cells grown in the presence of 12.5 mM sulfide a further correction to the dry weight was made for the small amount of precipitate (≤1.5 mg/100 ml) already in the medium. Phase-contrast microscopy was used to check that there was no increase in cell lysis at 12.5 mM sulfide.

Measurement of cellular ATP. Cells at the end of the logarithmic stage of growth were aggregated into a loose floc by inverting the growth flask and were then removed by a syringe with a wide-bore (16-gauge) needle and transferred to a vessel placed in ice. An even suspension of cells was then prepared using a Teflon piston homogenizer. Duplicate 1-ml volumes of cell suspension were then transferred to 4-ml volumes of 0.04 M tris(hydroxymethyl)aminomethane buffer (pH 7.8) just off the boil. Adenosine triphosphate (ATP) was extracted from cells by boiling for 5 min, quickly cooling to 0 to 2°C, and centrifuging at 8,000 × *g* for 10 min at 0 to 2°C. ATP in the supernatant was determined by the firefly bioluminescence technique described by Forsberg and Lam (9), using an ATP photometer (JRB model 100, SAI Industries, San Diego, Calif.). Controls showed that the growth medium had no significant effect on luciferase activity ($P < 0.05$). Internal ATP standards were used to account for the loss of ATP (28 ± 3%) during the extraction procedure. Correcting for losses in ATP due to extraction, the efficiency of the above extraction procedure was 70% when compared to the cold H_2SO_4 extraction procedure also described by Forsberg and Lam (9).

Redox potential. The redox potential of the growth medium was determined with a platinum electrode and a calomel reference electrode. The platinum electrode was cleaned by immersing in 50% (vol/vol) sulfuric acid for 1 min and then in 10% (vol/vol) sulfuric acid in 0.125 M potassium dichromate for 20 min. The electrodes were rinsed with distilled water before use and were tested using the redox buffer of Zobell (33). Readings were taken when the drift was less than 1 mV/min.

Chemicals. Firefly lantern extract (FLE-50) and ATP were obtained from Sigma Chemical Co. All other chemicals were of reagent grade and were obtained from commercial sources.

RESULTS

Growth of organism on methanol. In complex medium (routinely used in growth experiments) with methanol (1%, vol/vol) as the growth substrate full growth was attained 8 days after inoculation (Fig. 1). The doubling time during logarithmic growth was 28 h. Total methane (12 mmol) corresponded to a total production of cell mass of 52 mg (dry weight) of cells. In media with methanol omitted methane production after the same period was less than 2% of that with substrate added (results not shown). When yeast extract and Trypticase were omitted from the culture medium, the doubling time increased to 64 h, indicating that yeast extract and/or Trypticase stimulated growth.

Sulfide requirement and levels for optimum growth. Minimal growth of cells occurred in media in which sulfide was replaced by either sodium sulfate or coenzyme M at 1.25 mM (Fig. 2). Cysteine, which is always present in the growth medium (3 mM), also could not serve as an alternative to sulfide in facilitating growth. The redox potential of growth media to which no sulfide was added was −300 ± 10 mV (results not tabulated), indicating that the medium was sufficiently reduced for the growth of the methanogen. Also, the redox indicator phenosafranine was colorless in the same media, indicating a low redox potential. Thus, the requirement for sulfide could not have been merely as a reducing agent. To determine the level of sulfide giving optimum growth, sulfide was added to culture media (30 ml) in the range 0 to 62.5 mM. At the higher sulfide levels (≥12.5 mM) the pH of the media required readjustment to 7.0. Figure 3 shows that after 8 days of incubation cell dry weight reached a maximum at 1.25 mM sulfide

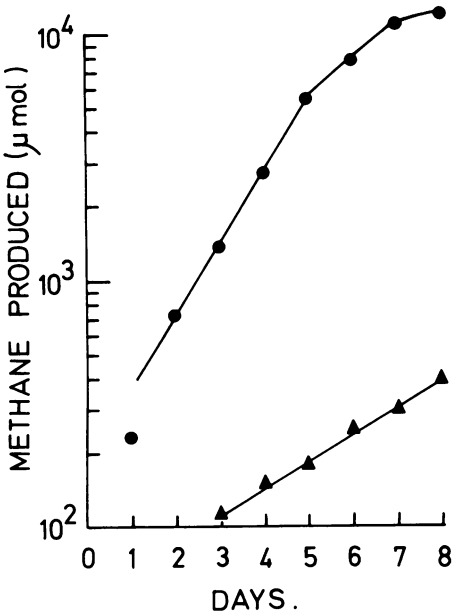

FIG. 1. *Representative plots of methane production by strain DM growing on methanol in defined and complex media (100 ml). Symbols:* (●) *complex medium (see text for composition of medium);* (▲) *medium in which Trypticase and yeast extract were omitted. In complex medium final methane production corresponded to a production of cell mass of 52 mg (dry weight) of cells. Media were inoculated with the equivalent of 6 mg (dry weight) of cells.*

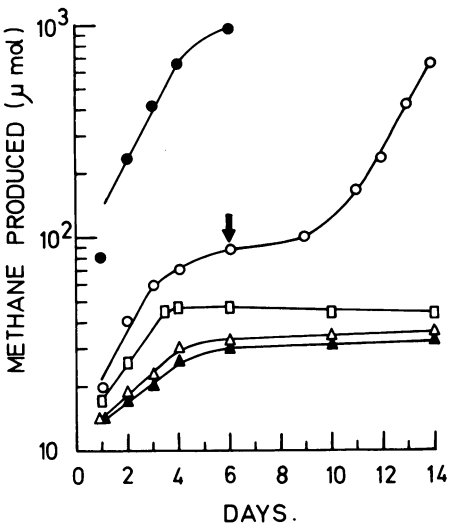

FIG. 2. *Effect of addition of sulfur compounds to media (7.5 ml) without sulfide. The following compounds were added to media before inoculation:* (□) *none;* (△) *1.25 mM coenzyme M;* (▲) *1.25 mM Na_2SO_4;* (●) *1.25 mM Na_2S. At the time shown by the arrow 1.25 mM sulfide was added to previously inoculated sulfide-free medium* (○). *Media were inoculated with cells equivalent to 0.5 to 1.0 mg (dry weight). Cells were washed in sulfide-free media before inoculation.*

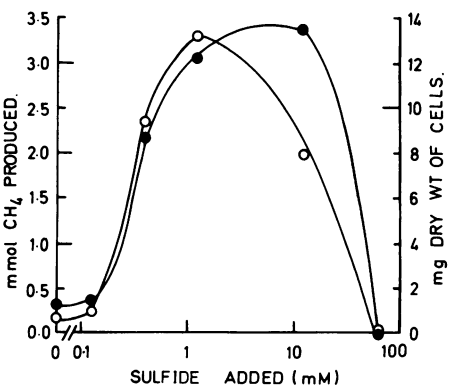

FIG. 3. *Effect of various sulfide concentrations on the growth of strain DM. Media (30 ml) were inoculated with cells equivalent to approximately 1 mg (dry weight), previously washed in sulfide-free media. Methane* (●) *and cell dry weight* (○) *were determined 8 days after inoculation. Values in the range 1.25 to 12.5 mM sulfide are averages of duplicate determinations.*

and declined at higher levels. At 12.5 mM sulfide, methane production clearly did not correspond with the decline in the production of cell mass.

Resolution of the effect of sulfide on growth at excess levels. The results for methane production and cell dry weight production in Fig. 3 indicated an anomaly in the molar growth yield for cells grown in the presence of 12.5 mM added sulfide. To resolve this, a detailed comparison was made with cells grown at 1.25 and 12.5 mM sulfide, using 100-ml cultures. The first parameter to be measured was growth rate. From the plots in Fig. 4 it was evident that whereas the relationship of methane production to cell mass changed at different sulfide levels, at any one level there was a linear relationship. Thus, methane production could justifiably be used to determine the growth rate. The specific growth rates (μ) for strain DM growing in the presence of 1.25 and 12.5 mM added sulfide were determined from plots similar to those in Fig. 5, using the following relationship: $\mu = 0.693/Td$, where Td is the doubling time. Table 1 shows that when sulfide was increased from 1.25 to 12.5 mM, μ decreased. Molar growth yields (Y_{CH_4}) were also determined for the same cells at the end of logarithmic growth and are also presented in Table 1. Results showed that Y_{CH_4} decreased by more than 50% when cells were grown at the 10-fold-higher sulfide level, thus supporting the earlier data of Fig. 3 (see also Fig. 4).

The data on Y_{CH_4} and μ were used to calculate

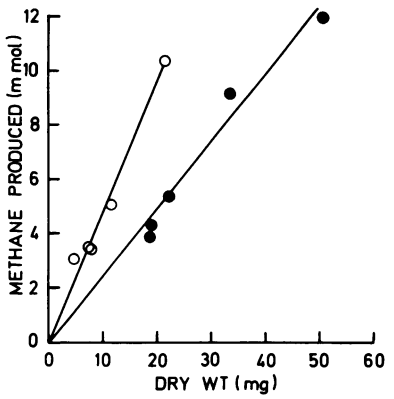

FIG. 4. *Relationship of methane production to dry weight of cells grown at different sulfide concentrations and at different growth stages. Symbols:* (○) *12.5 mM sulfide;* (●) *1.25 mM sulfide. Media (100 ml) were inoculated with cells equivalent to 4 to 6 mg (dry weight).*

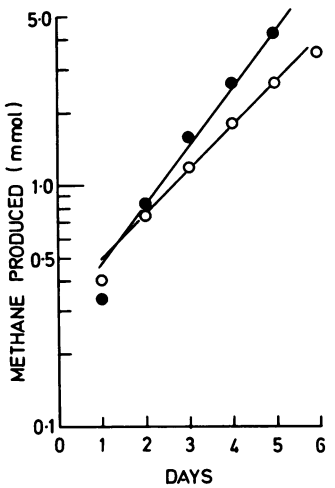

FIG. 5. *Exponential growth of strain DM in 100-ml cultures with different sulfide concentrations. Symbols:* (●) *1.25 mM sulfide;* (○) *12.5 mM sulfide. Values are averages of three determinations. For precision of measurements refer to data on specific growth rate in Table 1. Media were inoculated with the equivalent of 6 mg (dry weight) of cells.*

the specific rate of methane production (q CH_4) according to equation 2 (see Discussion). Table 1 shows that increasing the added sulfide levels from 1.25 to 12.5 mM resulted in an increase in the specific rate of methane production.

Stoichiometry of methanol fermentation. To eliminate the doubt that the effect of sulfide resulted from an alteration of the pathway(s) for methanol fermentation, the stoichiometry of methane production from methanol was investigated for cells grown at the two sulfide levels. Table 2 shows that the ratios of methane produced to methanol utilized for cells grown in the presence of the two sulfide concentrations were similar. Thus, the fate of methanol was largely unaffected by the change in sulfide levels. The range of 0.69 to 0.72 (CH_4/CH_3OH) approached the theoretical ratio of 0.75 (4).

ATP levels in cells grown in the presence of different sulfide levels. Previous studies with whole cells of methane bacteria have shown that methanogenesis is proportional to cellular ATP levels (19). To investigate whether the elevated q CH_4 in response to increased sulfide was associated with an increase in cellular ATP pools, ATP analyses were carried out in cells grown in the presence of 1.25 and 12.5 mM added sulfide. ATP levels were determined at the same growth stage (end of logarithmic growth) for the different cultures since previous studies have shown that cellular ATP levels may vary with growth (9). Table 1 shows that cellular ATP levels declined when cells were grown at the higher sulfide level (12.5 mM).

The data on ATP levels and q CH_4 showed that when added sulfide was increased from 1.25 to 12.5 mM the rate of methane production increased, whereas ATP levels decreased.

DISCUSSION

Minimal growth of *M. barkeri* occurred when sulfide was omitted from the growth medium, and addition of coenzyme M or sulfate would not restore growth (Fig. 2). The requirement for sulfide could not have been merely as a reducing agent since media prepared without it had a redox potential of −300 mV which would have been low enough to permit the growth of the methanogen. Thus, there appears to be a specific physiological requirement for sulfide by this organism. A similar requirement has also been shown for other methanogens (31). Not all strains of *Methanosarcina* have an absolute requirement for sulfide. Recently, Mah et al. (15) reported that *Methanosarcina* strain 227 could be grown in yeast extract medium supplemented with methanol in the absence of added sulfide.

Optimal growth occurred in the presence of 1.25 mM added sulfide, and Y_{CH_4} was 4.4 mg (dry weight) of cells per mmol of methane. At 12.5 mM sulfide Y_{CH_4} decreased to 1.9 mg (dry weight) of cells per mmol of methane. This decrease may have been a result of a larger part of the energy source being used for maintenance and uncoupling of growth and energy production. Growth yields are known to be dependent on μ, the maintenance coefficient (m), and the molar growth yield corrected for energy of maintenance (Y^{max}) and are expressed by the following equation (17, 20, 22, 26):

TABLE 1. *Growth parameters and ATP levels for strain DM growing on methanol in the presence of different sulfide concentrations*[a]

Sulfide (mM)	ATP[b] ± SD[c] (μg/mg [dry wt] of cells)	Y_{CH_4}[b] ± SD (mg [dry wt]/mmol of CH_4)	μ ± SD (h^{-1})	q CH_4 ± SD (mmol of CH_4/g [dry wt] per h)
1.25	0.56 ± 0.08	4.36 ± 0.36	0.025 ± 0.0016	5.7 ± 0.33
12.5	0.18 ± 0.007	1.90 ± 0.42	0.018 ± 0.002	9.5 ± 1.3

[a] Values at each sulfide concentration represent the mean of three determinations and for each column are significantly different ($P < 0.02$).
[b] Determined at the end of the exponential phase of growth (approximately 5 days).
[c] SD, Standard deviation.

TABLE 2. *Ratio of methane produced to methanol utilized for cells growing in 100-ml cultures with different sulfide concentrations*[a]

Sulfide (mM)	CH_3OH utilized (mmol)	CH_4 produced (mmol)	CH_4/CH_3OH
1.25	15.4	10.7	0.72
12.5	17.6	11.4	0.69

[a] Values at each sulfide concentration are averages of three determinations. Analyses were carried out on cultures near the end of the growth phase.

$$\frac{1}{Y} = \frac{m}{\mu} + \frac{1}{Y^{max}} \quad (1)$$

Thus, low growth yields would result from low growth rates, and the relative energy needed for maintenance would increase. The expression for q (specific rate of substrate utilization or product formation) may be obtained by multiplying equation 1 by μ. Thus, for growth during methanogenesis

$$\frac{\mu}{Y_{CH_4}} = \frac{\mu}{Y^{max}_{CH_4}} + m = q\ CH_4 \quad (2)$$

By rearrangement of equation 2 it follows that the molar growth yield is inversely proportional to q CH_4. In *M. barkeri* grown at elevated sulfide levels (12.5 mM added sulfide) both the decrease in μ and the increase in q CH_4 contributed to the decreased Y_{CH_4}. This suggests that uncoupling between growth and the energy from methanol fermentation occurred in addition to an increase in the requirement for maintenance energy. Stouthamer and Bettenhaussen (21) have previously reported that sulfide uncoupled growth in *Proteus mirabilis* because of the low growth yield obtained when sulfide was present. However, this result was later interpreted to be mainly due to an increase in the energy needed for cell maintenance because of the decrease in μ (22).

The observation reported here that increased q CH_4 values and decreased ATP levels occurred at elevated sulfide levels in the growth medium suggests uncoupling of ATP production from methane production. As far as we are aware, there have been no detailed reports on the effects of known uncouplers on growing methanogenic cultures. However, studies by Roberton and Wolfe (19) have shown that pentachlorophenol, dinitrophenol, and cyanide *m*-chlorophenylhydrazone decreased intracellular ATP levels in cell suspensions of *Methanobacterium* strain M.o.H. and simultaneously inhibited methanogenesis. Thauer et al. (24) have recently suggested that this was an indirect inhibition because of the requirement for ATP in methanogenesis demonstrated in studies with cell-free extracts (4, 8, 10, 19, 29). However, the same uncouplers were found to inhibit methanogenesis in cell-free extracts to which ATP was added (19). It therefore appears that the classical uncouplers can directly inhibit methanogenesis. This view is further supported by the recent studies of Gunsalus and Wolfe (11), who showed that the formation of methane from methyl coenzyme M in cell-free extracts of *Methanobacterium thermoautotrophicum* was inhibited by dinitrophenol and various halogenated compounds. In the studies reported here for growing cells, methanogenesis was stimulated at 12.5 mM sulfide whereas ATP levels decreased. This suggests that sulfide may have uncoupled ATP production from methane production. More study is required before it can be unequivocally established that sulfide acts as an uncoupler at elevated levels.

It is not known whether the effects of sulfide reported here are direct or indirect or both. A small amount (<5%) of 12.5 mM added sulfide precipitated in the medium. Thus, it is also possible that sulfide exerted an indirect effect by removing metal ions essential for growth. This possibility has also been proposed by Winfrey and Zeikus (28) to explain the inhibitory effect of sulfide on methanogenesis in freshwater lake sediments.

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