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# Nutritional Requirements of *Methanosarcina* sp. Strain TM-1

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***Methanosarcina* sp. strain TM-1, an acetotrophic, thermophilic methanogen isolated from an anaerobic sludge digestor, was originally reported to require an anaerobic sludge supernatant for growth. It was found that the sludge supernatant could be replaced with yeast extract (1 g/liter), 6 mM bicarbonate–30% $CO_2$, and trace metals, with a doubling time on methanol of 14 h. For growth on either methanol or acetate, yeast extract could be replaced with $CaCl_2 \cdot 2H_2O$ (13.6 μM minimum) and the vitamin *p*-aminobenzoic acid (PABA, ca. 3 nM minimum), with a doubling time on methanol of 8 to 9 h. Filter-sterilized folic acid at 0.3 μM could not replace PABA. The antimetabolite sulfanilamide (20 mM) inhibited growth of and methanogenesis by *Methanosarcina* sp. strain TM-1, and this inhibition was reversed by the addition of 0.3 μM PABA. When a defined medium buffered with 20 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid was used, it was shown that *Methanosarcina* sp. strain TM-1 required 6 mM bicarbonate–30% $CO_2$ for optimal growth and methanogenesis from methanol. Cells growing on acetate were less dependent on bicarbonate-$CO_2$. When we used a defined medium in which the only organic compounds present were methanol or acetate, nitrilotriacetic acid (0.2 mM), and PABA, it was possible to limit batch cultures of *Methanosarcina* sp. strain TM-1 for nitrogen at $NH_4^+$ concentrations at or below 2.0 mM, in marked contrast with *Methanosarcina barkeri* 227, which fixes dinitrogen when grown under $NH_4^+$ limitation.**

There is considerable diversity in the nutritional requirements of methanogenic bacteria. Some, such as *Methanobacterium thermoautotrophicum*, are capable of synthesizing all of their cell components in a completely inorganic growth medium in which carbon dioxide is the carbon source, ammonium ion is the nitrogen source, and sulfide serves as the sulfur source (27). Other methanogens have more complex nutritional requirements. *Methanobrevibacter ruminantium* was originally described as requiring rumen fluid for growth (2). It was eventually resolved that rumen fluid was supplying acetate, branched-chain fatty acids (3), and coenzyme M (2-mercaptoethanesulfonic acid) (22). *Methanococcus voltae* requires pantothenic acid, acetate, and either branched-chain amino acids or branched-chain fatty acids for growth (24), whereas *Methanomicrobium mobile* requires the aromatic amino acid tryptophan, the vitamins thiamine, pyridoxine, and *p*-aminobenzoic acid (PABA), branched-chain fatty acids, acetate, and an unknown factor (R. S. Tanner and R. S. Wolfe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, I90, p. 109). The methanogens also require a variety of metal ions, with an especially high requirement for nickel (5, 20), which is presnt in $F_{430}$ (4, 6, 25), hydrogenase (8, 10, 13), and carbon monoxide dehydrogenase (9).

The thermophilic, acetotrophic methanogen *Methanosarcina* sp. strain TM-1 was isolated from a 55°C anaerobic sludge digestor by Zinder and Mah (29). This organism was capable of growth on acetate, with doubling times as short as 12 h when grown at 50°C. Recent phylogenetic studies by Sowers et al. (21) with DNA-DNA and DNA-rRNA hybridizations indicated that *Methanosarcina* sp. strain TM-1 represents a species separate from other methanosarcinae, and the species epithet *Methanosarcina thermophila* TM-1 is proposed (S. H. Zinder, K. R. Sowers, and J. G. Ferry, Int. J. Syst. Bacteriol., in press).

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The culture medium originally described for *Methanosarcina* sp. strain TM-1 was complex and included 1 g of yeast extract per liter and 5% (vol/vol) digestor sludge supernatant (29). Most *Methanosarcina* cultures have been found to lack organic nutritional requirements, except for one strain studied by Scherer and Sahm (18) which required riboflavin. The development of a defined culture medium is essential for studies of the growth physiology of *Methanosarcina* sp. strain TM-1. We have been especially interested in the nutritional conditions which may favor the accumulation of a polyglucose reserve polysaccharide, since glycogen-like granules were present in thin-section electron micrographs of *Methanosarcina* sp. strain TM-1 that we examined. Since nitrogen limitation of growth typically triggers glycogen accumulation in bacteria, elimination of the N-containing yeast extract, sludge supernatant, and cysteine in the growth medium was essential to limiting the cells for nitrogen. We report here on the nutritional requirements of *Methanosarcina* sp. strain TM-1 and describe a chemically defined medium which supports rapid and extensive growth of the culture and enables us to limit cells for ammonium ion.

(A preliminary report on this material was presented previously [P. A. Murray and S. H. Zinder, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, I8, p. 141].)

## MATERIALS AND METHODS

**Growth media and conditions.** The basal medium contained the following: $NH_4Cl$, 1.0 g/liter; $K_2HPO_4$, 0.34 g/liter; $MgCl_2 \cdot 6H_2O$, 0.1 g/liter; resazurin, 0.001 g/liter; and trace metals solution (as described in reference 26, with the addition of 0.02 g of $NiCl_2 \cdot 6H_2O$ per liter), 10 ml/liter. The pH of the medium was adjusted to 6.7 with 1.2 N HCl. The medium was boiled under $N_2$ scrubbed with hot copper coils to remove oxygen, and 0.5 g of neutralized cysteine hydrochloride per liter was added. The medium was cooled under $N_2$, dispensed through an anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.) in 50-ml amounts into 118-ml glass serum vials sealed with butyl rubber stoppers

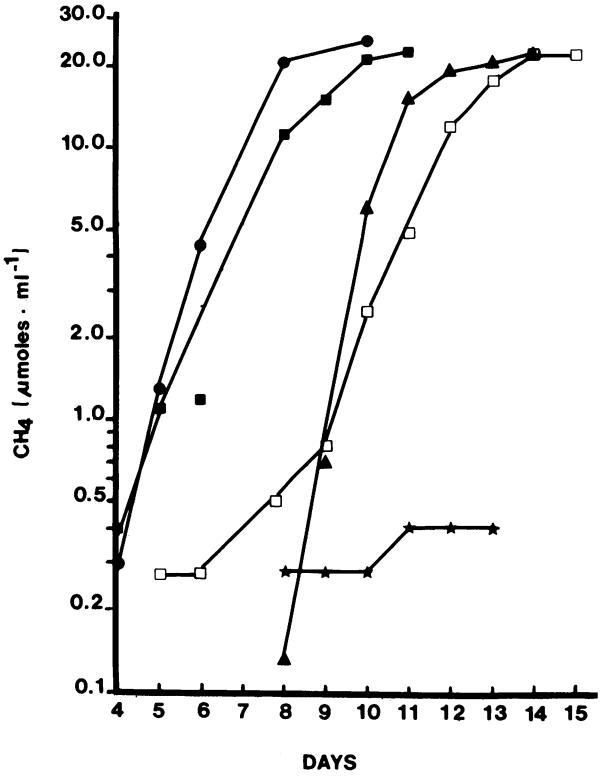


FIG. 1. Production of $CH_4$ by *Methanosarcina* sp. strain TM-1 grown on 24 mM methanol in the presence of different concentrations of yeast extract. Vials received a 1% inoculum of methanol-grown cells previously transferred twice in medium with no yeast extract. Symbols: ●, 1.0 g of yeast extract per liter; ■, 0.5 g of yeast extract per liter; □, 0.25 g of yeast extract per liter; ▲, 0.1 g of yeast extract per liter; ★, 0.0 g of yeast extract per liter.

(1), and autoclaved. The following sterile anaerobic solutions were added (final concentrations): $Na_2S \cdot 9H_2O$, 0.1 g/liter; $NaHCO_3$, 0.5 g/liter (6 mM); and 70% $N_2$–30% $CO_2$ (headspace). The following sterile anaerobic solutions were added when needed (final concentrations): yeast extract (Difco Laboratories, Detroit, Mich.), 0.0 to 1.0 g/liter; vitamin mixture (1), 1% (vol/vol); PABA, 0.3 nM to 0.3 $\mu$M; filter-sterilized folic acid, 0.3 $\mu$M; $CaCl_2 \cdot 2H_2O$, 0.1 g/liter; and sulfanilamide, 20 mM.

To test for $Ca^{2+}$ limitation of growth, we washed all glassware in 0.1 M HCl and rinsed it with high-purity deionized water (resistance, greater than 18 M$\Omega$). High-purity deionized water was used to make the basal medium in this experiment. In the ammonium limitation experiments, $NH_4Cl$ and cysteine hydrochloride were deleted from the basal medium, and sterile anaerobic $Na_2S \cdot 9H_2O$ (final concentation, 0.4 g/liter) was added instead to reduce the medium after it had been autoclaved. Sterile anaerobic $NH_4Cl$ was added to yield a final concentration of 0.0 to 1.0 g/liter (0 to 18 mM) as needed. In these experiments, since methanol concentrations greater than 24 mM appeared to be toxic for *Methanosarcina* sp. strain TM-1, 12 mM concentrations were added to culture vials as the methanol was depleted. Consequently, the final amount of methanol added to the vials with 20.0 or 2.0 mM $NH_4^+$ was 132 mM. In the vials with 1.0 mM $NH_4^+$, the final amount of methanol added was 72 mM. In the vials with 0.5 or 0 mM $NH_4^+$, the final amount of methanol added was 48 mM.

In experiments to determine the requirement for bicarbonate-$CO_2$, sterile anaerobic $NaHCO_3$ was added to vials at concentrations of 0.0 to 0.6 mM. These vials were flushed with either sterile 100% $N_2$ or $N_2$-$CO_2$ as needed. The *Methanosarcina* sp. strain TM-1 cells used as inocula (1% [vol/vol]) in these experiments were washed before use to prevent the carry-over of bicarbonate with the inoculum. This was done aseptically and anaerobically in sterile anaerobic glassware with basal medium supplemented with 20 mM BES (*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid) buffer (Sigma Chemical Co., St. Louis, Mo.) at pH 6.7.

**Analysis of gases.** Methane was determined with a model 550 thermal conductivity gas chromatograph (Gow-Mac Instrument Co., Bound Brook, N.J.) as outlined by Zinder et al. (28).

**Methanogenesis as a measure of cell growth.** *Methanosarcina* sp. strain TM-1 forms macroscopic aggregates ca. 1 mm or more in diameter (29), preventing a turbidimetric estimation of cell growth. Estimation of growth by removal of a small sample from the cultures for analysis of dry weight or cell protein was also infeasible because it was difficult to obtain a representative sample. Methanogenesis was generally used as a qualitative measure of overall cell growth, and the doubling time for methanogenesis, during periods when methane production was clearly exponential, was used to determine culture doubling times, as has been done previously with strain TM-1 (29). There was always good agreement between overall methanogenesis and visually estimated culture growth.

**Cell yield determinations.** Final cell dry weight was measured by filtering 10 ml of early-stationary-phase cells through a 0.4-$\mu$m-pore polycarbonate membrane filter (Nucleopore Corp., Pleasanton, Calif.) and drying the filters overnight in a dessicator at 50°C with silica gel desiccant (6/16 mesh; Fisher Scientific Co., Rochester, N.Y.). The filters were weighed with an analytical balance (Mettler Instrument Co., Hightstown, N.J.) sensitive to 0.1 mg.

**Chemicals.** All chemicals used were at least reagent grade.

## RESULTS

**Yeast extract requirement.** To enable us to study the physiology of *Methanosarcina* sp. strain TM-1, a completely defined growth medium was necessary. It was initially found that the requirement for 5% (vol/vol) anaerobic sludge supernatant was eliminated when yeast extract (1 g/liter), 6 mM bicarbonate–30% $CO_2$, and a trace metals solution were added to the growth medium. We wanted to determine if, in fact, yeast extract was required for the growth of *Methanosarcina* sp. strain TM-1. A 1% inoculum of methanol-grown cells previously transferred twice in medium with yeast extract deleted to eliminate nutrient carry-over was used. As the concentration of yeast extract in the medium was decreased, the lag period before methanogenesis from methanol began to increase by several days (Fig. 1). When no yeast extract was added to the growth medium, virtually no $CH_4$ was produced by these cells.

**Replacement of yeast extract with PABA and $Ca^{2+}$.** Various potential components of the yeast extract were tested for their ability to replace yeast extract in the growth medium. It was found that a vitamin mixture (1) alone could usually replace the yeast extract, but methanogenesis and growth were often inconsistent. Since the trace metal solution used provided only 1.3 $\mu$M $Ca^{2+}$ (26), we supplemented the vitamin-containing growth medium with 0.1 g of $CaCl_2 \cdot 2H_2O$ per liter (680 $\mu$M $Ca^{2+}$), which allowed con-

sistent growth in the absence of yeast extract (data not shown).

The individual vitamins in the mixture were each tested in $Ca^{2+}$-supplemented growth medium at the concentrations at which they were present in the mixture. PABA alone at 0.3 μM supported methanogenesis from methanol by *Methanosarcina* sp. strain TM-1 as well as the vitamin mixture did (Fig. 2). The doubling time for methanogenesis from methanol by the culture in medium with $Ca^{2+}$ and either vitamins or PABA was 7 to 9 h. PABA is a precursor in the synthesis of folic acid (14), but freshly prepared, filter-sterilized folic acid at 0.3 μM did not greatly stimulate methanogenesis, as compared with cultures to which no vitamins were added (Fig. 2). It was important that the folic acid was freshly prepared and filter sterilized, because older or autoclaved solutions stimulated some growth, presumably because of the breakdown to PABA.

To determine the concentrations of either PABA or $Ca^{2+}$ required for growth and methanogenesis by *Methanosarcina* sp. strain TM-1, we needed to transfer the cultures several times in medium lacking these to eliminate nutrient carryover. For a culture limited for PABA in this manner, decreasing the PABA concentration in the growth medium to 3 nM caused some decrease in the rate of methanogenesis from methanol but no decrease in final methane accumulation, and both the rate of methanogenesis and the final amount of methane accumulated significantly decreased when the concentration of PABA in the medium was 0.3 nM (data not shown). When cells were cultured in methanol-containing medium supplemented with PABA but not $Ca^{2+}$, no significant methanogenesis occurred (Fig. 3). Cultures supplemented with 13 μM $Ca^{2+}$ produced methane at rates (doubling time, ca. 7.5 h) similar to those in cultures supplemented with 680 μM $Ca^{2+}$. A limitation in the rate of methanogenesis, as well as a lag of several days, was seen in cultures supplemented with 6.8 μM $Ca^{2+}$, as compared with growth in the presence of 13.6 μM $Ca^{2+}$. Similar requirements for PABA and $Ca^{2+}$ were found for cultures of *Methanosarcina* sp. strain TM-1 grown on 40 mM sodium acetate (data not shown).

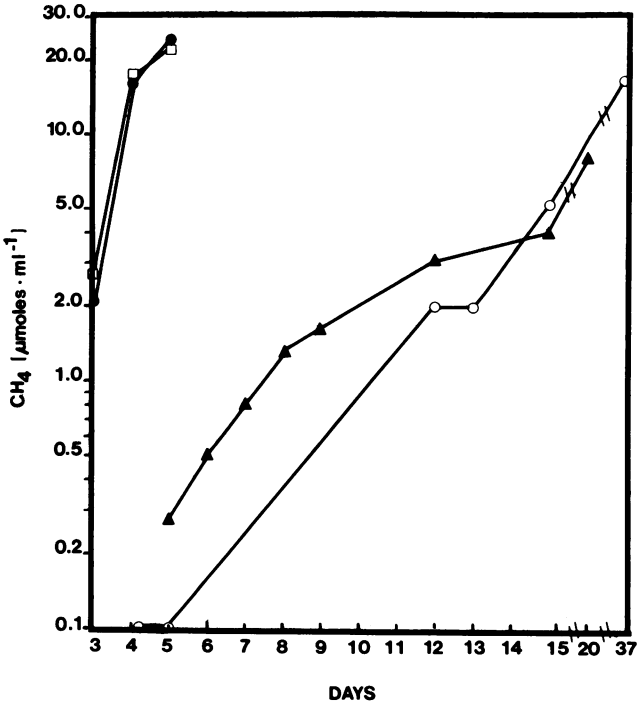


FIG. 2. PABA requirement for methanogenesis from 24 mM methanol by *Methanosarcina* sp. strain TM-1. $CaCl_2 \cdot 2H_2O$ (680 μM) was added to all culture vials. Vials received a 1% inoculum of methanol-grown cells previously transferred four times in medium lacking vitamins and yeast extract. Symbols: □, 1.0% (vol/vol) vitamin mixture; ●, 0.3 μM PABA; ▲, 0.3 μM filter-sterilized folic acid; ○, no vitamins.

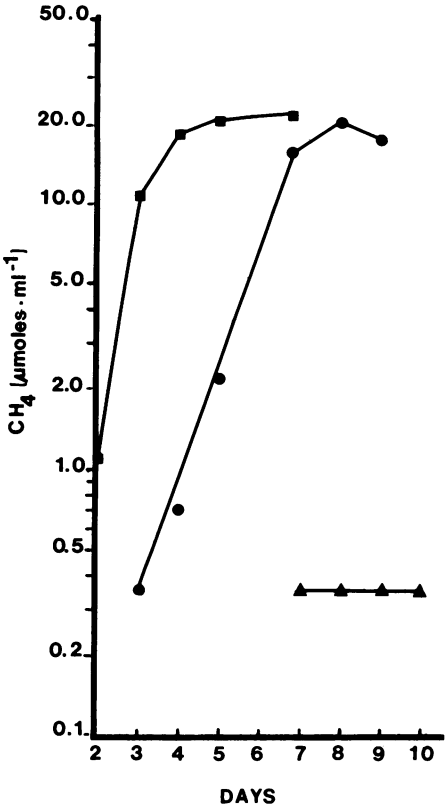


FIG. 3. Effect of calcium ion concentration on methanogenesis from 24 mM methanol by *Methanosarcina* sp. strain TM-1. A vitamin mixture (1.0% [vol/vol]) was added to all culture vials. A trace metal solution lacking $CaCl_2 \cdot 2H_2O$ and made with high-purity deionized water (see text) was used in this experiment. Vials received a 1% inoculum of methanol-grown cells previously transferred several times in medium lacking added $Ca^{2+}$. Symbols: ■, 13.6 μM $Ca^{2+}$; ●, 6.8 μM $Ca^{2+}$; ▲, no $Ca^{2+}$.

**Inhibition by sulfanilamide.** Sulfanilamide, or *p*-aminobenzenesulfonamide, is an analog of PABA. It can inhibit cell growth by blocking the synthesis of folic acid from its precursor PABA. Experiments with sulfanilamide were performed in methanol-containing medium lacking yeast extract and supplemented with 680 μM $Ca^{2+}$. When 20 mM sulfanilamide was added to culture vials, methanogenesis by *Methanosarcina* sp. strain TM-1 was severely inhibited, as compared with a culture to which no sulfanilamide was added (Fig. 4). When 0.3 μM PABA was added to culture vials along with 20 mM sulfanilamide, inhibition by sulfanilamide was reversed, and methanogenesis proceeded at a rate nearly equal to that in the control culture to which no sulfanilamide was added. Lower concentrations of PABA, such as 30 nM or 3 nM, did not as effectively reverse the inhibition caused by sulfanilamide.

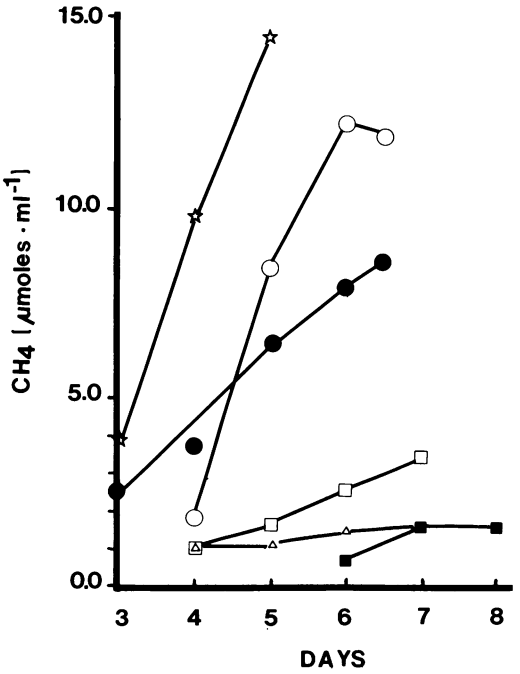


FIG. 4. Inhibition of methanogenesis from 24 mM methanol by 20 mM sulfanilamide and its reversal by PABA. Symbols: ☆, no sulfanilamide and 0.3 μM PABA; ○, 20 mM sulfanilamide and 0.3 μM PABA; ●, no sulfanilamide and no PABA; □, 20 mM sulfanilamide and 0.03 μM PABA; △, 20 mM sulfanilamide and 3 nM PABA; ■, 20 mM sulfanilamide and no PABA.

**$CO_2$ requirement for growth.** We also investigated the requirement of *Methanosarcina* sp. strain TM-1 for bicarbonate-$CO_2$ for growth on methanol and acetate. As the concentration of bicarbonate-$CO_2$ in medium buffered to pH 6.7 with 20 mM BES was decreased from the control level of 6 mM bicarbonate–30% $CO_2$ to 0.6 mM bicarbonate, there was an increase in the lag before methanogenesis began as well as an apparent decrease in the rate of methane production (Fig. 5). In addition, cultures with only 0.6 mM bicarbonate in the medium showed roughly a 0.04-fold decrease in the final yield of $CH_4$. When no bicarbonate-$CO_2$ was added to the methanol-containing medium, *Methanosarcina* sp. strain TM-1 produced no $CH_4$. These results were not a consequence of pH effects, since the final pH values of the media in these culture vials all fell within a range (6.3 to 6.65) which is well within the permissible range for the growth of *Methanosarcina* sp. strain TM-1 (29). *Methanosarcina* sp. strain TM-1 cells also showed a requirement for bicarbonate-$CO_2$ for growth on acetate. However, both the initial lag before methanogenesis and the decreased final level of methane produced were not as severe as with methanol-grown cultures (Fig. 6).

**Effects of $NH_4^+$ concentration on growth and methanogenesis.** Since it was possible that the cysteine used to reduce the medium could serve as a nitrogen source, it was important to remove it from our defined medium. Without cysteine as a reducing agent, it was necessary to increase the concentration of sodium sulfide added to the culture vials fourfold. This change did not affect growth of or methanogenesis by *Methanosarcina* sp. strain TM-1 (data not shown).

Methanol-grown *Methanosarcina* sp. strain TM-1 showed a significant limitation of final growth and methanogenesis when the concentration of $NH_4^+$ in the medium was 2.0 mM or less, as compared with control cultures provided with 20.0 mM $NH_4^+$ (Fig. 7). Results similar to these were seen with acetate-grown cells (data not shown). To determine that growth and methanogenesis were coupled under $NH_4^+$ limitation, we measured final cell dry weights. Table 1 shows that as the concentration of ammonium ion in the medium was decreased, the cellular dry weight in micrograms per milliliter correspondingly decreased.

## DISCUSSION

A chemically defined medium for the rapid growth of *Methanosarcina* sp. strain TM-1 was developed. The sludge supernatant which was originally reported to be a requirement for the growth of *Methanosarcina* sp. strain TM-1 (29) was replaced with yeast extract (1 g/liter), trace metals, and 6 mM bicarbonate–30% $CO_2$. It was found that $Ca^{2+}$ ions and the vitamin PABA were the required components provided by the yeast extract. The concentrations of these components which allowed complete growth of *Methanosarcina* sp. strain TM-1 on 24 mM methanol were 0.3 μM PABA and 13.6 μM $Ca^{2+}$.

The composition of yeast extract can be variable, but analysis of a typical batch (17) showed that 1 g/liter would provide ca. 0.15 μM PABA; the vitamin solution provided 0.3 μM PABA. These levels of PABA are adequate for optimal growth of and methanogenesis by *Methanosarcina*

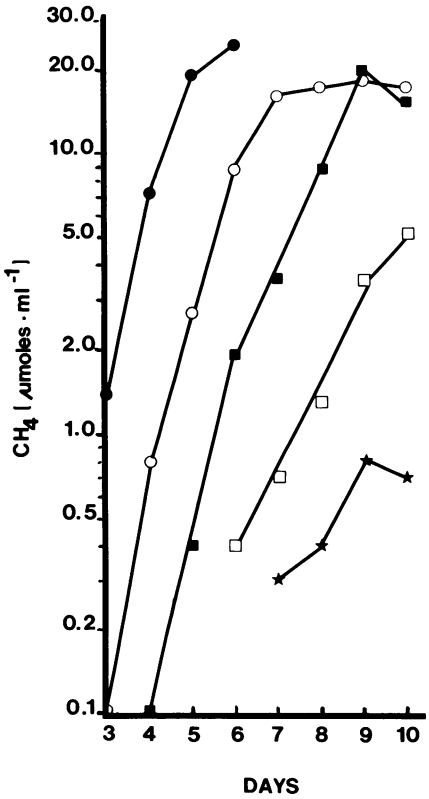


FIG. 5. Bicarbonate-$CO_2$ requirement for growth and methanogenesis from 24 mM methanol by *Methanosarcina* sp. strain TM-1. BES buffer (20 mM; pH 6.7) was used to buffer the system. The headspace consisted of 100% $N_2$ unless otherwise stated. Symbols: ●, 6 mM $NaHCO_3$ and 30% (17 mmol/liter) $CO_2$ added to the headspace; ○, 6 mM $NaHCO_3$; ■, 2.4 mM $NaHCO_3$; □, 1.2 mM $NaHCO_3$; ★, 0.6 mM $NaHCO_3$.

sp. strain TM-1. One other methanogen, *Methanomicrobium mobile*, has been shown to require PABA for growth (Tanner and Wolfe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982). Folic acid could not replace PABA in this organism either.

The antimetabolite sulfanilamide inhibited growth of and methanogenesis by *Methanosarcina* sp. strain TM-1, and this inhibition could be reversed by the addition of 0.3 μM PABA to the growth medium. In other bacteria, sulfanilamide specifically inhibits growth by blocking the synthesis of folic acid from its precursor PABA. Therefore, one might speculate that folic acid, rather than PABA, is the vitamin which is important in the metabolism of *Methanosarcina* sp. strain TM-1 and that the demonstrated inability of folic acid to eliminate the PABA requirement simply suggests the failure of *Methanosarcina* sp. strain TM-1 to transport folate into the cell. However, Leigh (15) has shown that in *Methanobacterium thermoautotrophicum* and *Methanococcus voltae*, folic acid is present in only minute quantities. He suggested that methanopterin, shown to serve as a $C_1$ carrier during methanogenesis (7), may replace folic acid as the pterin of the methanogens. This might explain the requirement for the folate precursor PABA by *Methanosarcina* sp. strain TM-1. However, it is unclear whether PABA is the precursor of the aromatic ring in methanopterin because that ring does not have the carboxyl group (23) present in PABA and folic acid. It is also possible that the inability of folic acid to substitute for PABA was because *Methanosarcina* sp. strain TM-1 was unable to reduce folic acid to tetrahydrofolate, the active coenzyme.

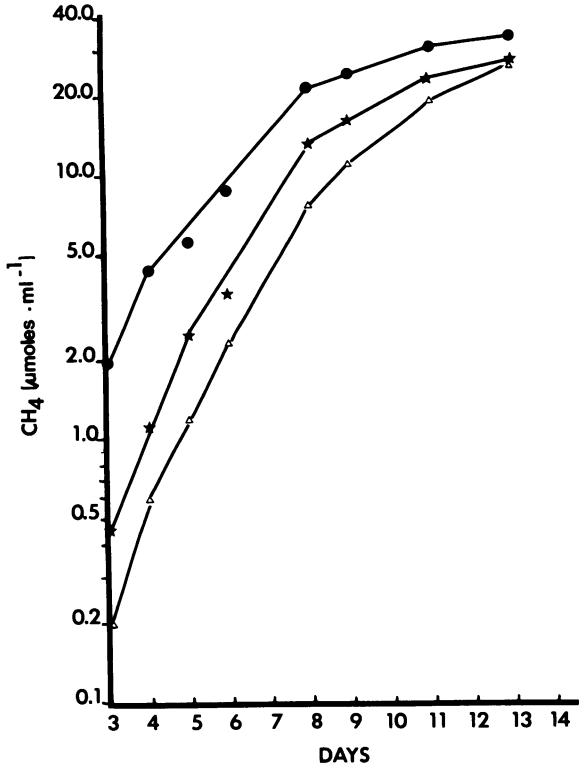


FIG. 6. Bicarbonate-$CO_2$ requirement for growth and methanogenesis from 40 mM sodium acetate by *Methanosarcina* sp. strain TM-1. BES buffer (20 mM; pH 6.7) was used to buffer the system. The headspace consisted of 100% $N_2$ unless otherwise stated. Symbols: ●, 6 mM $NaHCO_3$ and 30% (17 mmol/liter) $CO_2$ added to the headspace; , 0.6 mM $NaHCO_3$; △, no $NaHCO_3$.

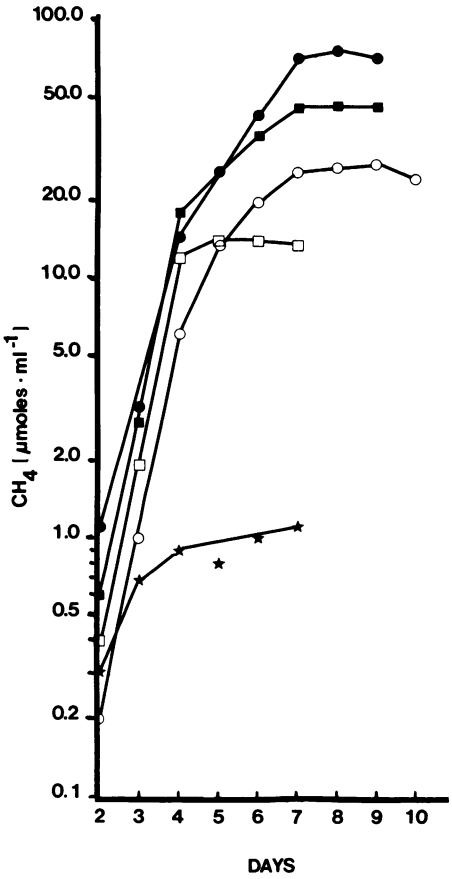


FIG. 7. Effect of $NH_4^+$ concentration on methanogenesis from methanol by *Methanosarcina* sp. strain TM-1. Vials received a 1% inoculum of methanol-grown cells previously transferred in medium lacking $NH_4^+$. Symbols: ●, 20.0 mM $NH_4CL$; ■, 2.0 mM $NH_4Cl$; ○, 1.0 mM $NH_4Cl$; □, 0.5 mM $NH_4Cl$; , no $NH_4Cl$.

Calcium ions were also provided by the yeast extract. A typical batch of yeast extract (17) would provide ca. 25 μM $Ca^{2+}$. *Methanosarcina* sp. strain TM-1 required 13.6 μM $Ca^{2+}$ for growth and methanogenesis. Another methanogen, *Methanococcus voltae*, has been shown to require 1 mM $Ca^{2+}$ for optimal growth in a defined medium (24). The high level of $Ca^{2+}$ required by *Methanococcus voltae* may be explained by the fact that this bacterium is a marine organism. It has been mentioned that a mesophilic strain of *Methanosarcina barkeri* (19) requires $Ca^{2+}$ for optimal growth, but no minimal concentration was reported.

In our defined medium, buffered with 20 mM BES, *Methanosarcina* sp. strain TM-1 required bicarbonate-$CO_2$ for optimal growth and methanogenesis. Methanol-grown

TABLE 1. Final cellular dry weights and methane accumulation in cultures of *Methanosarcina* sp. strain TM-1 grown in a medium containing different concentrations of $NH_4^+$

| $NH_4^+$ (mM) | Dry wt (μg/ml) | $CH_4$ (μmol/ml) |
|---|---|---|
| 20.0 | 310 | 76.8 |
| 2.0 | 120 | 48.5 |
| 1.0 | 60 | 27.5 |
| 0.5 | 40 | 13.8 |
| 0.0 | 15 | 1.1 |

cells required a considerably higher concentration of $CO_2$ initially added to the medium than did acetate-grown cells. In part, this may be due to the fact that once *Methanosarcina* sp. strain TM-1 begins to utilize acetate for growth and methanogenesis, one molecule of bicarbonate is produced for each molecule of $CH_4$ formed from acetate, thus quickly increasing the bicarbonate available to these cells. Methanol-grown cells, however, produce only one molecule of bicarbonate for every three molecules of $CH_4$ formed from methanol. There is another possible reason for the higher $CO_2$ requirement of methanol-grown cells. In studying anabolism in *Methanosarcina barkeri*, Kenealy and Zeikus (12) demonstrated that when this methanogen was grown on methanol and $CO_2$, only the C-2 of acetate (or acetyl coenzyme A) was derived from methanol. $CO_2$ was the precursor of the C-1 of acetate. They suggested that this C-2 intermediate (i.e., acetate or acetyl coenzyme A) was then carboxylated to form pyruvate, which is necessary for cell carbon synthesis. Consequently, *Methanosarcina* sp. strain TM-1 grown on methanol would require two $CO_2$ molecules for every methanol molecule incorporated into cell carbon. However, acetate-grown cells, which have acetate available in the medium, would only need to carboxylate this acetate to obtain the necessary pyruvate. Therefore, only one $CO_2$ molecule per acetate molecule would be required to form cell carbon. It is likely that the requirement for bicarbonate-$CO_2$ is universal in acetotrophic and methyltrophic methanogens and that bicarbonate-$CO_2$ is usually carried over the inoculum. In light of this $CO_2$ requirement, bicarbonate-$CO_2$ should routinely be added to media for the growth of *Methanosarcina* sp. on acetate or methanol, instead of using medium without bicarbonate-$CO_2$ under an atmosphere of 100% $N_2$. Bicarbonate-$CO_2$ also provides an additional buffering capacity to the medium.

The limitation of growth and methanogenesis by $NH_4^+$ concentrations lower than 2.0 mM is similar to results obtained for *Methanobacterium thermoautotrophicum* (11) and *Methanobrevibacter ruminantium* (3). These results are in marked contrast with results obtained recently for *Methanosarcina barkeri* 227 (16), which, instead of showing a limitation of growth and methanogenesis in low $NH_4^+$ concentrations, actively fixed dinitrogen. With a defined growth medium and the ability to limit *Methanosarcina* sp. strain TM-1 for $NH_4^+$, the physiology of this thermophilic methanogen can be studied further.

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