

Isolation and Characterization of a Thermophilic Strain of *Methanosarcina* Unable to Use H₂-CO₂ for Methanogenesis

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A thermophilic strain of *Methanosarcina*, designated *Methanosarcina* strain TM-1, was isolated from a laboratory-scale 55°C anaerobic sludge digester by the Hungate roll-tube technique. Penicillin and D-cycloserine, inhibitors of peptidoglycan synthesis, were used as selective agents to eliminate contaminating non-methanogens. *Methanosarcina* strain TM-1 had a temperature optimum for methanogenesis near 50°C and grew at 55°C but not at 60°C. Substrates used for methanogenesis and growth by *Methanosarcina* strain TM-1 were acetate (12-h doubling time), methanol (7- to 10-h doubling time), methanol-acetate mixtures (5-h doubling time), methylamine, and trimethylamine. When radioactively labeled acetate was the sole methanogenic substrate added to the growth medium, it was predominantly split to methane and carbon dioxide. When methanol was also present in the medium, the metabolism of acetate shifted to its oxidation and incorporation into cell material. Electrons derived from acetate oxidation apparently were used to reduce methanol. H₂-CO₂ was not used for growth and methanogenesis by *Methanosarcina* strain TM-1. When presented with both H₂-CO₂ and methanol, *Methanosarcina* strain TM-1 was capable of limited hydrogen metabolism during growth on methanol, but hydrogen metabolism ceased once the methanol was depleted. *Methanosarcina* strain TM-1 required a growth factor (or growth factors) present in the supernatant of anaerobic digester sludge. Growth factor requirements and the inability to use H₂-CO₂ are characteristics not found in other described *Methanosarcina* strains. The high numbers of *Methanosarcina*-like clumps in sludges from thermophilic digesters and the fast generation times reported here for *Methanosarcina* TM-1 indicate that *Methanosarcina* may play an important role in thermophilic methanogenesis.

The microbiology of thermophilic methanogenesis is poorly understood but has recently received increased attention. Ward (26) showed that microbial methanogenesis occurred at temperatures of up to 65°C in anaerobic layers of algal mats in neutral-pH hot springs in Yellowstone National Park, Wyo. Zeikus (32) isolated a thermophilic hydrogen-using methanogen from Yellowstone hot spring waters gassed with geothermal hydrogen and carbon dioxide. Pfeffer (20) reported that thermophilic methanogenic fermentation of urban refuse (primarily cellulose) was more efficient than the mesophilic process and that the optimum temperature for digestion of these wastes was 60°C. Varel et al. (25) showed rapid formation of methane from beef cattle manure at 60°C, with methane production rates as high as 4.5 liters per liter of digester volume per day and retention times as low as 3 days. Thermophilic digestion of raw sewage sludge has been in full-scale operation

for years and has been reviewed by Buhr and Andrews (7).

The only pure culture of a thermophilic methanogen previously described is *Methanobacterium thermoautotrophicum* (33). This organism grew at temperatures as high as 75°C and used H₂-CO₂ as a sole source of energy and cell carbon. It did not use other substrates such as formate, methanol, or acetate as sole energy sources for growth (33). Coolhaas (8) described an enrichment culture derived from canal mud which grew and produced methane at 60°C in a medium which contained calcium acetate as the sole organic constituent. The culture was dominated by a long thin rod. Pankhava and Pchelkina (19) described *Methanobacillus kuznetsovii*, a thermophilic rod which produced methane and acetate from methanol, but this culture may be impure (15).

It is presently unknown whether the flow of carbon to methane is the same in thermophilic

fermentations as in mesophilic ones. Preliminary results in our laboratory using ^{14}C -labeled acetate indicate that acetate was a major source of methane in a laboratory-scale thermophilic digester (55°C) and that acetate was split to methane and carbon dioxide in a manner similar to that found in mesophilic digestors (15). This report describes some of the properties of a thermophilic strain of *Methanosarcina*, designated *Methanosarcina* strain TM-1, isolated from such a thermophilic digester.

MATERIALS AND METHODS

Source of *Methanosarcina* strain TM-1. The laboratory-scale thermophilic digester was a 2-liter conical filter flask containing 1.5 liters of sludge, incubated in a Thelco 6M incubator (Scientific Products, McGaw Park, Ill.) at 55°C . The original inoculum for the digester was digested sludge from a thermophilic digester (51°C) operated at the Hyperion sewage treatment plant, El Segundo, Calif. The laboratory-scale digester was fed 100 ml of raw sludge (approximately 4% volatile solids) per day to give a retention time of 15 days. Gas production was measured by displacement of acidic brine and averaged 2.5 liters/day. The gas contained 60 to 70% methane.

Media and conditions for isolation and cultivation of strain TM-1. The medium used for the isolation of *Methanosarcina* strain TM-1 was as follows (g/liter of distilled water): NH_4Cl , 1.0; $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.4; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1; sodium acetate $\cdot 3\text{H}_2\text{O}$, 5; L-cysteine hydrochloride (Sigma Chemical Corp., St. Louis, Mo.; neutralized to pH 7), 0.5; yeast extract (Difco Laboratories, Detroit, Mich.) 0.2; resazurin, 0.001; and agar (Difco, purified), 15.0. The pH of the medium was adjusted to 6.5 with 1.2 N HCl, and the medium was then boiled under N_2 (scrubbed free of trace quantities of O_2 by hot-copper turnings) until the resazurin was reduced. The medium was cooled to 50°C , and 5-ml aliquots were dispensed by Cornwall syringe into screw-cap test tubes (16 by 150 mm), which were continuously flushed with N_2 . The tubes were sealed with butyl rubber Hungate-type stoppers (Bellco Glass Inc., Vineland, N.J.). Stoppers were used only once because they tended to leak after several punctures with a syringe needle. Since the cultures were never under a partial vacuum, leakage was not usually a problem. The tubes were autoclaved at 121°C for 20 min. Before inoculation, the following solutions were injected into each tube: 0.05 ml of a sterile anaerobic solution of 2% (vol/vol) methanol (final concentration = $5 \mu\text{mol/ml}$); 0.05 ml of sterile, anaerobic 1% $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ neutralized with sterile, anaerobic 6 N HCl; 0.25 ml of sterile anaerobic supernatant from the thermophilic digester. The supernatant was prepared by centrifugation as described below. The final pH of the medium was 6.5 to 6.8.

Cultures were grown routinely in a liquid medium of a composition similar to that described above, except for deletion of sodium acetate and agar and an increase of the yeast extract concentration to 1.0 g/liter. This medium was dispensed in 50-ml amounts

into serum vials (total vial volume = 124 ml) under an N_2 atmosphere. The vials were sealed with the crimp-on rubber stoppers described by Balch and Wolfe (1) and autoclaved at 121°C for 20 min. Before inoculation, sterile, anaerobic solutions of substrate, 0.5 ml of 1.0% $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (pH 7), and 2.5 ml of sludge supernatant were injected into the vials.

Cultures were routinely maintained at 50°C in medium containing both $38 \mu\text{mol}$ of sodium acetate and $5 \mu\text{mol}$ of methanol per ml as growth substrates. After 3 days of growth in this medium, the culture metabolized all of the methanol (see Results), and in late growth phase it used acetate for methanogenesis. Inoculum for most experiments was 1 to 2% of a 3- to 7-day-old culture. Unless stated otherwise, data points for the experiments presented in this paper represent averages of duplicate samples.

Attempts to grow cultures on $\text{H}_2\text{-CO}_2$ included addition of 0.5 ml of a sterile anaerobic 5% solution of NaHCO_3 and pressurizing the vials to 1-atm (101 kPa) overpressure with a 70% $\text{H}_2\text{-30% CO}_2$ gas mixture (Matheson, Joliet, Ill.) using an apparatus similar to that described by Balch and Wolfe (1).

Preparation of SS. Digested sludge was collected in a 20-liter plastic carboy from a thermophilic digester (51°C) at the Hyperion sewage treatment plant, El Segundo, Calif. On the day of collection, it was centrifuged at $4,000 \times g$ for 15 min in a large rotor using an IEC B-20 refrigerated centrifuge (International Equipment Company, Needham, Mass.). The partially clarified sludge was bubbled with nitrogen gas for 15 min and stored frozen under N_2 in rubber-stoppered glass bottles. Bottles of the sludge were thawed as needed and were centrifuged at $29,000 \times g$ for 20 min. This clarified sludge supernatant (SS) was placed in 124-ml serum vials, bubbled with N_2 , sealed with butyl rubber stoppers, and autoclaved for 20 min at 121°C . After autoclaving, 0.5 ml of 1% $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ was added per 50 ml of SS. The SS was a clear to slightly opalescent yellow liquid which contained $7 \mu\text{mol}$ of acetate per ml, $0.5 \mu\text{mol}$ of propionate per ml, and trace amounts of butyrate.

Analysis of gases and volatile fatty acids. Methane and carbon dioxide were quantified by gas chromatography as outlined by Baresi et al. (2). In most instances, hydrogen was only qualitatively measured since helium was the carrier gas. Acetate and other volatile fatty acids were quantified by the gas chromatographic method described by Baresi et al. (2), except that the carrier gas was saturated with formic acid to prevent ghosting and peak tailing.

Determination of radioactivity. The radioactivity in ^{14}C -labeled methane was quantified using the gas chromatographic-proportional counter technique described by Nelson and Zeikus (18). Radioactive carbon dioxide was determined at the end of an experiment by acidifying the culture medium and sampling the headspace. The total radioactivity could then be calculated using Bunsen absorption coefficients (24). Label incorporated into the cells was determined by filtering a sample of the culture medium through a Millipore HAWP 0.45- μm membrane filter (Millipore Corp., Bedford, Mass.). The filter was counted in Aquasol (New England Nuclear Corp., Boston, Mass.)

using a Beckman LS 100 C liquid scintillation counter (Beckman Instruments, Irvine, Calif.). Aqueous radioactivity was also counted in Aquasol. Quench correction was accomplished by the channels ratio method.

Cell yield determinations. Cell yields were determined by the method of Mah et al. (14). A Cahn model 25 electrobalance (Cahn/Ventron, Cerritos, Calif.) was used to weigh the filters.

Determination of K_s value. The K_s for growth on acetate was determined using the method described by Smith and Mah (21).

Chemicals and radiochemicals. Sodium [$1\text{-}^{14}\text{C}$]acetate ($\text{CH}_3^{14}\text{COO}^-$) (specific activity, 2.5 mCi/mmol) and sodium [$2\text{-}^{14}\text{C}$]acetate ($^{14}\text{CH}_3\text{COO}^-$) (specific activity, 4.7 mCi/mmol) were purchased from New England Nuclear Corp. Sodium [^{14}C]carbonate (called $^{14}\text{CO}_2$) (specific activity, 53 mCi/mmol) and [^{14}C]methanol ($^{14}\text{CH}_3\text{OH}$) (specific activity, 3.4 mCi/mmol) were purchased from Schwarz/Mann, Orangeburg, N.Y.

All routine chemicals used were of reagent grade. Sodium coenzyme M was kindly provided by R. E. Hungate, University of California, Davis, Calif., and 2-methylbutyric acid was purchased from Eastman Chemical Corp., Rochester, N.Y. Clarified rumen fluid was obtained from P. H. Smith, University of Florida, Gainesville, Fla.

RESULTS

Isolation of *Methanosarcina* strain TM-1. Sludge from a 55°C laboratory digester was inoculated into roll tubes containing 5 μmol of methanol and 40 μmol of sodium acetate per ml as growth substrates and a low concentration (0.02%) of yeast extract. After 3 days of incubation at 55°C, 10 to 50 large, smooth, yellow to brown colonies formed in the 10^{-1} dilution tubes and occasionally in the 10^{-2} dilution tubes. Large numbers of smaller, whitish colonies also formed. Substantial quantities of methane were produced in tubes containing the yellowish colonies; gas bubbles and gas splits were occasionally associated with these colonies. When examined by phase microscopy, such colonies were found to contain large refractile aggregates resembling *Methanosarcina* surrounded by confluent growth of small rod-shaped contaminants.

Such colonies could not be successfully cultured unless sludge supernatant from the laboratory digester was also added to the medium (final concentration of 5%, vol/vol). After 4 to 7 days of incubation at 55°C, 10 to 25 of the large yellowish colonies formed along with approximately 10^3 contaminant colonies. This low yield of methanogenic colonies was most likely due to the highly aggregated state of the *Methanosarcina*-like organism. Attempts to free this organism from the contaminants by using the usual methods of picking and diluting colonies proved futile. To achieve isolation, penicillin G and D-cycloserine were added to the medium at final concentrations of 2 g/liter (3,338 U/ml) and 0.1

g/liter, respectively. Since *Methanosarcina* and other methanogens do not contain peptidoglycan (13), their growth should not be affected by these inhibitors of peptidoglycan synthesis. Jones et al. (12) found that *Methanococcus vaniellii* readily grew in the presence of either antibiotic. Because of the lability of penicillin G, especially at high temperatures, growth of contaminants was suppressed for only 2 to 3 days if penicillin was added alone.

After one transfer into roll tubes with medium containing both antibiotics, colonies containing the *Methanosarcina*-like organism still formed, whereas contaminant colonies did not. The morphology of the *Methanosarcina*-like organism was unchanged, and no contaminants were microscopically visible in the colonies. The colony morphology changed from smooth to a rough, granular consistency once the contaminating organisms were removed. The colonies were transferred three times into antibiotic-containing medium and then were transferred back into medium without antibiotics. No contaminants appeared when the antibiotics were removed. A colony was transferred into liquid medium and was designated *Methanosarcina* strain TM-1. No growth of contaminating heterotrophs was obtained when the culture was inoculated into glucose-nutrient broth medium incubated aerobically or anaerobically at 55°C. Contaminants were never observed when the culture was examined using phase-contrast microscopy.

Figure 1 is a phase-contrast photomicrograph of a clump of *Methanosarcina* strain TM-1 grown in liquid medium containing a methanol-acetate mixture. The organism, during growth, formed large phase-refractile clumps, corresponding to biotype 1 of Zhilina (34). Tetrads were rare, and single cell bodies were never observed. If the wet mount of the organism was allowed to dry partially, the large clumps tended to disaggregate and lost their refractility. Figure 2 is a phase-contrast micrograph (at higher magnification than Fig. 1) of a clump of *Methanosarcina* subjected to such a procedure. Many division planes, which are clearly non-perpendicular, are evident.

Substrates used for methanogenesis by *Methanosarcina* strain TM-1. Various substrates were added to the basal medium to determine whether they would support methanogenesis and growth. If no substrate was added, little or no methane was formed, and no visible growth occurred (Table 1). The methane that did form was most likely derived from the 0.45 to 0.95 μmol of acetate present per ml in the basal medium, which was contributed by the yeast extract (0.1 $\mu\text{mol}/\text{ml}$), the SS, (0.35 $\mu\text{mol}/\text{ml}$), and the inoculum (0.0 to 0.5 $\mu\text{mol}/\text{ml}$). The

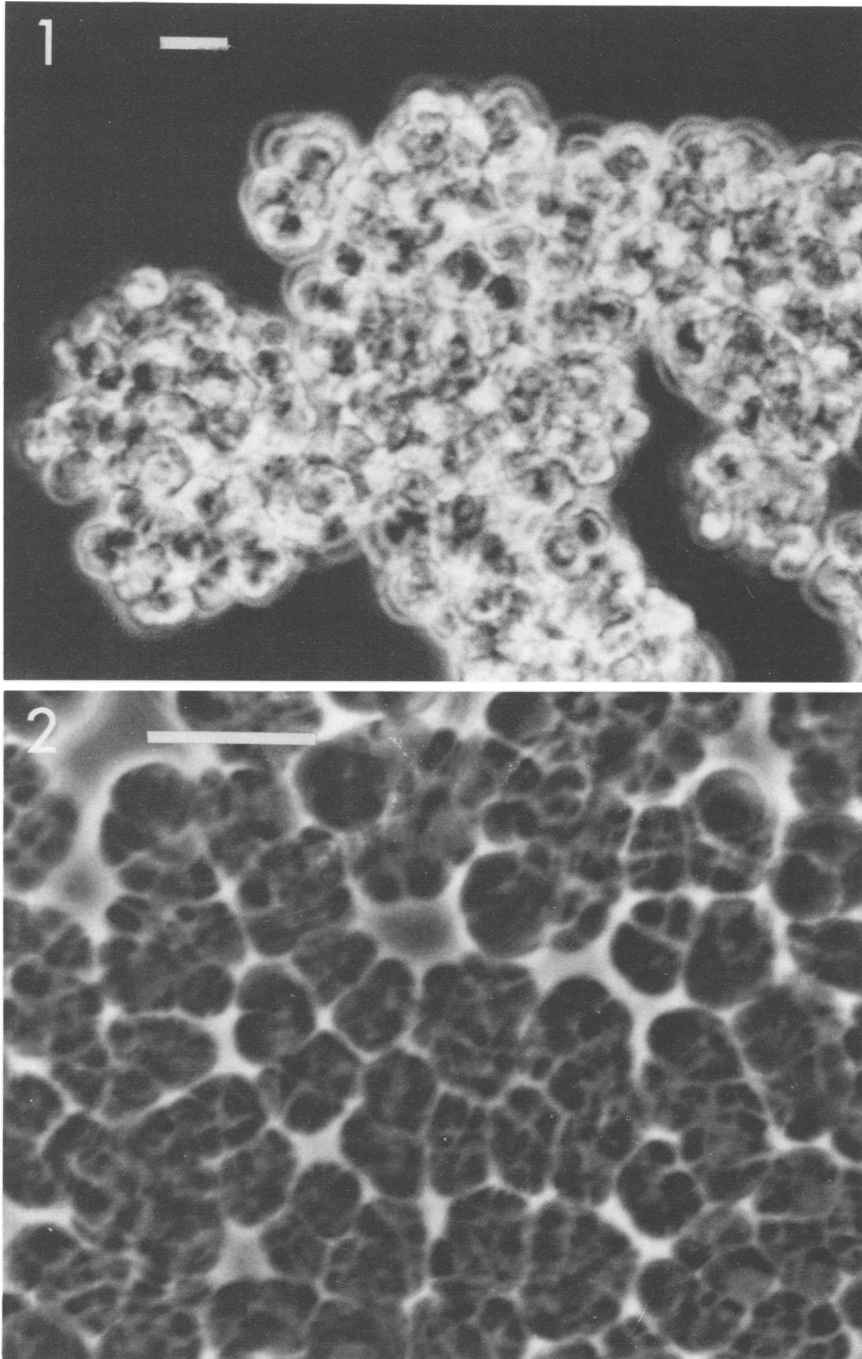


FIG. 1. Phase-contrast micrograph (40 \times dry objective, wet mount) of a refractile clump of *Methanosarcina* strain TM-1 grown on a methanol-acetate mixture at 50 $^{\circ}$ C. Bar, 10 μ m.

FIG. 2. Phase-contrast micrograph (100 \times oil objective) of a clump of *Methanosarcina* strain TM-1 which was allowed to partially dry out so that cells partially disaggregated and lost their refractility. Bar, 10 μ m.

sodium, calcium, and ammonium salts of acetate were all converted to quantities of methane nearly equal to the quantity of substrate added.

Somewhat more than three quarters of the methanol added to the medium was converted to methane. The production of substantial quan-

tities of methane was always accompanied by visible growth of the culture.

Methylamine and trimethylamine were both converted to methane by *Methanosarcina* strain TM-1. The yield of methane was close to that expected from the stoichiometry, and all three methyl groups of trimethylamine were apparently used. The rate of methanogenesis was slower from methylamine than from trimethylamine, and the utilization rate of either was slower than that for methanol.

Substrates which were not converted to methane after 30 days of incubation included H₂-CO₂ mixtures, sodium formate, sodium oxalate, sodium butyrate, ethanol, trimethylamine-*N*-oxide, and methane thiol. If methanol was added to cultures along with methane thiol, the amount of methane produced by those cultures was roughly the amount expected if methanol alone was added. Therefore, the methane thiol was not inhibitory to the cultures and also apparently was not significantly cometabolized with methanol.

Figure 3A shows that growing cultures of *Methanosarcina* strain TM-1 produced methane from acetate exponentially at 50°C, indicating that growth was exponential since methane production and cell yields on acetate are proportional to each other (21). On the last day shown, the cells were entering stationary phase. The doubling time for methanogenesis from acetate was approximately 12 h. Production of ¹⁴CH₄ from ¹⁴CH₃COO⁻ paralleled total methanogene-

sis (Fig. 3A), and all methanogenesis could be accounted for by acetate dissimilation. ¹⁴CO₂ production from ¹⁴CH₃COO⁻ was approximately 1% of that converted to methane (Table 2). CH₃¹⁴COO⁻ was converted solely to ¹⁴CO₂ (Table 2). The *K_s* for growth on acetate was approximately 4.5 μmol/ml.

TABLE 1. Methanogenesis from various substrates by *Methanosarcina* strain TM-1

Substrate	Substrate added per vial (μmol)	CH ₄ (μmol) per vial	
		Expected ^a	Produced ^b
None	0		0.0-17.0
Sodium acetate	2,000	2,000	1,700
Calcium acetate	500	500	470
Ammonium acetate	1,000	1,000	930
Methanol	625	470	490
Methyl amine	1,000	750	766
Trimethylamine	500	1,125	1,114
H ₂ -CO ₂	2,000-875 ^c	500	2.0
H ₂ -CO ₂	270-116 ^d	68	1.3
Sodium formate	500	125	0.0
Sodium oxalate	500	125	3.0
Sodium butyrate	500	1,250	5.7
Ethanol	500	750	18.0
Trimethylamine- <i>N</i> -oxide	500	1,125	2.5
Methane thiol	200	150	0.0
Methane thiol + methanol	200 + 500	150 + 375	380

^a Assuming total conversion to CH₄ and CO₂.

^b Measured after up to 30 days of incubation at 50°C. Culture volume was 50 ml.

^c 0.7 atm-0.3 atm (71-30 kPa).

^d 0.1 atm-0.04 atm (10-4 kPa).

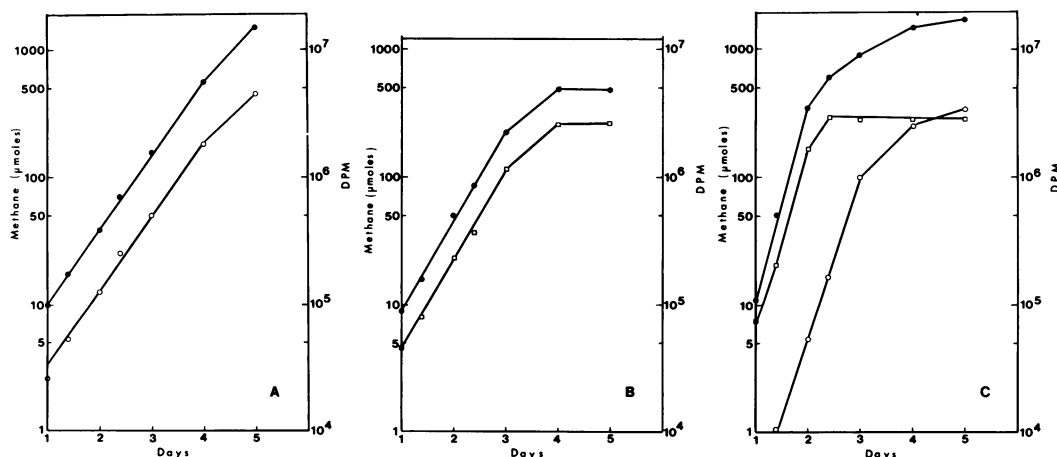


FIG. 3. Time courses for methanogenesis by *Methanosarcina* strain TM-1 growing on acetate, methanol, or a methanol-acetate mixture. The growth temperature was 50°C, and the culture volume was 50 ml. (A) Methanogenesis when 40 μmol of sodium acetate per ml (2,000 μmol per vial) and 6.7×10^6 dpm of ¹⁴CH₃COO⁻ were added. (B) Methanogenesis when 12.5 μmol of methanol per ml (625 μmol per vial) and 4.3×10^6 dpm of ¹⁴CH₃OH were added. (C) Methanogenesis when 12.5 μmol of methanol and 40 μmol of sodium acetate were added per ml. Either 6.7×10^6 dpm of ¹⁴CH₃COO⁻ or 4.3×10^6 dpm of ¹⁴CH₃OH was also added. Symbols: CH₄ (μmol per vial), ●, ¹⁴CH₄ (dpm per vial) from ¹⁴CH₃COO⁻, ○; ¹⁴CH₄ from ¹⁴CH₃OH, □.

TABLE 2. Incorporation and oxidation to $^{14}\text{CO}_2$ of radiolabeled substrates by *Methanosarcina* strain TM-1 grown on acetate, methanol, or both^a

Substrate added ^b	Label ^c	CH ₄ per vial (μmol)	Incorporation per vial		I/C ^d	$^{14}\text{CH}_4/^{14}\text{CO}_2$
			dpm ($\times 10^3$)	μmol		
Acetate	$^{14}\text{CH}_3\text{COO}^-$	1,500	128	38	0.029	85
Acetate	$\text{CH}_3^{14}\text{COO}^-$	1,200	135	48	0.049	<0.001
Methanol	$^{14}\text{CH}_3\text{OH}$	490	145	22	0.045	4.0
Methanol	$^{14}\text{CH}_3\text{COO}^-$ ^e	450	842	15	0.640	0.20
Methanol + acetate ^f	$^{14}\text{CH}_3\text{COO}^-$	475	112	32	0.370	1.3
Methanol + acetate	$^{14}\text{CH}_3\text{COO}^-$	1,650	155	47	0.059	22
Methanol + acetate	$^{14}\text{CH}_3\text{OH}$	1,700	91	14	0.030	8.0

^a 50-ml culture volume and 5-day incubation at 50°C, except where noted.

^b Acetate, 40 $\mu\text{mol}/\text{ml}$ (2,000 μmol per vial); methanol, 12.5 $\mu\text{mol}/\text{ml}$ (625 μmol per vial).

^c $^{14}\text{CH}_3\text{COO}^-$, 6.7×10^6 dpm per vial; $\text{CH}_3^{14}\text{COO}^-$, 5.2×10^6 dpm per vial; $^{14}\text{CH}_3\text{OH}$, 4.2×10^6 dpm per vial.

^d Disintegrations per minute (dpm) incorporated/dpm catabolized to $^{14}\text{CH}_4$ and $^{14}\text{CO}_2$.

^e 2.2×10^6 dpm per vial.

^f 2.4 days of incubation.

When $^{14}\text{CH}_3\text{COO}^-$ was added to cells growing on acetate, 2.9% of the amount of label catabolized to CH_4 and CO_2 was incorporated into cells (Table 2). $\text{CH}_3^{14}\text{COO}^-$ was incorporated to a slightly greater extent, 4.1% of that catabolized, indicating that some of the label converted to $^{14}\text{CO}_2$ might subsequently be assimilated. This may not be surprising since pathways involving acetate assimilation, especially those involving pyruvate synthetase, require carbon dioxide uptake (28).

Methanosarcina TM-1 had a doubling time of approximately 10 h when methanol served as the methanogenic substrate (Fig. 3B). Doubling times as low as 7 h have been observed for cultures growing with methanol as the sole addition to the basal medium. The ratio of $^{14}\text{CH}_4$ to $^{14}\text{CO}_2$ produced from $^{14}\text{CH}_3\text{OH}$ was approximately 4:1 (Table 2), rather than 3:1 as would be expected from the previously reported stoichiometry of this reaction (15, 32). In other experiments this ratio varied between 3.5:1 and 4.5:1. This discrepancy could be explained if the acetate in the basal medium (0.85 $\mu\text{mol}/\text{ml}$) was taken into account. When this acetate was labeled with 2.2×10^6 dpm of $^{14}\text{CH}_3\text{COO}^-$ (Table 2) during growth on methanol, 1.1×10^6 dpm (0.43 $\mu\text{mol}/\text{ml}$) was converted to $^{14}\text{CO}_2$ and only 0.22×10^6 dpm (0.08 $\mu\text{mol}/\text{ml}$) was converted to $^{14}\text{CH}_4$ ($^{14}\text{CO}_2/^{14}\text{CH}_4 = 5.0$). Thus, the electrons generated from the oxidation of acetate could reduce an extra 0.43 μmol of methanol per ml. The extra methanol reduced to methane was 0.62 $\mu\text{mol}/\text{ml}$, slightly greater than the reduction predicted from the amount of acetate oxidized to $^{14}\text{CO}_2$. This discrepancy may be due to $^{14}\text{CO}_2$ assimilation (27) or use of components in the yeast extract for reducing equivalents.

Incorporation of $^{14}\text{CH}_3\text{OH}$ into cells was 4.5% of that catabolized to CH_4 and CO_2 (Table 2). The small amount of acetate in the medium was extensively incorporated; 64% of the amount of acetate catabolized was taken up (Table 2).

Methanogenesis by *Methanosarcina* strain TM-1 was biphasic in medium in which both methanol and acetate were added (Fig. 3C). During the first phase (2.4 days), methanol metabolism accounted for over 90% of the methane and the doubling time was approximately 5 h. Once the methanol was depleted, methanogenesis occurred at a much slower doubling rate, with acetate serving as the methanogenic substrate. Methanogenesis by cultures growing on methanol-acetate mixtures usually ceased before the acetate was completely used, most likely due to nutrient depletion. Although methanol was the preferred methanogenic substrate during the first phase, both methanol and acetate dissimilation paralleled total methanogenesis.

The ratio of $^{14}\text{CH}_4$ to $^{14}\text{CO}_2$ produced from $^{14}\text{CH}_3\text{OH}$ after growth on methanol-acetate medium was approximately 8:1 rather than 3:1. Approximately 4.5% of the amount of $^{14}\text{CH}_3\text{COO}^-$ that was metabolized to $^{14}\text{CH}_4$ was oxidized to $^{14}\text{CO}_2$, as compared to 1% for cultures without methanol. Only 0.1% of the $\text{CH}_3^{14}\text{COO}^-$ that was added to the medium was reduced to $^{14}\text{CH}_4$ (data not shown). The extra electrons generated from acetate oxidation could reduce 1.6 extra μmol of $^{14}\text{CH}_3\text{OH}$ per ml, and 1.8 $\mu\text{mol}/\text{ml}$ extra was reduced.

When incorporation of these substrates into cellular material was examined at 2.4 days, just when the methanol was depleted, it was found that 37% of the $^{14}\text{CH}_3\text{COO}^-$ catabolized was incorporated (Table 2). Thus, a high percentage

of the acetate metabolized was incorporated during growth on methanol in medium containing either small or large amounts of acetate. After methanol depletion, acetate was used primarily for methanogenesis, and the ratio of incorporation to catabolism decreased accordingly. The presence of 40 μmol of acetate per ml in the medium depressed methanol incorporation from 22 to 14 μmol per vial while acetate incorporation (methyl group) increased from 15 to 32 μmol per vial.

The growth yield for *Methanosarcina* strain TM-1 was 1.8 ± 0.2 mg (dry weight) per mmol of CH_4 on 40- $\mu\text{mol}/\text{ml}$ acetate. The yields on methanol (12.5 $\mu\text{mol}/\text{ml}$) or methanol (same concentration) plus acetate (40 $\mu\text{mol}/\text{ml}$) were 4.5 ± 0.03 and 4.5 ± 0.6 mg of cells per mmol of CH_4 , respectively.

Hydrogen metabolism of *Methanosarcina* strain TM-1. As previously stated, methanogenesis and growth were not obtained in medium containing $\text{H}_2\text{-CO}_2$ mixtures as the methanogenic substrate. Since other strains of *Methanosarcina* can use hydrogen (6, 15, 32), this phenomenon was investigated further.

The apparatus used for pressurizing the vials with the $\text{H}_2\text{-CO}_2$ mixture, similar to that described by Balch and Wolfe (1), had been used

successfully in this laboratory to culture mesophilic *Methanosarcina* strains as well as other methanogens. *Methanobacterium thermoautotrophicum* and *Methanosarcina* strain 227 grew readily in the standard $\text{H}_2\text{-CO}_2$ medium. Several medium modifications, such as changing the yeast extract or bicarbonate concentrations, were employed without success. A batch of medium used by T. Ferguson in our laboratory to culture *Methanosarcina* strain 227 on $\text{H}_2\text{-CO}_2$ failed to support growth of *Methanosarcina* strain TM-1 when supplemented with 5% SS. Neither acetate-grown nor methanol-grown cells of *Methanosarcina* strain TM-1 grew on $\text{H}_2\text{-CO}_2$.

A more convincing test to determine the ability of *Methanosarcina* strain TM-1 to metabolize hydrogen was to present cultures with both $\text{H}_2\text{-CO}_2$ and methanol simultaneously. If the cultures used the methanol but still did not use the $\text{H}_2\text{-CO}_2$, this would be significant, since other *Methanosarcina* strains can use both substrates simultaneously and will readily use $\text{H}_2\text{-CO}_2$ alone when methanol is depleted (27; T. Ferguson and R. A. Mah, unpublished data). Such an experiment is presented in Fig. 4.

In Fig. 4A, the course of metabolism of $^{14}\text{CH}_3\text{OH}$ (12.5 $\mu\text{mol}/\text{ml}$) was followed in the

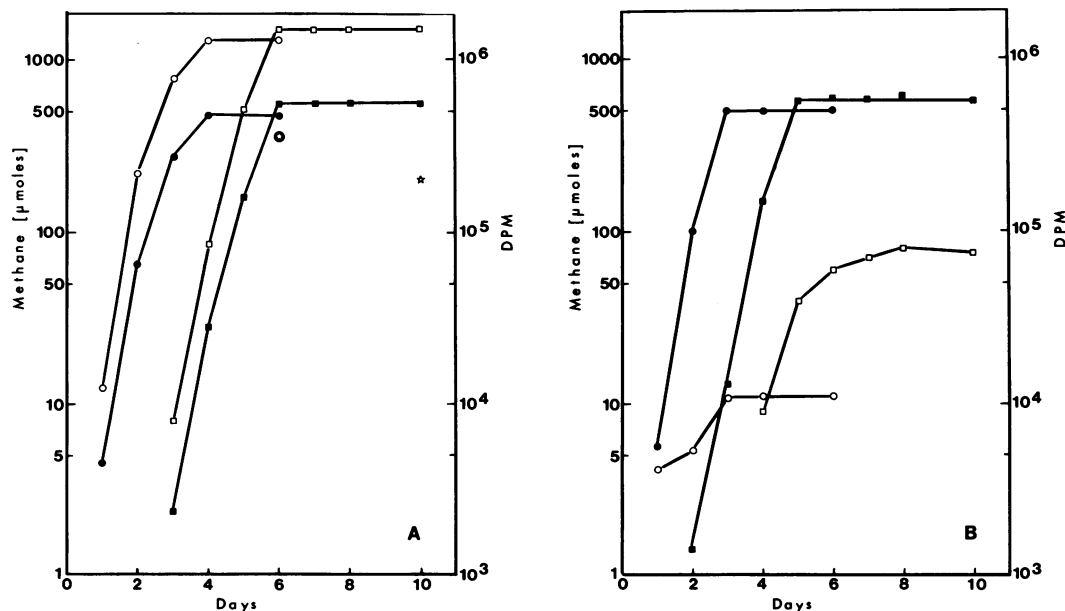


FIG. 4. Effect of hydrogen on methanol metabolism by *Methanosarcina* strain TM-1 at 50°C. The methanol concentration was 12.5 $\mu\text{mol}/\text{ml}$ (625 μmol per vial), and the headspaces over the cultures contained 1-atm overpressure of either 70% $\text{N}_2\text{-30\% CO}_2$ (control) or 70% $\text{H}_2\text{-30\% CO}_2$. The culture volumes were 50 ml, and the headspace volumes were 74 ml. (A) 2.1×10^6 dpm of $^{14}\text{CH}_3\text{OH}$ added; (B) 4.2×10^6 dpm of $^{14}\text{CO}_2$ added. Symbols: CH_4 produced under $\text{N}_2\text{-CO}_2$, ●; CH_4 produced under $\text{H}_2\text{-CO}_2$, ■; $^{14}\text{CH}_4$ produced under $\text{N}_2\text{-CO}_2$, ○; $^{14}\text{CH}_4$ produced under $\text{H}_2\text{-CO}_2$, □; $^{14}\text{CO}_2$ produced under $\text{N}_2\text{-CO}_2$, ●; $^{14}\text{CO}_2$ produced under $\text{H}_2\text{-CO}_2$, ☆.

presence of either 70% N₂-30% CO₂ (control) or 70% H₂-30% CO₂. The gases were added at 1-atm overpressure to a headspace already containing 1 atm of N₂. In the presence of 70% N₂-30% CO₂, methanol was metabolized rapidly without any lag. ¹⁴CH₄ production paralleled total methanogenesis, the specific activity of the ¹⁴CH₄ was as expected, and the final ratio of ¹⁴CH₄ to ¹⁴CO₂ produced was 3.6, similar to that found under an atmosphere containing N₂ alone. If the culture headspace contained 1 atm of 70% H₂-30% CO₂, the cultures tended to lag 2 to 4 days. After this lag period, methanogenesis was nearly as rapid as that in medium without hydrogen, and there was visible growth of the culture. Since the lag times were variable, these data points represent single culture vials rather than duplicates, but similar results were obtained in all cases. Methane production was slightly greater in the presence of H₂ by about 75 μmol per vial. There was sufficient H₂ present to produce over 500 μmol of extra CH₄ per vial. The ratio of ¹⁴CH₄ to ¹⁴CO₂ produced by cultures with added ¹⁴CH₃OH was 7.2 in the presence of hydrogen, with approximately 60 μmol of extra methane produced per vial from methanol reduction.

For Fig. 4B the cultures were grown on 12.5 μmol of methanol per ml in the presence of either N₂-CO₂ or H₂-CO₂, and the label added was ¹⁴CO₂ so that CO₂ reduction to CH₄ could be quantified. Under 70% N₂-30% CO₂, only 0.25% of the ¹⁴CO₂ was reduced to ¹⁴CH₄ (less than 3 μmol of CH₄ per vial). In the presence of 70% H₂-30% CO₂, more ¹⁴CO₂ was reduced to ¹⁴CH₄, equivalent to 20 μmol of CH₄ per vial. The rate of ¹⁴CO₂ reduction gradually decreased once methanol was depleted. No further methanogenesis was detected in cultures incubated for another 10 days. Hydrogen was never appreciably depleted under these conditions. No methanogenesis or growth was detected in control vials to which H₂CO₂ but not methanol was added. Thus *Methanosarcina* strain TM-1 could metabolize hydrogen for limited reduction of methanol and carbon dioxide during growth on methanol but appeared to be unable to use it once methanol was depleted.

If the gas pressure was reduced to 0.1 atm of H₂-0.04 atm (4 kPa) of CO₂, cultures grew on methanol without a lag but still did not use appreciable H₂. If 40 μmol of sodium acetate per ml was added instead of methanol, no growth or methanogenesis occurred in the presence of hydrogen at either 0.7 or 0.1 atm (71 or 10 kPa).

Temperature and pH range for growth of *Methanosarcina* TM-1. Figure 5 shows the specific rate of methanogenesis (0.69/doubling

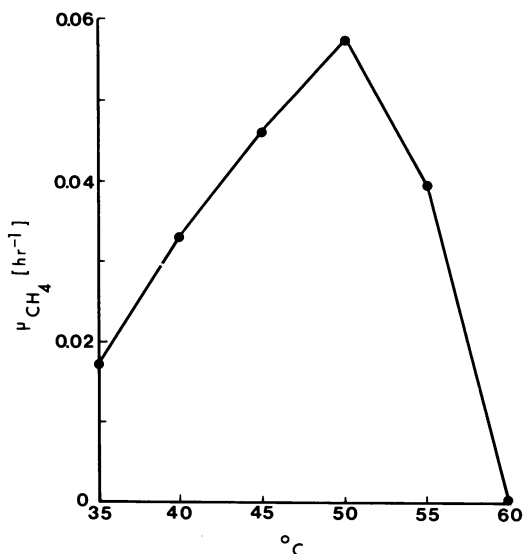


FIG. 5. Specific rate of methanogenesis ($\mu\text{CH}_4 = 0.69/\text{doubling time [h]}$) for cultures of *Methanosarcina* strain TM-1 growing on 40 μmol of sodium acetate per ml at various temperatures.

time) for *Methanosarcina* strain TM-1 growing exponentially in basal medium supplemented with 40 μmol of sodium acetate per ml. The maximum growth temperature was near 50°C, with a doubling time of 12 h. There was slower growth at 35°C (doubling time = 36 h), and the culture grew well at 55°C, the temperature at which it was isolated, but did not grow at 60°C. Similar results were obtained for growth on methanol or methanol-acetate mixtures. When grown in acetate medium at temperatures below 50°C, the cells would often completely lyse once stationary phase was reached. Partial lysis was evident when cultures were examined by phase-contrast microscopy, and if lysis was total, the cell mass completely disappeared from the culture medium. There was occasionally slight cell lysis at 50°C on acetate but none at 55°C. Lysis in methanol-containing medium, except for very old cultures, was rare. The optimum growth temperature of 50°C was used in all other experiments.

Methanosarcina strain TM-1 had a wide pH range for growth, as demonstrated in Fig. 6. All cultures grew in methanol-acetate medium ranging in initial pH from 5.5 to 8.0. Once some growth occurred, the pH of the medium changed, so results are only shown for day 1. Methanogenesis was somewhat slower at the extreme pH values. There was a similar range for growth in media containing either acetate or methanol alone.

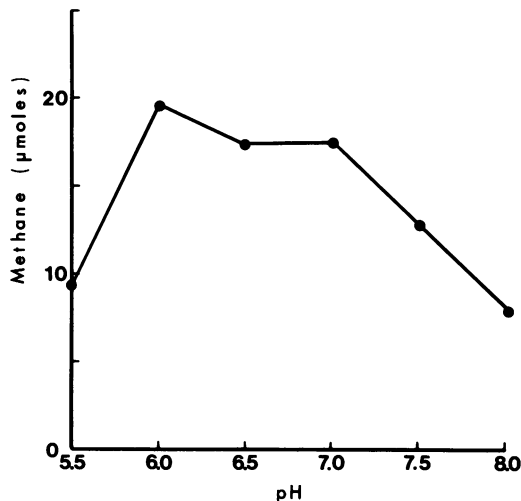


FIG. 6. Methane production (micromoles per milliliter) at 50°C by cultures of *Methanosarcina* strain TM-1 inoculated into media of various initial pH values. The culture volume was 50 ml, and the medium contained 40 μmol of sodium acetate and 12.5 μmol of methanol per ml. The pH of the medium changed less than 0.1 unit when the temperature was increased to 50°C.

Growth factor requirement of *Methanosarcina* strain TM-1. During the course of the isolation of *Methanosarcina* strain TM-1, it was noted that colonies would only transfer to tubes containing SS, usually at a concentration of 5% (vol/vol). Once the organism was isolated, it was desirable to study this growth factor requirement more closely (the singular term growth factor will be used for simplicity, even though it has not been established that there is only one growth factor). SS from the laboratory digester from which *Methanosarcina* strain TM-1 was isolated was initially used, but several difficulties made the use of this supplement undesirable. Because of the small size of the digester, only 100 ml of SS could be generated per day, and the potency of this SS varied from batch to batch, such that some batches did not support growth at all whereas others were so potent that the carryover with the inoculum would support growth of a new culture. A similar situation was reported by Bryant (5) in initial experiments on the growth of *Methanobacterium ruminantium* grown in medium supplemented with rumen fluid. To alleviate this situation, 10 liters of digested sludge was obtained from a thermophilic digester at the Hyperion sewage treatment plant. The potency of this large quantity of SS could be determined, and uniform results were obtained during the course of these experiments.

Figure 7 shows the effect of adding various

concentrations of SS on methanogenesis from 25 μmol of methanol per ml by *Methanosarcina* strain TM-1. With no SS added, only slightly more than 1 μmol of methane was produced after 6 days, and no visible growth occurred. If 1% SS was added, methane was produced at a depressed rate; 2% SS markedly increased methanogenesis, and nearly all the methanol was used after 6 days. Increasing the SS concentration to 4 or 8% further increased methanogenesis. Similar results were obtained with cultures growing on acetate or methanol-acetate mixtures. A final concentration of 5% (vol/vol) SS was used routinely for culturing *Methanosarcina* TM-1.

DISCUSSION

Methanosarcina strain TM-1 is a moderate thermophile, since its optimum temperature for methanogenesis was near 50°C and its maximum temperature was below 60°C. *Methanobacterium thermoautotrophicum* is a more extreme thermophile with a maximum temperature of 75°C (33). We have recently isolated other thermophilic strains of *Methanosarcina* which grow

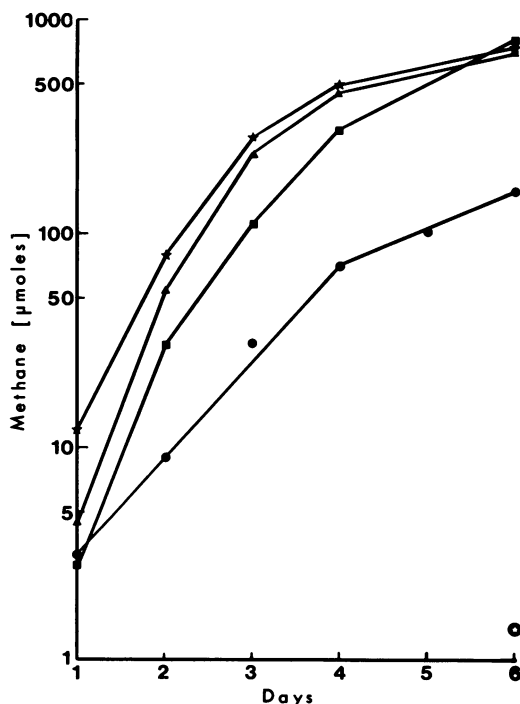


FIG. 7. Methanogenesis by *Methanosarcina* strain TM-1 in the presence of various concentrations of SS. The growth temperature was 50°C, and the medium contained 25.0 μmol of methanol per ml (1,250 μmol per vial). Symbols: 0% (vol/vol) SS (★); 1% SS (●); 2% SS (■); 4% SS (▲); 8% SS (☆).

optimally at 55°C and can grow at 60°C, and we are presently in the process of characterizing these strains (Zinder and Mah, unpublished data).

Methanosarcina TM-1 grew more rapidly at 50°C than its mesophilic counterparts do at 35°C, with doubling times of 12 h on acetate, 7 to 10 h on methanol, and 5 h on methanol-acetate mixtures. Smith and Mah (21) reported maximum doubling times for *Methanosarcina* 227 on acetate of 24 to 36 h and a doubling time of 10 to 12 h for *Methanosarcina* strains MS and 227 on methanol (21, 27). It is of interest that *Methanosarcina* strain TM-1 had a doubling time on acetate at 35°C of 36 h, quite close to that of strain 227. One may speculate that mesophilic strains of *Methanosarcina* are, in effect, temperature-sensitive mutants of thermophilic strains.

When acetate was the sole methanogenic substrate added to the basal medium, it was primarily split to methane and carbon dioxide, as previously described in other strains of *Methanosarcina* (14, 21, 28). Only a small fraction of the acetate metabolized was incorporated or oxidized. Both the K_s (4.5 $\mu\text{mol/ml}$) and the growth yield of *Methanosarcina* strain TM-1 on acetate were similar to those reported for other strains (21, 28).

When methanol and acetate were both added to the growth medium, the pattern of acetate utilization changed dramatically. The methyl group of acetate served as a minor source of methane compared to methanol. Acetate was now largely incorporated into cell mass or oxidized. High ratios of assimilation versus catabolism of acetate were also reported for *Methanosarcina* strain MS growing on methanol-acetate mixtures (28).

Addition of acetate to the medium enhanced growth rates on methanol, and it is likely that the variability of the doubling time in medium containing methanol (7 to 10 h) reflected small variations in the acetate concentration in the basal medium. Stimulation of growth by acetate has been noted in other methanogens (28, 32). Although growth on methanol was much faster in the presence of added acetate, the growth yields were the same, 4.5 mg of cells per mmol of CH_4 . This yield is lower than that reported for methanol-grown mesophilic strains, 5.1 to 7.2 mg of cells per mmol of CH_4 (11, 21, 26).

When acetate was added to methanol-containing medium, the methyl group was significantly oxidized. Ratios of $^{14}\text{CO}_2$ to $^{14}\text{CH}_4$ from $^{14}\text{CH}_3\text{COO}^-$ during growth on methanol by various strains of *Methanosarcina* varied from 0.8 (28) and 2.0 (14) to 5.0 (this study). The lower

the acetate concentration, the greater the relative oxidation, probably because the low concentrations were below the K_s for methanogenesis from acetate (4 to 5 $\mu\text{mol/ml}$) so that oxidation competed more effectively with methanogenesis for acetate. When acetate was oxidized, methanol was reduced via some unknown mechanism by a quantity of electrons equivalent to that generated from oxidation of the methyl group of acetate to CO_2 . It is unlikely that carbon dioxide produced from methanol was the electron acceptor, since very little $^{14}\text{CO}_2$ reduction was observed in cultures grown on methanol medium without H_2 in the headspace (Fig. 4B), even though low levels of acetate were present in the medium. The reduction of methanol via the equation: $\text{CH}_3\text{COO}^- + 4\text{CH}_3\text{OH} \rightarrow 2\text{HCO}_3^- + 4\text{CH}_4 + \text{H}^+$ has a $\Delta G'_0$ of -20.7 kcal (ca. -86.7 kJ) per mol of CH_4 , using the free energies of formation quoted by Thauer et al. (22), so the reaction is energetically favorable and similar to that for fermentation of methanol (-25.6 kcal [ca. -107.2 kJ] per mol of CH_4).

To calculate the amount (micromoles) of extra $^{14}\text{CH}_3\text{OH}$ reduced to methane due to oxidation of the methyl group of acetate, one must consider the number of electrons produced from each substrate. In the exclusive fermentation of methanol, oxidation of one methanol molecule to CO_2 yields six electrons, which are used to reduce three other methanol molecules to methane. However, oxidation of one acetate molecule to CO_2 yields eight, instead of six, electrons and consequently provides two additional electrons for reduction of an additional methanol to methane. Thus, the net result is the appearance of one extra methane molecule for each acetate molecule oxidized during methanogenesis from methanol.

Extensive oxidation of the methyl group of acetate to carbon dioxide occurred simultaneously with extensive reduction of methanol to methane. This implies that the cell could discriminate between methanol and the methyl group of acetate for purposes of oxidation. If a common intermediate (called $^*\text{CH}_3\text{-X}$) was involved in both the oxidation and reduction of $^*\text{CH}_3\text{COOH}$ and $^*\text{CH}_3\text{OH}$, as has been suggested by Weimer and Zeikus (28) extrapolating from Baker's scheme (4), then one should expect the two substrates to share a similar fate. The results from three strains of *Methanosarcina* do not support this conclusion (14, 28), since the oxidation of the methyl group of acetate was always greater than that expected for methanol ($^{14}\text{CO}_2/^{14}\text{CH}_4 = 0.33$). Among the possible mechanisms for acetate oxidation, the tricarboxylic acid cycle and the glyoxylate cycle are unlikely since the

enzymes of these pathways may not all be present in the methanogens (29). Other possibilities include the following. (i) The presence of two separate intermediates, one for oxidation and the other for reduction of the methyl group of acetate and methanol; acetate may compete more effectively for formation of the intermediate involved in oxidation. (ii) Reversal of an acetate synthetase system: Fuchs et al. (10) suggested that de novo acetate synthesis is the major mechanism of carbon fixation by methanogenic bacteria. Reversal of such a reaction could provide carbon dioxide and reducing equivalents from acetate.

Except for the inability to utilize H_2-CO_2 , *Methanosarcina* strain TM-1 grew on the same substrates, namely acetate, methanol, methylamine, and trimethylamine, that were previously shown to support growth of mesophilic *Methanosarcina* strains (11, 14). Growth and methanogenesis from methylamine and trimethylamine were similar to those described by Hippe et al. (11); interestingly, trimethylamine-*N*-oxide was not a substrate for methanogenesis. Methane thiol, shown by Zinder and Brock (35) to be converted to methane by anaerobic lake sediments, did not support growth or methanogenesis by *Methanosarcina* strain TM-1 or strain MS (27). It is still possible that cometabolism of this substrate to form methane may occur under the appropriate physiological conditions.

Our inability to demonstrate growth or substantial methanogenesis from H_2-CO_2 by *Methanosarcina* strain TM-1 is unique (6, 15, 30, 32) since all previously described strains will normally use H_2-CO_2 for methanogenesis. However, there are two possible exceptions to this presumption: (i) the filamentous acetate-using rod mentioned by Zehnder and Brock (31) was unable to use H_2-CO_2 ; and (ii) *Methanosarcina* strain 227, when grown in the mineral-acetate medium described by Baresi et al. (2), apparently did not metabolize hydrogen added to the headspace during growth on acetate (L. Baresi, D.P.H. thesis, University of California, Los Angeles, 1978). Evidently, *Methanosarcina* strain TM-1 was capable of limited hydrogen metabolism during growth on methanol but was unable to metabolize it once the methanol was depleted. In complex medium, *Methanosarcina* strain 227 will utilize H_2-CO_2 and methanol concurrently (Ferguson and Mah, unpublished data), as will strain MS (27).

Methanogenesis from methanol occurred after a lag in the presence of 0.7 atm of H_2 but was uninhibited by 0.1 atm of H_2 . Acetate metabolism was inhibited at both hydrogen pressures. The mechanism of this inhibition is obscure and

is presently being investigated.

At present we have no explanation for the apparent inability of *Methanosarcina* strain TM-1 to metabolize H_2-CO_2 extensively. A lesion in electron transport pathways or in biosynthetic pathways may be involved. The possibility remains that the appropriate conditions for growth on H_2-CO_2 were not achieved, but it should be reemphasized that the culture did grow on methanol in the presence of H_2-CO_2 , and that other *Methanosarcina* cultures readily used the two substrates concurrently.

The requirement of a growth factor (or growth factors) from SS is a characteristic of *Methanosarcina* strain TM-1 not shared by other described strains of *Methanosarcina* (6, 14, 15, 28). Preliminary characterization of this factor indicates that it is found in both thermophilic and mesophilic anaerobic digester SS, rumen fluid, and extracts of methanogenic bacteria, but not in yeast, beef, or *Escherichia coli* extracts. The SS in the medium could not be replaced with a mixture containing coenzyme M, 2-methylbutyrate (factors required by *Methanobacterium ruminantium* [1, 5]), and beef extract, a rich source of vitamins and other common cofactors. Thus, it seems likely that this factor is one which has not been previously described for methanogens. It is possible that there is some relationship between this growth factor requirement and the inability to use hydrogen. Further characterization of this factor is in progress.

Phase-contrast microscopy of the thermophilic laboratory digester sludge from which *Methanosarcina* strain TM-1 was isolated disclosed large, refractile masses of *Methanosarcina*-like clumps. These clumps showed the characteristic blue-green fluorescence of F_{420} (17) when viewed with an epifluorescence microscope. A mesophilic digester (37°C) receiving the same batch of raw sludge at the same retention time (15 days) did not contain microscopically visible *Methanosarcina*-like clumps; instead, filaments resembling *Methanobacterium sohngeii* were present. *Methanosarcina*-like clumps were also observed in sludge from a thermophilic laboratory digester operated at 55°C on cellulosic wastes and from a Hyperion sewage treatment digester operated at 51°C. The biovolume of the clumps in the Hyperion sludge was estimated by using a Petroff-Hausser counting chamber and an eyepiece micrometer and was approximately equivalent to that of 1.0×10^8 to 5.0×10^8 *E. coli*-sized cells per ml. Consequently, thermophilic *Methanosarcina* strains may play an important role in thermophilic digestors since they are among the predominant bacteria in terms of cell mass.

However, *Methanosarcina* may not be the only acetate-using methanogen in thermophilic digestors, since some active acetate-fermenting thermophilic enrichments maintained in our laboratory do not contain *Methanosarcina* and instead are dominated by a rod similar to that described by Coolhaas (8). It is unlikely that the cells observed in the sludge were not *Methanosarcina*, since no other reported organisms have the characteristic pseudosarcina shape or blue-green fluorescence (6) of the methanosarcinas. Occasionally, clumps of round yeastlike cells may be seen in thermophilic digestors, but these do not resemble the pseudosarcina.

The natural habitat for thermophilic methanosarcinas may be in the anaerobic layers of hot-spring algal mats such as those described by Ward (26) or in other anaerobic geothermal environments. It is possible that the thermophilic strains could compete successfully in mesophilic habitats, since the doubling times on acetate are similar at 35°C for *Methanosarcina* strains 227 and TM-1.

Because of the unmistakable pseudosarcina morphology (3, 16) and methanogenesis from acetate and methanol, the thermophilic isolate should be placed in the genus *Methanosarcina*. The criteria for generic status of methanogenic bacteria are morphology and production of significant quantities of methane from certain substrates (6). However, a species epithet will await further characterization and isolation of other strains from other locations and habitats. It is possible that other thermophilic strains may not require a growth factor or can use H₂-CO₂.

The phylogenetic relationships between thermophilic and mesophilic methanosarcinae may be clarified by examining the guanine plus cytosine content of the deoxyribonucleic acid and by fingerprint analysis of the ribosomal ribonucleic acid (9). Comparison of the lipids of the thermophilic methanosarcinae with those of their mesophilic counterparts may also be taxonomically interesting, especially since the lipids of methanogens are glycerol-linked phytanyl ethers of the "archaeobacterial" type (23).

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