APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1983, p. 265–274
0099-2240/83/010265-10$02.00/0

Vol. 45, No. 1

Isolation and Characterization of an H_2-Oxidizing Thermophilic Methanogen

THOMAS J. FERGUSON* AND ROBERT A. MAH

Division of Environmental and Nutritional Sciences, School of Public Health, University of California, Los Angeles, California 90024

Received 18 June 1982/Accepted 16 September 1982

A thermophilic methanogen was isolated from enrichment cultures originally inoculated with sludge from an anaerobic kelp digester (55°C). This isolate exhibited a temperature optimum of 55 to 60°C and a maximum near 70°C. Growth occurred throughout the pH range of 5.5 to 9.0, with optimal growth near pH 7.2. Although 4% salt was present in the isolation medium, salt was not required for optimal growth. The thermophile utilized formate or H_2-CO_2 but not acetate, methanol, or methylamines for growth and methanogenesis. Growth in complex medium was very rapid, and a minimum doubling time of 1.8 h was recorded in media supplemented with rumen fluid. Growth in defined media required the addition of acetate and an unknown factor(s) from digester supernatant, rumen fluid, or Trypticase. Cells in liquid culture were oval to coccoid, 0.7 to 1.8 μm in diameter, often occurring in pairs. The cells were easily lysed upon exposure to oxygen or 0.08 mg of sodium dodecyl sulfate per ml. The isolate was sensitive to tetracycline and chloramphenicol but not penicillin G or cycloserine. The DNA base composition was 59.69 mol% guanine plus cytosine.

Although thermophilic anaerobic digestion processes are used for the treatment of organic wastes (12, 26, 27), little is known about the bacteria in these systems. In 1928, Coolhaas (6) reported the utilization of formate, acetate, and higher fatty acids as substrates for methane production by enrichment cultures incubated at 60°C. The first axenic culture of a thermophilic methanogen was the H_2-oxidizing *Methanobacterium thermoautotrophicum* reported by Zeikus and Wolfe in 1972 (33). Strains of this species were isolated from other thermophilic anaerobic environments (18, 19, 31). Two other thermophilic strains of *Methanobacterium* were reported and partially characterized (14). The only acetate-utilizing thermophilic methanogen in axenic culture, *Methanosarcina* sp. strain TM1, was isolated from a 55°C anaerobic digester (34). Recently, an extreme thermophile, *Methanothermus fervidus*, was isolated from an Icelandic volcanic hot spring (23). This rod-shaped methanogen utilized H_2-CO_2 and had a temperature optimum near 83°C.

We describe here the isolation and characterization of a previously unreported thermophilic H_2-CO_2- and formate-using methanogen.

This work was presented in part at the 1982 meeting of the American Society for Microbiology (T. J. Ferguson and R. A. Mah, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, I98, p. 110).

MATERIALS AND METHODS

Inoculum. A thermophilic anaerobic digester was established by introducing 2 liters of raw ground sea kelp (*Macrocystis pyrifera*) into a 3-liter flask connected to an acid-brine reservoir as previously described (34). The pH was adjusted to 6.7 to 7.2. The vessel was incubated at 55°C for several weeks before methane was produced. It was then batch fed at approximately weekly intervals. The pH was maintained near 7.0 by the addition of NaOH. Acetate enrichments were initiated by using sludge from this digester.

Culture methods. The anaerobic techniques of Hungate were used throughout these investigations (10). An anaerobic glovebox (Coy Lab Products, Ann Arbor, Mich.) was used during the preparation of culture media and anaerobic stock solutions of reagents. The serum vial technique of Balch et al. was used (1, 2), and average values for duplicate or triplicate vessels were reported unless otherwise noted. Stock cultures were incubated unshaken at 55°C, but experimental vessels were shaken at 55 to 60°C.

Culture media. Complex media consisted of the following additions per 900 ml of Milli-Q deionized water (resistivity = 17 MΩ cm): clarified rumen fluid (RF) or kelp digester supernatant (KS), 100 ml; vitamin solution (29), 5 ml; trace minerals solution (per liter of Milli-Q water: H_2SeO_3, 0.01 g; $MnCl_2 \cdot 4H_2O$, 0.10 g; $FeSO_4 \cdot 7H_2O$, 0.10 g; $CoCl_2 \cdot 6H_2O$, 0.15 g; $ZnCl_2$, 0.10; H_3BO_3, 0.01 g; $NaMoO_4 \cdot 2H_2O$, 0.01 g; $CuCl_2 \cdot 2H_2O$, 0.02 g; $NiSO_4 \cdot 6H_2O$, 0.02 g; $AlCl_3 \cdot 6H_2O$, 0.04 g; disodium EDTA dihydrate, 0.50 g), 5 ml; NH_4Cl, 1.0 g; $K_2HPO_4 \cdot 3H_2O$, 0.5 g; KH_2PO_4, 0.5 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; KCl, 20.0 g; NaCl, 10.0 g; yeast extract (Difco Laboratories, De-

troit, Mich.), 0.2 g; Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 0.2 g; cysteine-hydrochloride, 0.5 g; resazurin (0.1% [wt/vol]), 1 ml. Defined medium was composed of the same additions per liter of Milli-Q water except for the deletion of KS or RF, vitamin solution, Trypticase, and yeast extract. Solid media were prepared by the addition of 2.5% agar (Difco).

The medium was mixed and boiled under O_2-free N_2 for 5 min after the reduction of resazurin and cooled for 10 min at room temperature under N_2. The flask was stoppered and transferred to an anaerobic glovebox; 30-ml portions of media were dispensed into 120-ml serum vials (Wheaton Scientific Co., Millville, N.J.) with a Repipet Junior (Labindustries, Berkeley, Calif.) dispenser. The vials were stoppered with butyl rubber closures (Bellco Glass, Inc., Vineland, N.J.) and removed from the glovebox. The serum vials were outgassed for 3 min with N_2-CO_2 (70:30) and sealed with aluminum crimp seals (Wheaton). After autoclaving and before inoculation, the serum vials were injected with 0.30 ml of 1% $Na_2S \cdot 9H_2O$ and 0.30 ml of 10% Na_2CO_3. The final pH of the media was 6.9 to 7.1. Roll tube media were prepared in the same manner, dispensed into Pyrex culture tubes (Bellco) in 4.5-ml quantities, and sealed with no. 00 butyl rubber stoppers (Arthur H. Thomas Co., Philadelphia, Pa.). The tubes were outgassed with N_2-CO_2 for 1 min and autoclaved in a culture tube press. After the media were autoclaved and before inoculation, 0.05 ml of 1% $Na_2S \cdot 9H_2O$ and 0.05 ml of 10% Na_2CO_3 were added to obtain a final pH near 7.0.

KS and RF. Kelp digester effluent was clarified by centrifugation (10,000 rpm), autoclaving, and recentrifuging to obtain a clear yellow KS solution. Effluent prepared in this manner was stored frozen and added to the complex media before boiling. RF was obtained by filtering the entire stomach contents of a freshly slaughtered steer (ACME Meat Packers, Vernon, Calif.) through cheesecloth. The resulting liquid was processed in the same manner as KS, and a clear, dark brown liquid was obtained. Sterile anaerobic solutions of KS were prepared by dispensing 50-ml portions of the fluid into 120-ml serum vials, outgassing with N_2 for 15 to 20 min at 50°C, and autoclaving for 20 min at 15 lb/in^2 and 120°C. Before use, these stock solutions were injected with sterile 1% $Na_2S \cdot 9H_2O$ to obtain a final Na_2S concentration of 0.01%.

Analytical techniques. Culture headspace gases were analyzed by gas chromatography as previously described (4). Optical density was determined at 660 nm by using matched culture tubes (1 cm path length) and a Spectronic 21 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). Cell counts were determined with a Petroff-Hausser counting chamber (Hausser Scientific Co., Bluebell, Pa.). Molar growth yields were calculated from gravimetric determinations of cells concentrated by membrane filtration (34). Routine mathematical analyses were performed on a programmable calculator (Texas Instruments, Dallas, Tex.; model 59) equipped with a PC-100C printer. Regression analyses (linear, exponential, logarithmic, and power) were obtained by using a four-curve regression analysis program provided by Texas Instruments (PPX59-208011). Radioactivity was followed by using the gas chromatographic-gas proportional counting technique as previously reported (4).

DNA base composition. Whole-cell DNA was extracted and purified by the method of Price et al. (17). The buoyant density of the purified DNA was determined by ultracentrifugation in a cesium chloride gradient (17).

Microscopy. A Zeiss Universal Research Microscope (Carl Zeiss, West Germany) equipped with phase optics, epifluorescence, and an automatic photomicrographic exposure system was used to examine samples at 420 to 430 nm. Methanogenic cells and colonies exhibited a blue-green fluorescence under UV epi-illumination. Kodak Ektachrome film (ASA 400) was used for phase and epifluorescent photomicrography.

Samples for transmission electron microscopy were prepared by fixing with glutaraldehyde and osmium and were treated in a standard manner with Spurrs plastic (R. Robinson, personal communication).

Reagents, chemicals, and gases. All chemicals were of reagent quality unless otherwise noted. Mercaptoethanesulfonic acid was obtained as a 3 N solution from Pierce Chemicals (Rockford, Ill.). Gas mixtures were purchased from Matheson (Searle Medical Products, Cucamonga, Calif.). Other gases were purchased from Liquid Carbonics, Los Angeles, Calif. Radioactive [^{14}C]sodium carbonate (53.5 mCi/μmol) was purchased from Amersham Corp. (Arlington Heights, Ill.) and prepared as an anaerobic stock solution at a final concentration of 1 μCi/ml.

RESULTS

Isolation of thermophilic methanogens. Thermophilic acetate enrichment cultures were initiated by inoculating complex KS liquid medium (20 mM sodium acetate) with sludge from the 55°C anaerobic kelp digester. After 1 week of incubation at 55°C, both H_2 and CH_4 were detected in the headspaces of these enrichments, and microscopic examination of samples revealed numerous nonfluorescent rod-shaped and filamentous bacteria. Fluorescent *Methanosarcina*-type clumps surrounded by fluorescent coccoid bacteria were also present. The cocci were so small that they were difficult to distinguish without epifluorescence microscopy. The *Methanosarcina* sp. was eventually isolated in complex acetate medium by using the antibiotic roll tube technique of Zinder and Mah (34). It utilized acetate but not H_2-CO_2 for methanogenesis and closely resembled *Methanosarcina* sp. strain TM1 in morphology (34).

The coccoid organism was isolated by picking and diluting fluorescent colonies from H_2-CO_2 roll tube dilutions from the original acetate enrichments. The UV fluorescence technique of Edwards and McBride (8), as modified by Doddema and Vogels (7), facilitated identification of methanogenic colonies in roll tubes. Colonies of the thermophilic methanogen were observable after 12 to 30 h of incubation. An axenic culture was isolated from these dilutions in H_2-CO_2 roll tube media without the addition of antibiotics. Culture purity was established through direct

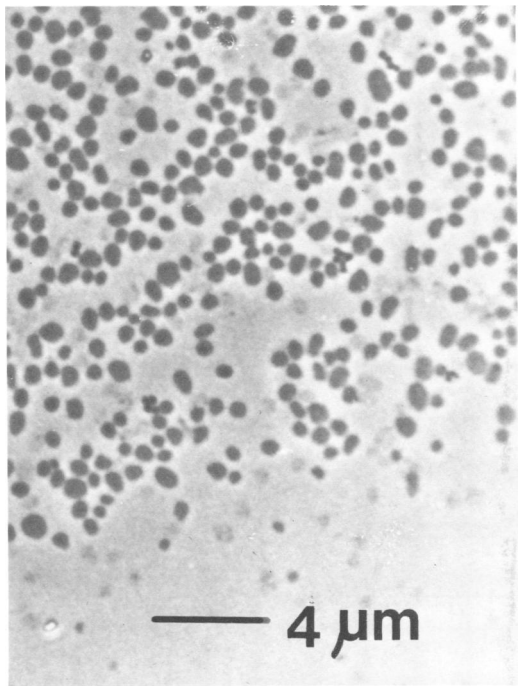

FIG. 1. Phase-contrast photomicrograph of the thermophilic isolate. Cells were pregrown in H_2-CO_2 in yeast extract- and Trypticase-supplemented liquid defined medium. Culture fluid was spread on a thin surface of agar and examined immediately.

microscopic examination of liquid cultures, examination of colonies present in roll tube dilutions, and by inoculation of anaerobic and aerobic glucose-supplemented nutrient broth incubated at 55°C. Stock cultures were inoculated into liquid KS media pressurized to 20 to 30 lb/in^2 with H_2 and incubated at 55°C without shaking. Stock liquid cultures were transferred at 24- to 48-h intervals, and culture purity was checked periodically through roll tube dilution and inoculation of glucose-supplemented anaerobic nutrient broth.

Morphology of the H_2-oxidizing methanogen. In pure culture, colonies of the methanogen were small (1 to 3 mm in diameter), convex, white to tan in color, and smooth with entire margins. These colonies fluoresced blue-green at 420-430 nm and were enumerated by microscopic examination of the agar roll tubes. When wet mounts of whole colonies were examined under epifluorescence microscopy, individual cocci with an average diameter of 0.7 to 1.8 μm (Fig. 1) were observed. In liquid media, the cells frequently occurred in pairs and were oval to short rods or coccobacilli. The coccobacilli were feebly motile in wet mounts, but motility rapidly diminished upon exposure to air. A well-defined cell wall was evident in transmission electron micrographs of the isolate, but no flagella were observed (Fig. 2). Flagellar and other staining procedures were not successful, primarily be-

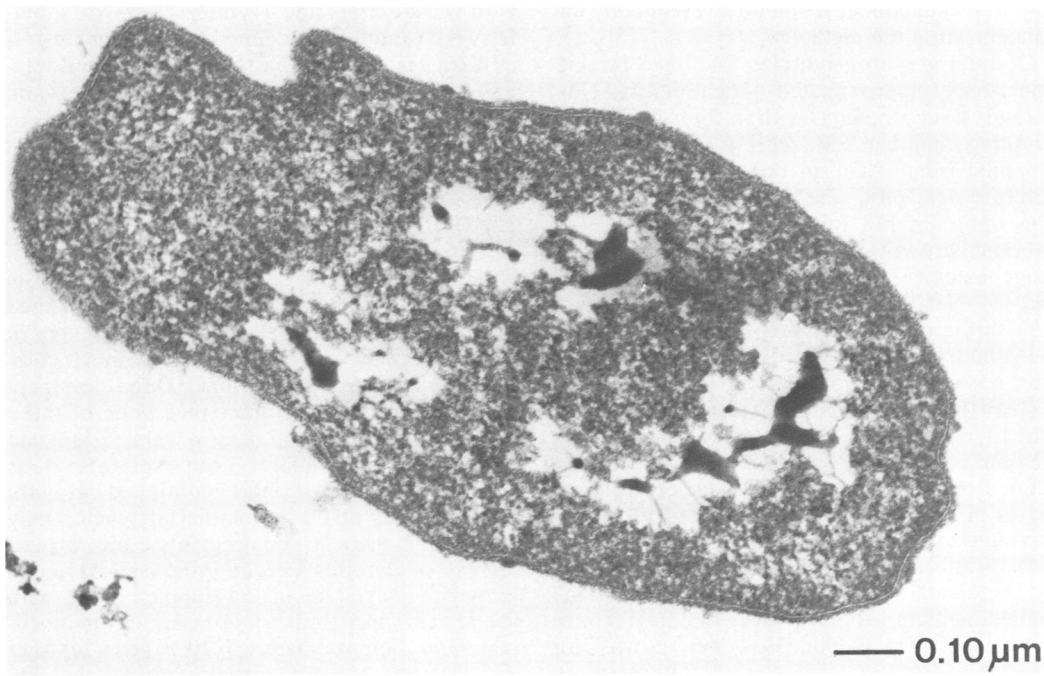

FIG. 2. Transmission electron micrograph of the thermophilic isolate. Note the well defined plasma membrane. The electron-dense nuclear region is probably due to the fixation process.

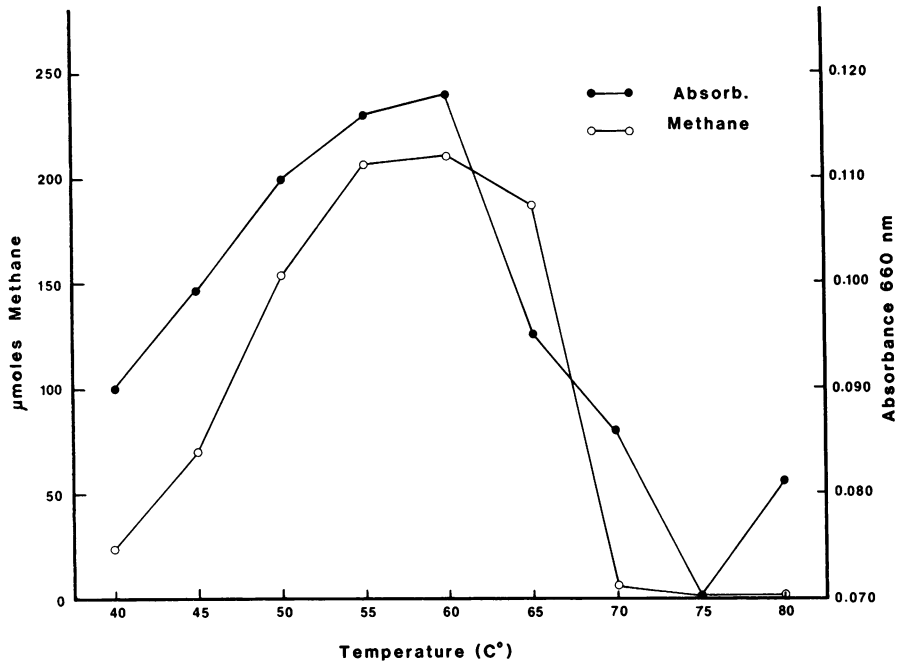

FIG. 3. Growth and methane production versus temperature. H_2-CO_2 complex RF medium was incubated without shaking for 24 h at the temperatures indicated. The increased absorbance at 80°C may be due to factors other than microbial activity, since no methane was produced and cultures were not contaminated.

cause the cells were extremely fragile and lysed when heat fixed. Lysis also occurred upon exposure to sodium dodecyl sulfate at a relatively low concentration (80 mg/liter).

Optimal growth conditions. Methane production closely approximated optical density (Fig. 3) and direct counts with a Petroff-Hausser counting chamber cell (see Fig. 7A). The thermophilic isolate had an optimum growth temperature near 60°C and a maximum near 70°C. The optimum pH for growth was about 7.2, although growth occurred throughout the pH range tested (Fig. 4). Although 3% salt (2% KCl and 1% NaCl) was included in most media, a high salt concentration was not required, since the isolate grew in RF media without added salt after a short adaptation period (Fig. 5).

Growth requirements. Although excellent growth on H_2-CO_2 was observed in complex medium containing RF, poor growth occurred in defined medium without RF. Nutrient requirements of the thermophile were studied by observing growth in defined medium supplemented with various nutrient additions. The results (Table 1) indicated that the addition of acetate to the defined medium supported growth equivalent to that on complex media. However, it was not possible to transfer such acetate cultures more than twice in succession on defined medium. Thus, acetate alone was not sufficient to support continued growth in defined medium. Since growth was observed in medium supplemented with yeast extract and Trypticase, growth kinetics were examined in defined media supplemented with Trypticase, yeast extract, RF, and vitamins (Fig. 6). Growth in yeast extract and Trypticase medium was usually exponential for 12 to 24 h, followed by linear growth until exhaustion of substrate. The addition of a vitamin solution had little or no effect. RF at a concentration of 5% was highly stimulatory. Trypticase alone was as stimulatory as Trypticase and yeast extract mixtures. The factor from Trypticase which supported growth in defined media was not identified, but it was not one of the amino acids or peptides found in vitamin-free Casamino acids, peptone (Difco), neopeptone, or proteose peptone, since none of these supported growth. The data in Table 2 indicate that acetate is stimulatory, but acetate alone is not sufficient to support continued growth. Combinations of these volatile fatty acids may satisfy the growth requirements, and experiments to test this are in progress. Although growth occurred in media supplemented with acetic acid, growth factors present in the inoculum fluid (approximately 0.10% yeast extract and Trypticase) were carried over into the new medium. The increased growth in defined medium with acetate compared to defined medium

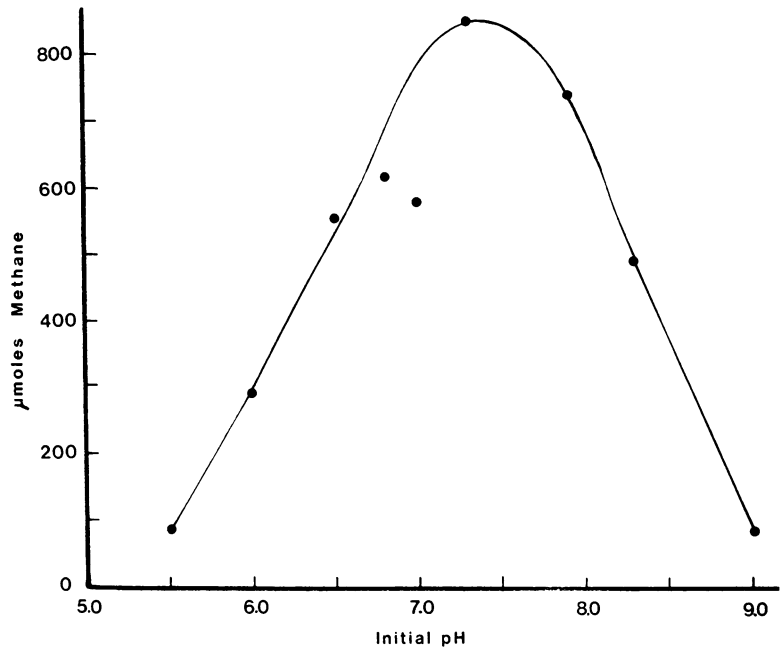

FIG. 4. pH versus methane production. H_2-CO_2 complex RF medium was incubated for 24 h with shaking at 55°C. The pH was adjusted with anaerobic solutions of Na_2CO_3 and $NaHCO_3$.

without acetate supported the hypothesis that acetate may satisfy part of the growth requirement. Yeast extract and Trypticase contained sufficient basal quantities of acetate to satisfy this growth requirement. Furthermore, the addition of acetate to medium containing 0.10% yeast extract and Trypticase did not stimulate methane production above the control yeast extract and Trypticase cultures (data not shown). Cell morphology differed with the medium composition of liquid cultures. In RF medium, the individual coccobacilli were of regular size and shape and often occurred in pairs; in Trypticase medium, the cells usually occurred singly, were irregular in shape, and lost fluores-

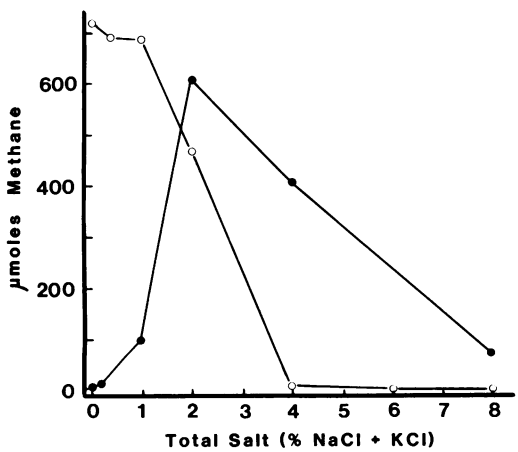

FIG. 5. Salt concentration versus methane production. H_2-CO_2 complex RF medium was supplemented with salt (3 parts KCl to 1 part NaCl) as indicated and incubated with shaking at 55°C. ○, Inoculum pregrown with three transfers in complex RF medium without salt; ●, inoculum pregrown in complex RF medium (3% salt).

TABLE 1. Nutrient requirements[a]

Added nutrient	Concentration	Methane (μmol)
Cattle RF	5%	1,027
Sheep RF	5%	1,024
KS	5%	946
Wooly colony supernatant[b]	5%	961
Sodium acetate	20 mM	911
Yeast extract and Trypticase	0.40% each	839
Coenzyme M[c]	1.6 mM	139
Vitamin solution[d]	1%	75
Propionic and butyric acids	3 mM each	64
None		90

[a] The basal medium for these experiments was defined medium (see the text) plus H_2-CO_2. Nutrients were added as indicated. Methane production was determined after 10 days of incubation at 55°C.

[b] Prepared from cultures of a mesophilic nonmethanogenic anaerobic rod pregrown in complex medium.

[c] 2-Mercaptoethanesulfonic acid (24).

[d] As reported by Wolin et al. (29).

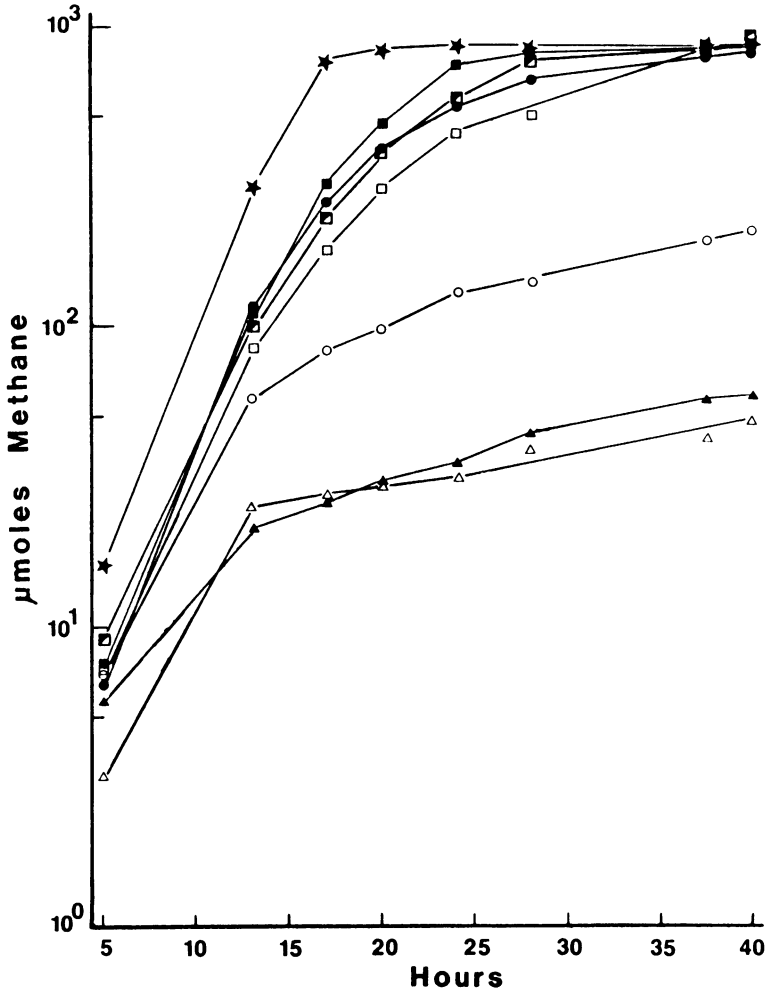

FIG. 6. Growth requirements: effect of nutrient additions on growth rate. H_2-CO_2 defined medium was incubated at 55°C with shaking. ★, 5% RF; ■, 0.20% yeast extract and Trypticase; ◩, 0.10% yeast extract and Trypticase; □, 0.05% yeast extract and Trypticase; ○, 0.10% yeast extract; ●, 0.10% Trypticase; ▲, 0.50% vitamin solution; △, no additions.

cence quickly. Excellent growth of the coccobacillus was obtained by transferring from Trypticase to RF medium.

Substrate utilization. Substrate utilization was studied in complex liquid medium supplemented with the organic nutrients listed in Table 3. The coccus produced methane from H_2-CO_2 or formate but none of the other substrates tested. Of the thermophilic methanogens in axenic culture (23, 25, 31, 33), only one coccoid isolate (C. J. Rivard and P. H. Smith, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, I99, p. 111) and perhaps two rods (14) were previously reported capable of using both H_2-CO_2 and formate for methanogenesis.

The stoichiometry of the methanogenic reactions was studied in liquid RF medium by the addition of known quantities of substrate and the recovery of the gaseous methane end product upon completion of growth. The results are summarized in Table 4. The experimental results closely fit those predicted from the standard methanogenic reactions, although slightly more (2 to 4%) substrate was consumed than was accounted for in CH_4. This small difference may be due to assimilation of substrate for cell carbon synthesis. The growth yield obtained for the thermophilic coccus (Table 4) was similar to that reported for other hydrogen-oxidizing methanogens (20, 25) and probably reflected similarities in metabolic pathways and substrate conversion efficiency.

Growth on either H_2-CO_2 or formate in KS or RF medium was exponential, with little or no lag

TABLE 2. Nutrient requirements: fatty acids[a]

Addition	Methane (μmol)	Turbidity
2% RF	849.0	+++++
Acetic acid	345.6	+++
Propionic acid	50.7	+
n-Butyric acid	13.8	−
iso-Butyric acid	0	−
n-Valeric acid	96.0	+
iso-Valeric acid	46.4	+
2-Methylbutyric acid	93.9	+
None	90.0	−

[a] Growth of the thermophilic coccobacillus on H_2-CO_2 in defined media. Fatty acids were added to final concentrations of approximately 0.8 mM, and growth determinations were made after 5 days of incubation at 55°C.

when exponentially growing inocula were used (Fig. 7). Generation times varied from 2.5 to 3.5 h, but values as low as 1.8 h were obtained on H_2-CO_2 in complex RF medium. A methane production rate of 39.3 nmol/min per ml was calculated for a 1-liter shake flask pressurized with H_2 (above the amount of 70% N_2–30% CO_2 initially present) and incubated at 55°C.

When $^{14}CO_2$ was added to a formate-utilizing culture under 70% N_2–30% CO_2, the final specific activity of $^{14}CO_2$ was the same as the specific activity in $^{14}CH_4$ at the exhaustion of the formate substrate. Thus, methanogenesis from formate by the thermophilic isolate most likely occurred via reduction of CO_2 with reducing equivalents derived from formate.

Effects of oxygen, antibiotics, and sulfide on growth and methanogenesis. The effect of oxygen on methanogenesis from H_2-CO_2 was studied by adding various amounts of O_2 to active cultures. Even at the lowest concentration tested, 4.1 μmol of O_2 per 120-ml vial, methane production and growth were completely inhibited. This inhibition was partially reversed by outgassing with N_2 and regassing with H_2-CO_2. When 4.1 or 20.5 μmol of O_2 was added to the 120-ml vials, the oxidation-reduction indicator (resazurin) remained reduced, and there was no indication that the medium was oxidized sufficiently to prevent growth of the methanogen. Even though the resazurin was not oxidized, the Eh of the medium could be high enough to prevent growth of obligate anaerobes.

Due to the unusual composition of the archaebacterial cell wall (1, 16), inhibitors of peptidoglycan synthesis are not effective antibiotics against methanogenic bacteria. We found that penicillin G and cycloserine did not inhibit growth or methanogenesis by the thermophilic isolate. However, growth and methanogenesis were completely inhibited by two inhibitors of protein synthesis, tetracycline and chloramphenicol. Gramicidin, a cyclic polypeptide, exhibited limited effectiveness at the concentration tested, but this may reflect limited uptake by growing cells, since this antibiotic was relatively insoluble in the culture media.

Bromoethanesulfonic acid, a structural analog of coenzyme M and a potent inhibitor of methanogenesis (3), completely inhibited methanogenesis and growth at a concentration of 0.50×10^{-6} M. This inhibition diminished with the addition of 4.0×10^{-6} M coenzyme M.

Since sulfide is usually added to reduce culture media for methanogenic bacteria (1) and since sulfide may stimulate growth of *M. thermoautotrophicum* (18), we examined the effect of sulfide concentration on the present isolate. Excellent growth was obtained in media without added sulfide (sulfide concentration = 0.007 mM carried over with inoculum); the addition of as much as 0.832 mM sulfide did not stimulate growth. High sulfide concentrations, at or above 1.25 mM sulfide in yeast extract and Trypticase medium at pH 6.9 to 7.1, increased the lag but did not significantly affect the rate of methane production.

DNA base composition. The bouyant density of purified DNA from the thermophilic isolate was 1.7185 g/cm^3, which corresponded to 59.69 mol% guanine plus cytosine.

DISCUSSION

Two thermophilic methanogenic bacteria were isolated from a 55°C anaerobic kelp digester. One of these isolates used acetate but not H_2-CO_2 for methanogenesis and closely resembled *Methanosarcina* sp. strain TM1 in morphology. The *Methanosarcina* sp. was not further charac-

TABLE 3. Substrate Utilization[a]

Substrate	Substrate (μmol)	Methane produced (μml)
H_2 alone	205	49.4
H_2 + sodium formate	500	165.2
H_2 + sodium formate	2,000	523.4
H_2 + sodium acetate	500	48.5
H_2 + propionic acid	500	47.0
H_2 + butyric acid	500	44.3
H_2 + methanol	500	41.1
H_2 + ethanol	500	41.1
H_2 + methylamine	500	45.5
H_2 + D-glucose	500	47.3

[a] Culture medium contained RF plus 5 ml of H_2 per vial (205 μmol). Methane production was determined by gas chromatography after 3 and 25 days of incubation at 55°C. All culture vessels contained 205 μmol of H_2 with substrate added in excess of this concentration at the values shown.

TABLE 4. Stoichiometry and growth yield

Methanogenic reaction	Substrate consumed/methane produced		Y_{CH_4}
	Predicted	Observed	
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	4.00	4.15	0.90 ± 0.21 mg/mmol
$4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$	4.00	4.09	ND[a]

[a] ND, Not determined.

terized. The second isolate was a coccobacillus which utilized H_2-CO_2 or formate but not acetate for methanogenesis. This isolate was morphologically similar to *Methanococcus vannielii* (22) but differed in other characteristics (see below). Although one other thermophilic methanogenic coccobacillus has been isolated from ocean sediments (C. J. Rivard, and P. H. Smith, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, I99, p. 111), this is the first report of such an organism from an anaerobic thermophilic digester.

A growth temperature optimum of 60°C and limited methane production below 50°C indicated that the coccobacillus is an obligate thermophile, although not as extreme a thermophile as *M. thermoautotrophicum*, whose temperature optimum ranges between 65 to 70°C (31, 33), nor *M. fervidus*, with an 83°C optimum (23). Its growth versus pH relationship resembled that of *M. thermoautotrophicum*, except that growth occurred throughout a wider range for the coccobacillus.

The limited substrate utilization of the present isolate was not surprising, since few known axenic species use substrates other than H_2-CO_2 or formate (13, 30, 34), and only one is thermophilic (34). The two recently reported strains of thermophilic methanogenic bacteria which utilized H_2-CO_2 and formate (14) resembled *M.*

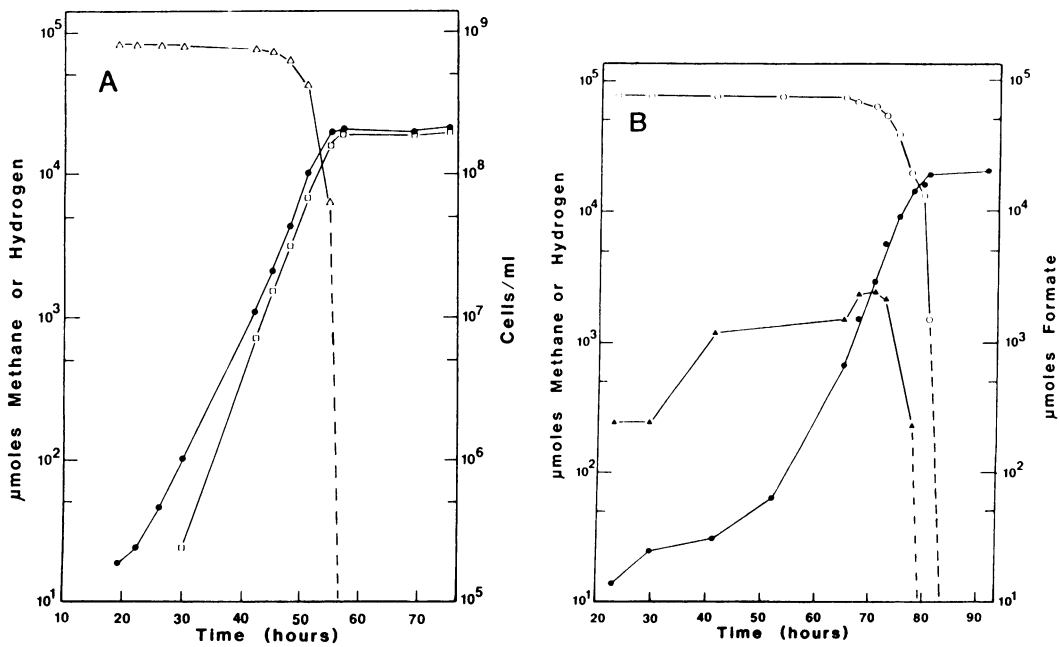

FIG. 7. (A) H_2-CO_2 growth curve for the thermophilic coccobacillus. Two-liter shake flasks contained 1 liter of complex KS medium pressurized (253 kPa) with H_2-CO_2 and incubated at 55°C. ●, Total methane produced; □, Petroff-Hausser cell counts; △, calculated H_2 consumption (from the stoichiometry presented in Table 4). Doubling time of the culture based on methane production (30 to 55 h) = 3.23 h; based on cell counts = 2.67 h. (B) Formate growth curve for the thermophilic coccobacillus. Culture vessels were 2-liter shake flasks with 1 liter of complex KS medium (80 mM sodium formate) incubated at 55°C. ●, Total methane produced; ▲, H_2 produced; ○, calculated formate consumption. Doubling time of the culture based on methane production (57 to 77.5 h) = 3.16 h.

thermoautotrophicum in morphology but required growth factor(s) from yeast extract to support growth on H_2-CO_2 in defined medium. *M. thermoautotrophicum* grew auxotrophically on H_2-CO_2 but not formate in defined medium (33).

Generation times for the thermophilic coccobacillus on H_2-CO_2 in complex media were 1.8 to 3.2 h. *M. thermoautotrophicum* YTB had a generation time of 2.5 h (31) in complex medium compared to 5.0 h in defined medium (25, 33). These generation times are three to six times faster than those reported for most mesophilic methanogens (9, 32), except *Methanococcus voltae*, which exhibited a generation time of 1.2 h (28).

Generation times on formate (Fig. 7B) were very similar to those on H_2-CO_2 (Fig. 7A). In fact, radioisotopic experiments demonstrated that formate was converted to methane via reduction of CO_2. Furthermore, since H_2 was produced during utilization of formate, H_2 appeared to be formed from formate during methanogenesis.

H_2 formation from formate was also reported in *M. vannielli* (22), *Methanobacterium mobilis* (15), and *Methanobacterium formicicum* (20). However, although H_2 may be produced from formate, it is probably not an obligate intermediate, since the K_m for H_2 oxidation was much higher than the dissolved H_2 concentration of the formate culture medium (20). Thus, the mechanism of methanogenesis from formate is still unknown.

The thermophilic isolate was sensitive to the same antibiotics as other methanogenic isolates (11, 16). Chloramphenicol was completely inhibitory at the concentration tested, but inhibition by tetracycline diminished after approximately 8 h of incubation at 55°C. Penicillin G and cycloserine, inhibitors of peptidoglycan synthesis, were not effective at the concentrations tested. Similar results were reported for other methanogenic bacteria (11, 16) and may be attributed to the lack of peptidoglycan in the cell wall (1).

Growth of the thermophilic methanogen was inhibited by exposure to 0.10% O_2 in the gas phase compared to 0.03% O_2 for *Methanobacterium ruminantium* (21) and 0.10% O_2 for *M. mobilis* (15). The lower limit of O_2 sensitivity has not yet been determined for the thermophilic coccobacillus. However, sulfide could be omitted from the culture medium without adverse effect, unlike *M. thermoautotrophicum*, which requires sulfide for growth (18). These findings suggest that the present isolate required less sulfide than other methanogens and that cysteine may be a sufficient sulfur source for growth. In addition, the thermophile tolerated sulfide well and was not inhibited by sulfide concentrations approaching 1 mM.

Based on similarities in growth requirements, substrate utilization and other metabolic properties, DNA base ratio, and morphology, the current thermophilic methanogen probably belongs to the family *Methanomicrobiaceae* (1). This is supported by the results of immunological fingerprinting performed by E. Conway de Macario (personal communication), which showed no immunological relationship with methanogens outside this family for either the S or R probes (5). In fact, the strongest reaction was a moderate reaction with antiserum prepared from *Methanomicrobium mobile*.

The characteristics of the thermophilic isolate are summarized below.

An anaerobic 55°C kelp digester served as the inoculum source.

Very short rods to cocci, 0.7 to 1.8 μm in diameter, often occurring in pairs but not chains, were seen. Gram staining was inconclusive due to the fragile nature of cell wall.

Small (1 to 3 mm) colonies grew; they were white to tan in color, circular, convex with entire margins, and smooth. Cultures formed colonies within 18 to 30 h of incubation which were highly fluorescent (420 to 430 nm).

The isolate is thermophilic, with an optimum near 60°C, a maximum slightly above 65°C, and a minimum below 35°C.

The optimum pH for growth is near 7.2, with growth throughout a pH range of 5.5 to 9.0.

The DNA base composition was 59.69 mol% guanine plus cytosine.

There was no immunological reaction with the S probe (5); the R probe (5) showed weak reactions with members of *Methanomicrobiaceae*, especially *M. mobilis*.

The organism is a very strict anaerobe. Exposure to 0.10% O_2 stopped growth and methanogenesis immediately. It grew well in complex medium on H_2-CO_2 and formate but not acetate, methanol, methylamine, or higher fatty acids. Salt was not required for optimal growth in complex media, but 4% salt does not inhibit growth. It was sensitive to sodium dodecyl sulfate, chloramphenicol, and tetracycline but not to penicillin G or cycloserine.

Optimal growth was obtained in media supplemented with RF, digester effluent, or yeast extract and Trypticase. The organism requires an unidentified growth factor(s), which is probably not an amino acid, single fatty acid, common vitamin, or coenzyme M.

ACKNOWLEDGMENTS

We thank Ida Yu for excellent advice and assistance in the isolation and purification of bacterial DNA. We thank Paul Smith, Michael Henson, and Christopher Rivard for determining the bouyant density of the purified DNA. Ralph Robinson

prepared electron micrographs. Technical assistance was generously provided by Harvey Negoro and Mario Panaqua.

This research was supported in part by grant C820101 from the Gas Research Institute and a grant from the U.S. Department of Energy (DE-AT03-80ER10684).

LITERATURE CITED

1. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260–296.
2. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM) dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. **32**:781–791.
3. **Balch, W. E., and R. S. Wolfe.** 1979. Specificity and biological distribution of coenzyme M (2-mercaptoethanesulfonic acid). J. Bacteriol. **137**:256–263.
4. **Baresi, L., R. A. Mah, D. M. Ward, and I. R. Kaplan.** 1978. Methanogenesis from acetate: enrichment studies. Appl. Environ. Microbiol. **36**:186–197.
5. **Conway de Macario, E., M. J. Wolin, and A. J. L. Macario.** 1982. Antibody analysis of relationships among methanogenic bacteria. J. Bacteriol. **149**:316–319.
6. **Coolhaas, C.** 1928. Zur Kenntnis der Dissimilation fettsaurer Salze und Kohlenhydrate durch thermophile Bakterien. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2 **75**:161–170.
7. **Doddema, H. J., and G. D. Vogels.** 1978. Improved identification of methanogenic bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **36**:752–754.
8. **Edwards, T., and B. C. McBride.** 1975. New method for the isolation and identification of methanogenic bacteria. Appl. Microbiol. **29**:540–545.
9. **Ferry, J. G., P. H. Smith, and R. S. Wolfe.** 1974. *Methanospirillum*, a new genus of methanogenic bacteria, and characterization of *Methanospirillum hungatii*, sp. nov. Int. J. Syst. Bacteriol. **24**:465–469.
10. **Hungate, R. E.** 1969. A roll tube method for cultivation of strict anaerobes, p. 117–132. *In* R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 3B. Academic Press, Inc., New York.
11. **Jones, J. B., B. Bowers, and T. C. Stadtman.** 1977. *Methanococcus vannielii*: ultrastructure and sensitivity to detergents and antibiotics. J. Bacteriol. **130**:1357–1363.
12. **Mackie, R. I., and M. P. Bryant.** 1981. Metabolic activity of fatty acid-oxidizing bacteria and the contribution of acetate, propionate, butyrate and CO_2 to methanogenesis in cattle waste at 40 and 60°C. Appl. Environ. Microbiol. **41**:1361–1373.
13. **Mah, R. A.** 1980. Isolation and characterization of *Methanococcus mazei*. Curr. Microbiol. **3**:321–326.
14. **Marty, D. G., and A. J. M. Bianchi.** 1981. Isolement de deux souches methanogenes thermophiles appartenant au genre Methanobacterium. C. R. Seances Acad. Sci. Ser. III **292**:41–43.
15. **Paynter, M. J. B., and R. E. Hungate.** 1968. Characterization of *Methanobacterium mobilis* sp. n., isolated from the bovine rumen. J. Bacteriol. **95**:1943–1951.
16. **Pecher, T., and A. Bock.** 1981. In vivo susceptibility of halophilic and methanogenic organisms to protein synthesis inhibitors. FEMS Microbiol. Lett. **10**:295–297.
17. **Price, C. W., G. B. Fuson, and H. J. Phaff.** 1978. Genome comparison in yeast systematics: delimitation of species within the genera *Schwanniomyces*, *Saccharomyces*, *Debaryomyces*, and *Pichia*. Microbiol. Rev **42**:161–193.
18. **Ronnow, P. H., and L. A. H. Gunnarsson.** 1981. Sulfide-dependent methane production and growth of a thermophilic methanogenic bacterium. Appl. Environ. Microbiol. **42**:580–584.
19. **Sandbeck, K. A., and D. M. Ward.** 1981. Fate of immediate methane precursors in low-sulfate, hot-spring algal-bacterial mats. Appl. Environ. Microbiol. **41**:775–782.
20. **Schauer, N. L., and J. G. Ferry.** 1980. Metabolism of formate in *Methanobacterium formicicum*. J. Bacteriol. **142**:800–807.
21. **Smith, P. H., and R. E. Hungate.** 1958. Isolation and characterization of *Methanobacterium ruminantium* n. sp. J. Bacteriol. **75**:713–718.
22. **Stadtman, T. C., and H. A. Barker.** 1951. Studies on the methane fermentation. A new formate-decomposing bacterium, *Methanococcus vannielii*. J. Bacteriol. **62**:269–280.
23. **Stetter, K. O., M. Thomm, J. Winter, G. Wildgruber, H. Huber, W. Zillig, D. Janecovic, H. König, P. Palm, and S. Wunderl.** 1981. *Methanothermus fervidus*, sp. nov., a novel extremely thermophilic methanogen isolated from an Icelandic hot spring. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **C2**:166–178.
24. **Taylor, C. D., B. C. McBride, R. S. Wolfe, and M. P. Bryant.** 1974. Coenzyme M, essential for growth of a rumen strain of *Methanobacterium ruminantium*. J. Bacteriol. **120**:974–975.
25. **Taylor, G. T., and S. J. Pirt.** 1977. Nutrition and factors limiting the growth of a methanogenic bacterium (*Methanobacterium thermoautotrophicum*). Arch. Microbiol. **113**:17–22.
26. **Varel, V. H., A. G. Hashimoto, and Y. R. Chen.** 1980. Effect of temperature and retention time on methane production from beef cattle waste. Appl. Environ. Microbiol. **40**:217–222.
27. **Varel, V. H., H. R. Isaacson, and M. P. Bryant.** 1977. Thermophilic methane production from cattle waste. Appl. Environ. Microbiol. **33**:298–307.
28. **Whitman, W. B., E. Ankwanda, and R. S. Wolfe.** 1982. Nutrition and carbon metabolism of *Methanococcus voltae*. J. Bacteriol. **149**:852–863.
29. **Wolin, E. A., M. J. Wolin, and R. S. Wolfe.** 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238**:2882–2886.
30. **Zehnder, A. J. B., B. A. Huser, T. D. Brock, and K. Wuhrmann.** 1980. Characterization of an acetate-decarboxylating, non-hydrogen-oxidizing methane bacterium. Arch. Microbiol. **124**:1–11.
31. **Zeikus, J. G., A. Ben-Bassat, and P. W. Hegge.** 1980. Microbiology of methanogenesis in thermal, volcanic environments. J. Bacteriol. **143**:432–440.
32. **Zeikus, J. G., and D. L. Henning.** 1975. *Methanobacterium arbophilicum* sp. nov.: an obligate anaerobe isolated from wetwood of living trees. Antonie van Leeuwenhoek J. Microbiol. Serol. **41**:543–552.
33. **Zeikus, J. G., and R. S. Wolfe.** 1972. *Methanobacterium thermoautotrophicum* sp. nov.: an anaerboic autotrophic, extreme thermophile. J. Bacteriol. **109**:707–713.
34. **Zinder, S. H., and R. A. Mah.** 1979. Isolation and characterization of a thermophilic strain of *Methanosarcina* unable to use H_2-CO_2 for methanogenesis. Appl. Environ. Microbiol. **39**:996–1008.