Methanogenesis in a Thermophilic (58°C) Anaerobic Digestor: *Methanothrix* sp. as an Important Aceticlastic Methanogen

S. H. Zinder, S.C. Cardwell, T. Anguish, M. Lee and M. Koch
***Appl. Environ. Microbiol.* 1984, 47(4):796.**

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1984, p. 796–807
0099-2240/84/040796-12$02.00/0

Vol. 47, No. 4

Methanogenesis in a Thermophilic (58°C) Anaerobic Digestor: *Methanothrix* sp. as an Important Aceticlastic Methanogen

S. H. ZINDER,* S.C. CARDWELL, T. ANGUISH, M. LEE, AND M. KOCH†

Department of Microbiology, Stocking Hall, Cornell University, Ithaca, New York 14853

Received 30 September 1983/Accepted 30 January 1984

Aceticlastic methanogens and other microbial groups were enumerated in a 58°C laboratory-scale (3 liter) anaerobic digestor which was fed air-classified municipal refuse, a lignocellulosic waste (loading rate = 1.8 to 2.7 g of volatile solids per liter per day; retention time = 10 days). Two weeks after start-up, *Methanosarcina* sp. was present in high numbers (10^5 to 10^6 CFU/ml) and autofluorescent *Methanosarcina*-like clumps were abundant in sludge examined by using epifluorescence microscopy. After about 4 months of digestor operation, numbers of *Methanosarcina* sp. dropped 2 to 3 orders of magnitude and large numbers (most probable number = 10^6 to 10^7/ml) of a thermophilic aceticlastic methanogen morphologically resembling *Methanothrix* sp. were found. *Methanothrix* sp. had apparently displaced *Methanosarcina* sp. as the dominant aceticlastic methanogen in the digestor. During the period when *Methanothrix* sp. was apparently dominant, acetate concentrations varied between 0.3 and 1.5 μmol/ml during the daily feeding cycle, and acetate was the precursor of 63 to 66% of the methane produced during peak digestor methanogenesis. The apparent K_m value obtained for methanogenesis from acetate, 0.3 μmol/ml, indicated that the aceticlastic methanogens were nearly saturated for substrate during most of the digestor cycle. CO_2-reducing methanogens were capable of methanogenesis at rates more than 12 times greater than those usually found in the digestor. Added propionate (4.5 μmol/ml) was metabolized slowly by the digestor populations and slightly inhibited methanogenesis. Added *n*-butyrate, isobutyrate, or *n*-valerate (4.5 μmol/ml each) were broken down within 24 h. Isobutyrate was oxidized to acetate, a novel reaction possibly involving isomerization to *n*-butyrate. The rapid growth rate and versatile metabolism of *Methanosarcina* sp. make it a likely organism to be involved in start-up, whereas the low K_m value of *Methanothrix* sp. for acetate may cause it to be favored in stable digestors operated with long retention times.

The anaerobic digestor is a microbial ecosystem in which several groups of microorganisms must interact effectively if organic matter is to be efficiently converted to methane. Bryant (7) and McInerney and Bryant (26) have divided digestor microorganisms into three groups. The first group is the fermentative bacteria which hydrolyze polymers and ferment monomers and oligomers to acetate. H_2, CO_2, and varying amounts of short-chain fatty acids, alcohols, and other fermentation products such as lactate. Since the fermentative bacteria are usually coupled with hydrogen-consuming methanogens (26, 42), acetate is usually the primary product of carbohydrate fermentation. Amino acids are usually fermented to short-chain fatty acids. The second microbial group is the hydrogen-producing (proton-reducing) acetogenic bacteria. These convert acids and alcohols to acetic acid, H_2, and CO_2. Oxidation of fatty acids is only thermodynamically feasible if the H_2 partial pressure is kept extremely low by methanogens or other hydrogen-oxidizing bacteria (4, 7, 26, 38).

The end products of the first two microbial groups are acetate, H_2, and CO_2. These are the substrates for the third microbial group, the methanogens. The methanogens can be divided into two subgroubs, the CO_2-reducing (hydrogen-oxidizing) methanogens and the aceticlastic (acetate-splitting) methanogens (35). Several genera of CO_2-reducing methanogens are known (2), and two genera of aceticlastic methanogens have been described, *Methanosarcina* sp. (22, 34, 49) and *Methanothrix* sp. (14, 44). It is generally accepted that two-thirds or more of the methane produced in a digestor is derived from acetate (3, 20, 27, 37), and the remainder is produced by reduction of CO_2. Methane can also be produced from formate, methanol, and methylamines (2).

If the interaction among these microbial groups becomes unbalanced, digestor failure can occur. Besides carrying out the terminal reactions in anaerobic digestors, both types of methanogens play an important role in maintaining digestor stability. If acetic acid is produced more rapidly than it is consumed by aceticlastic methanogens, the digestor pH will eventually decrease to the point where failure occurs. If CO_2-reducing methanogens do not maintain H_2 partial pressures at a sufficiently low level, other fatty acids such as propionate and butyrate can accumulate. Aspects of the dynamics of carbon flow to methane have been studied in mesophilic (1, 3, 13, 18, 27, 37) and thermophilic (8, 20) digestors.

We have been studying the microbial ecology of methanogenesis in a laboratory-scale thermophilic (58°C) digestor converting a lignocellulosic waste (air-classified municipal refuse) to methane. We used cultural techniques, microscopy, and radiotracer techniques to determine the numbers and activities of microbial populations present in the digestor. The aceticlastic methanogen populations in the digestor were of particular interest. Initially, the digestor contained large numbers of *Methanosarcina* sp., as has been seen in other thermophilic digestors (6, 35, 49; S. Zinder, unpublished results), and it was concluded that *Methanosarcina* sp. was responsible for methanogenesis from acetate in the digestor. However, after ca. 4 months of digestor operation, the numbers of *Methanosarcina* sp. decreased markedly,

* Corresponding author.
† Present address: Abt. Gewasserschutz Neugasse, 8005 Zurich, Switzerland.

and a thermophilic *Methanothrix* sp. was found to be present in the digestor fluid. A thermophilic *Methanothrix* strain had not been described previously, but recently Nozhevnikova and Yagodina (30) obtained one in enrichment culture one from a thermophilic digestor in Russia. We report here on the microbiology and dynamics of methanogenesis in a thermophilic digestor containing *Methanothrix* sp.

MATERIALS AND METHODS

Digestor. A New Brunswick Microferm fermentor (New Brunswick Scientific Co., Edison, N.J.) was operated as a digestor in a manner similar to that described by Mackie and Bryant (20). The vessel volume was 5 liters, and the liquid volume was 3 liters. The operating temperature was 58°C. The digestor was stirred continuously at 250 rpm. Gas production was measured as displacement of acidic brine containing 200 g of NaCl and 5 g of citric acid per liter. Butyl rubber tubing was used for all connections and for sealing off outlets. The inoculation port was sealed with a rubber stopper which could be removed to allow addition and removal of sludge. The digestor was always under slight positive pressure from the hydraulic head of the acidic brine so that oxygen was prevented from entering during feeding and sampling.

Digestor feed. The feed used was the organic fraction of air-classified municipal refuse generated in Richmond, Calif., and was kindly supplied by R. A. Mah, University of California at Los Angeles. The material had been hammermilled to the point where it was of a fibrous nature with particle sizes of 0.2 to 2 cm. As received, the material contained ca. 35% water and was stored frozen. Batches of 1 to 2 kg were dried to 4% moisture in a 50°C oven and ground to a powder (particle size, 0.5 mm) with a Wiley mill. This made the material much more uniform and facilitated handling of the sludge. The material was analyzed by the methods of Goering and van Soest (12), courtesy of P. van Soest, Cornell University. The dry matter in the feed was analyzed as containing 57.2% volatile solids. Of the volatile solids, 45% were assayed as cellulose, 13% as hemicellulose, and 18% as lignin. Thus, 76% of the volatile solids were assayed as lignocellulosic fiber. The nonvolatile solids were assayed as containing 81% silicon dioxide. The nitrogen and phosphorous content of the material was not determined. These nutrients and others were not added since stable digestion was obtained without them.

Brief history of digestor operation. The inoculum for the digestor was started from a 55°C cattle manure digestor operated by the Department of Agricultural Engineering, Cornell University. The inoculum was built up from 50 ml to 500 ml to 3 liters. Once the digestor was started up, it was fed daily in the following manner. A slurry of the dried powdered feed material (10 or 15 g) in 300 ml of tap water was prepared daily. At feeding time, the impeller speed was briefly increased to 800 rpm, and 300 ml of sludge was removed from the digestor through rubber tubing (inner diameter, 6 mm) into a filter flask. A hand-operated vacuum pump (Nalgene, supplied by Fisher Scientific Co., Rochester, N.Y.) was used to pump the sludge up into the flask. Care was taken not to remove the liquid more rapidly than gas was escaping from the inoculation port to prevent the entry of oxygen into the digestor. The 300 ml of sludge removed was replaced with the 300 ml of fresh feed, giving a retention time of 10 days, which was used throughout these studies.

At the time of start-up (1 November 1981), the digestor was operated at a loading rate of 1.8 g of volatile solids per liter per day. Within 2 weeks, digestor gas production stablilized at 0.5 to 0.7 liters per liter of digestor volume per day, and the gas contained 55 to 59% CH_4. On 8 December 1981, the digestor temperature was accidentally shifted from 58°C to 64°C and was shifted back to 58°C the next day. This caused a drop in gas production and a large increase in acetic acid concentration. The digestor restabilized within 10 days as described in another publication (48).

During January 1982, digestor operation was stable with the following parameters: gas production, 0.5 to 0.7 liters per liter per day; CH_4, 55 to 59%; acetate (sample taken just before feeding), 1 to 2 μmol/ml; propionate, 2 to 3.5 μmol per ml; butyrate, 0.02 μmol/ml; isobutyrate, 0.6 μmol/ml; valerate and isovalerate, trace. During March 1982, the concentrations of volatile fatty acids decreased in the digestor until they reached the following levels: acetate, 0.2 to 0.5 μmol/ml; propionate, 0.1 to 0.2 μmol/ml; other acids, less than 0.01 μmol/ml. On 19 May 1982, the feed rate was increased 50% to 2.7 g of volatile solids per day. Within 2 days, methanogenesis increased accordingly.

The experiments presented in this publication were performed during the period of 15 June 1982 to 15 September 1982. The digestor was stable during this period, and the following parameters were obtained: gas production, 0.85 to 1.05 liters per liter per day; CH_4, 55 to 60%; conversion to CH_4, 0.20 liters of CH_4 per g of volatile solids added; pH, 6.1 to 6.3; temperature, 58°C; retention time, 10 days; impeller speed, 250 rpm.

Gas analysis. Methane and carbon dioxide were determined by using a Gow-Mac 550 thermal conductivity gas chromatograph (Gow Mac Instrument Co., Bound Brook, N.J.), operated under the following conditions: carrier gas, He; flow rate, 50 ml/min; column, copper (1 m by 2 mm [inner diameter]) packed with 120/140 Carbosieve S (Supelco, Inc., Bellefonte, Pa.); column temperature, 100°C. Hydrogen was quantified by using a Carle 8500 thermal conductivity gas chromatograph (Carle Instruments, Anaheim, Calif.) fitted with a similar column. The carrier gas was nitrogen, and the column was operated at 40°C.

Short-chain fatty acid analysis. Short-chain fatty acids were quantified by using a Varian 2400 gas chromatograph equipped with a flame ionization detector and on-column injection. It was operated under the following conditions: carrier gas, N_2; flow, 30 ml/min; oven temperature, 130°C; injector and detector temperatures, 180°C. The column usually used was Teflon (0.3 m by 2 mm [inner diameter]) packed with 80/100 mesh Porapak Q (Supelco). This column provided excellent separation between the injection peak and the acetate peak. Acetate concentrations as low as 0.1 μmol/ml could be quantified in aqueous samples. A portion of the column in the injector block (ca. 8 cm) was left without packing so that salts and complex organic molecules in samples would be deposited on the sides of the column. Periodically, the column was cleaned out by inserting a moistened delinted pipe cleaner through the injection port into the unpacked portion of the column. If this procedure was not followed, complex organic matter from samples would build up to a point where considerable problems with base-line drift were encountered. The Porapak Q column did not provide adequate resolution between butyric and isobutyric acids for quantitative studies, and a Teflon column (0.5 m by 2 mm) packed with Chromosorb 101 (Supelco) was used to quantify fatty acids longer than propionate. Samples were acidified by the addition of phosphoric acid to a final concentration of 0.15 M and were centrifuged, and 1-μl samples were injected. The columns were preconditioned

before use by multiple injections of 5 M formic acid and 0.15 M phosphoric acid. When these procedures were followed, there was no ghosting, and reproducibility and peak symmetry were excellent.

Radioisotope analysis. ^{14}C-label in aqueous samples was counted in ACS scintillant (Amersham Corp., Arlington Heights, Ill.) using a Beckman LS-230 liquid scintillation counter (Beckman Instruments, Fullerton, Calif.). Quench correction was by the external standard channel ratio method. ^{14}C-label in methane and carbon dioxide in the effluent of the gas chromatograph was quantified with a Packard gas proportional counter (Packard Instrument Co., Inc., Downers Grove, Ill.) according to the method of Nelson and Zeikus (28). Corrections were made for syringe dead volume. Methane partial pressures were less than 0.5 atm (50.65 kPa), so that quenching was not a problem (43). Total $^{14}CO_2$ in samples was estimated by determining the amount of CO_2 released from representative liquid samples after acidification and by employing the appropriate Bunsen absorption coefficient (39).

Serum vial experiments. Most of the experiments described were performed by using 10-ml samples of digestor sludge incubated in 37-ml serum vials. The gas phase was 70% N_2–30% CO_2 (Matheson Scientific, Inc., Joliet, Ill.), which was scrubbed of residual O_2 by passage over hot copper coils. All syringes and vials were flushed with this gas mixture. Sludge was removed from the digestor for these experiments just before feeding by using a 60-ml plastic syringe in which the Luer-lok hub was cored out to prevent clogging. A 30-cm piece of rubber tubing (inner diameter, 6 mm) was attached to the hub of the syringe. Sludge was deposited in 120-ml serum bottles. Sludge samples of 9 to 10 ml were then dispensed into the 37-ml serum vials by using a 10-ml plastic syringe with a cored Luer-lok hub. The vials were sealed with butyl rubber stoppers (Bellco Glass, Inc., Vineland, N.J.) and aluminum crimps as described by Balch et al. (2). Subsequent additions were made to the vials by syringe through the stoppers. The vials were then usually placed in a 58°C shaking water bath (Fisher Scientific) with the shaking speed set at 150 strokes per minute. To minimize the cooling and perturbation of the microorganisms in the sludge, these procedures were carried out as rapidly as possible, usually within 10 to 20 min.

Sludge in vial experiments was either "fed" or "unfed." Fed vials were those in which the diurnal cycle of the just-fed digestor was simulated by adding 1 ml of feed suspension (1.5 g of feed per 30 ml) along with 9 ml of digestor sludge. Unfed vials received 10 ml of digestor sludge and were used in experiments in which low acetate concentration or low rates of background metabolism were desired. Results presented represent the average of duplicate vials unless otherwise stated, and each experiment reported was performed at least two times with similar results.

During the course of the incubation, gas samples were taken by using 1-ml Glaspak syringes (Fisher Scientific) fitted with Mininert syringe valves (Supelco) to maintain gas pressure. Vials incubated in a static water were shaken vigorously before sampling to ensure adequate methane transfer from the liquid to the gas phase. As many as 10 syringes with Mininert valves were assembled and numbered so that samples could be taken from several vials simultaneously and subsequently analyzed. Teflon tape was wrapped around the Luer tip of each syringe to assure a tight fit between the syringe and the valve, and the syringe plungers were lubricated with silicone grease to assure a good seal.

Liquid samples were taken by inverting the vials briefly and removing a 100-μl sample with a 1-ml syringe fitted with a 20-gauge 25-mm needle. Inverting the vials allowed larger particles to settle so that the syringe needle, which was in the upper part of the liquid, would not clog. The 100-μl sample was injected into a polyethylene 250-μl microcentrifuge tube (Fisher Scientific) containing 5 μl of 3 M H_3PO_4 (final concentration, 0.15 M). The samples were then centrifuged by using a Fisher microcentrifuge and were stored at 4°C for gas chromatographic analysis.

Growth medium and conditions. The basal medium used for enumerating and culturing organisms from the digestor contained (grams per liter): NH_4Cl, 1.0; K_2HPO_4, 0.4; $MgCl_2 \cdot 6H_2O$, 0.1; yeast extract (Difco Laboratories, Detroit, Mich.), 0.2; resazurin, 0.001; trace metal solution, (as described in reference 45, except that 0.02 g of $NiCl_2 \cdot 6H_2O$ per liter was also added), 10 ml/liter. After the medium was boiled under N_2, neutralized cysteine-hydrochloride was added to a concentration of 0.5 g/liter, and the medium was boiled until the resazurin was reduced. After being dispensed in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.) and autoclaved, the following sterile anaerobic solutions were added to these final concentrations: $NaHCO_3$, 1 g/liter; $CaCl_2 \cdot 2H_2O$, 0.1 g/liter; $Na_2S \cdot 9H_2O$, 0.1 g/liter (0.2 g/liter for roll tubes); autoclaved digestor sludge supernatant, 5%. The headspaces were flushed with 70% N_2–30% CO_2. The medium was solidified when desired by the addition of 2.25% purified agar (Difco) before boiling. Most probable number (MPN) determinations were performed by using 10 ml of liquid medium in 27-ml tubes (Balch et al. [2]), whereas roll tubes for colony counts contained 5 ml of agar-solidified medium in 27-ml tubes. All incubations were at 56°C and were carried out for at least 90 days.

Various physiological types were enumerated by using three-tube MPN determinations (10-fold dilutions) by varying the energy source. Aceticlastic methanogens were enumerated by adding sodium acetate to a concentration of 40 μmol/ml. Butyrate and propionate oxidizers were enumerated in medium containing 20 μmol/ml of the appropriate substrates and 0.1 ml from a turbid culture of a *Methanobacterium* sp. isolated from the digestor. Tubes were considered positive if methanogenesis was significantly greater than that of controls without added substrate. Cellulolytic bacteria were enumerated by using medium to which 2.0 g of cellulose per liter (MN 300; Machery and Nagel, Duren, Federal Republic of Germany; crystallinity index = 58% [29]) was added. Tubes were considered positive if the cellulose was cleared. Total fermentative bacteria were estimated by adding to the medium 1 g of yeast extract per liter plus 0.25 g each of glucose, cellobiose, sucrose, and xylose per liter. MPN tables were consulted for MPN values obtained (9). *Methanosarcina* CFU were counted in roll tubes containing 25 μmol of methanol per ml, 40 μmol of acetate per ml, and 0.5 g of sodium ampicillin per liter (Sigma Chemical Co., St. Louis, Mo). *Methanosarcina* colonies were yellowish brown, 1 to 5 mm in diameter, and were easily recognized. CO_2-reducing methanogens were enumerated by using roll tubes with an 80% H_2–20% CO_2 (Matheson) atmosphere. The methanogens formed large yellow colonies, and bacteria from representative colonies autofluoresced when viewed by epifluorescence microscopy.

Microscopy. Phase-contrast and epifluorescence microscopy were performed by using a Zeiss model 18 standard microscope equipped with a mercury burner for epifluorescence and an automatic camera for photography. A 487704

filter combination and Planapochromat objectives were used for F_{420} autofluorescence. A 487702 filter combination and Neofluar objectives were used for F_{342} autofluorescence and for 4',6-diamidino-2-phenylindole (DAPI) fluorescence.

Sludge samples taken out for light microscopic observation were fixed by the immediate addition of formaldehyde to a final concentration of ca. 2%. The formaldehyde did not interfere with F_{420} autofluorescence. Samples were usually observed on the same day they were taken. Before observation, most samples were usually diluted 10-fold with 9 g of NaCl per liter and spread out on microscope slides coated with a thin layer of dried purified agar (Difco) to immobilize the cells. For DAPI staining, fixed cells were incubated at least 60 min at room temperature with 10 μM DAPI before observation, as described by Paul (31).

Samples taken for scanning electron microscopy were fixed with 2% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) and were prepared following the osmium-thiocarbohydrazide-osmium method outlined by Malick and Wilson (23), modified so that the procedure was carried out in centrifuge tubes. The samples were then filtered onto a 0.45-μm Nuclepore filter (Fisher Scientific) by using a slow vacuum and then dehydrated with a series of ethanol washes. The filters were then critical-point dried, glued onto stubs, and sputter-coated with gold-palladium (60:40). The samples were viewed with a Cambridge 254 scanning electron microscope equipped with a LaB_6 gun at an accelerating voltage of 20 kV.

Chemicals and radiochemicals. All chemicals used were at least reagent grade. [1-^{14}C]sodium acetate (61.6 mCi/mmol), [2-^{14}C]sodium acetate (58.9 mCi/mmol), and sodium bicarbonate (58 mCi/mmol) were obtained from Amersham, and stock solutions were anaerobic, filter sterilized, and stored at 4°C.

RESULTS

Microbial populations in the digestor. Soon after digestor start-up (1 November 1981), *Methanosarina* CFU were between 10^5 and 10^6/ml of sludge. Similar numbers were obtained through January 1982. Observations of sludge by using phase-contrast and epifluorescence microscopy revealed numerous *Methanosarcina*-like clumps (ca. one clump for every two fields [viewed at ×400] of undiluted sludge) which autofluoresced when illuminated with radiation near 420 or 350 nm, indicative of the presence of the methanogenic cofactors F_{420} and F_{342} (10). Figures 1a and 1b show a typical *Methanosarcina*-like clump in the digestor sludge. A *Methanosarcina* culture designated *Methanosarcina* sp. strain CALS-1 was isolated from the digestor by using previously described techniques (49). It was physiologically similar to *Methanosarcina* sp. strain TM-1 (49) except that it grew optimally at 55 to 58°C and could grow at 60°C (48).

Viable counts of *Methanosarcina* sp. declined to 2×10^3/ml during March 1982, and *Methanosarcina*-like clumps were difficult to find by microscopy (less than one clump in 25 fields viewed at ×400). On 28 March 1982, dilutions of sludge were made into liquid medium containing 40 μmol of sodium acetate per ml and were incubated for 30 days at 56°C. In 10^{-1} to 10^{-3} dilutions of sludge, *Methanosarcina* sp. was present, but in dilutions of 10^{-4} to 10^{-6}, an organism resembling *Methanothrix* sp. was found. In these tubes, the acetate was stoichiometrically converted to CH_4, and the culture grew when transferred into fresh acetate medium. The *Methanothrix*-like cells were clearly the predominant morphotype in the acetate enrichment cultures and MPN tubes. This morphotype only grew when acetate was present, and significant methanogenesis was only detected when the organism grew. The major product of $^{14}CH_3COO^-$ added to an enrichment culture was $^{14}CH_4$ ($^{14}CH_4/^{14}CO_2$ = 16), thus verifying that the aceticlastic reaction was occurring. Figure 1c is a phase-contrast micrograph of an early stationary-phase enrichment culture of the organism. The cells showed the typical morphology of the short cells of *Methanothrix* sp. (14, 44) rather than long filaments, and empty shealths were associated with some of the cells. Cells in stationary-growth phase usually contained phase-refractile granules which could be collapsed by hydrostatic pressure and thus presumably were gas vacuoles (41). Cells from growing cultures were two to five times longer than stationary-phase cells and contained fewer granules. The cells show the characteristic square ends of *Methanothrix* sp. (44). We have a stable enrichment culture of the organism growing at 60°C and are attempting isolation.

During the period of research presented in subsequent sections (15 June 1982 to 15 September 1982), the viable counts of *Methanothrix* sp. as determined by the MPN method were 8×10^6/ml (2 June 1982) and 2×10^6/ml (5 August 1982; Table 1). *Methanothrix*-like cells were numerous in sludge viewed by microscopy. Figure 1d and 1e show cells of the characteristic morphology of *Methanothrix* sp., including refractile granules. Figure 1f shows that the *Methanothrix*-like cells, similar to mesophilic strains (44), were not autofluorescent. The *Methanothrix*-like cells sometimes appeared as aggregates in sludge viewed by light or scanning electron microscopy, as shown in Fig. 1d and 2a. Note the different degrees of granule content of cells in Fig. 1d and the apparent associations with other cell types in Fig. 2a. Figure 2b shows a *Methanothrix*-like cell associated with a particle.

CO_2-reducing methanogens were found to be present in high numbers (Table 1), as expected. The only morphotype found in colonies in the roll tubes was an organism which resembled *Methanobacterium thermoautotrophicum* (46), except for being somewhat straighter. Autofluorescent *Methanobacterium*-like rods were numerous in the digestor sludge (Fig. 1f) and were the only autofluorescent morphotype except for *Methanosarcina* sp. Coccoid organisms similar to the thermophilic organism described by Ferguson and Mah (11) were not detected in roll tubes or in sludge viewed by epifluorescence microscopy. A culture isolated from the digestor, designated *Methanobacterium* sp. strain CALS-1, showed the physiological characteristics of *Methanobacterium thermoautotrophicum* (46), including growth only on H_2-CO_2, growth up to 75°C, and lack of growth factor requirements.

Other organisms found to be present in the digestor included propionate and butyrate oxidizers (Table 1). The counts of the propionate oxidizers were lower than those for butyrate oxidizers, and the cultures grew more slowly than the butyrate oxidizers. The estimated doubling time for a propionate enrichment culture derived from the digestor was over 4 days (data not shown). The predominant organisms in both butyrate and propionate enrichments were rods. It would appear from the enumerations that cellulolytic organisms represented only a small fraction of the total fermentative organisms present. However, we found that fibrous particles in the digestor sludge were usually heavily colonized by rod-shaped organisms (Fig. 2c and 2d). An organism isolated from a 10^{-7} dilution of the sludge was a spore-forming rod resembling *Clostridium thermocellum* (29).

Digestor cycle after feeding. Figure 3A depicts a typical

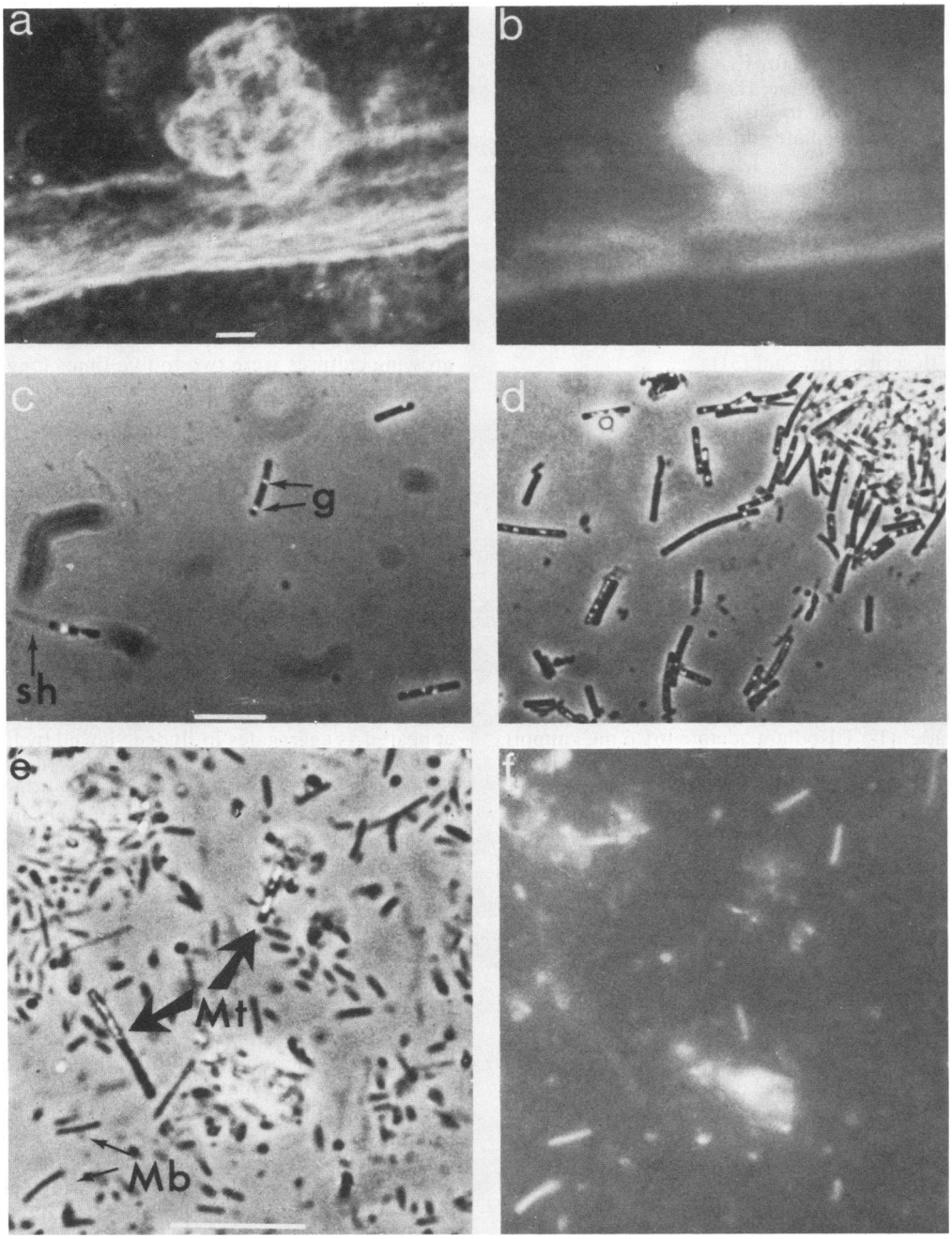

FIG. 1. Photomicrographs of microorganisms found in a 58°C anaerobic digestor. (a) Phase-contrast micrograph of a *Methanosarcina*-like clump associated with a fiber particle in undiluted digestor sludge. Marker bar = 10 μm. (b) Epifluorescence micrograph of the same field as (a) illuminated to stimulate F_{420} autofluorescence. (c) Cells from an early stationary-phase acetate enrichment culture of a thermophilic *Methanothrix* sp. g, Granules; sh, sheath; marker bar = 10 μm. (d) Phase-contrast micrograph of an aggregate of *Methanothrix*-like cells found in a diluted sample of digestor sludge. Marker bar = 10 μm. (e) Phase-contrast micrograph of microorganisms present in digestor sludge in July 1982; Mt, *Methanothrix*-like cells; Mb, autofluorescent *Methanobacterium*-like cells; marker bar = 10 μm. (f) Epifluorescence micrograph of the same field as (e) illuminated to stimulate F_{420} autofluorescence.

daily cycle in the digestor after feeding. The acetate concentration immediately after feeding was 0.3 μmol/ml. Within 4 h the acetate concentration increased to 1.5 μmol/ml and then gradually returned to the initial value. Propionate showed a similar but attenuated cycle. Hydrogen was never detected in the digestor headspace (detection limit = 50 Pa). Methanogenesis was linear for the first 12 h, after which the rate gradually decreased.

Our ability to simulate events in the digestor by incubating 10-ml sludge samples in serum vials is shown in Fig. 3B, which presents results obtained with duplicate vials shaken at 150 rpm in a 58°C water bath, and Fig. 3C, which shows

TABLE 1. Estimation of microbial populations found in a thermophilic anaerobic digestor[a]

Microbial population	No.	Method used
Aceticlastic methanogens	2.3×10^6	MPN
Methanosarcina sp.	8.0×10^2	Colony counts
CO_2-reducing methanogens	3.3×10^8	Colony counts
Butyrate oxidizers	5.0×10^6	MPN
Propionate oxidizers	9.0×10^4	MPN
Cellulolytic bacteria	2.3×10^7	MPN
Fermentative bacteria	2.1×10^9	MPN

[a] The counts were performed on 5 August 1982. The numbers were either obtained from three-tube MPN determinations or from colony counts in duplicate roll tubes as described more completely in the text. All of these counts were performed at other times during the research period, and all other counts agreed with these counts within a factor of 10.

results for vials which were not shaken. The results obtained were qualitatively similar to those obtained from the digestor for methanogenesis and acetate concentration. Acetate tended to accumulate to a higher concentration (2.0 versus 1.5 μmol/ml) but still returned to its original concentration within 24 h. Somewhat more methane accumulated in the vials. It is not clear whether this represents a true increase in methanogenesis or a systematic error in sample preparation or analysis. Propionate concentrations increased during the entire incubation period in vials, indicating that propionate utilizing populations were perturbed.

Methanogenesis from acetate and carbon dioxide: turnover and saturation. The fates of methyl- and carboxyl-labeled [^{14}C]acetate in digestor sludge during peak methanogenesis (4 h after feeding) were examined to determine whether the aceticlastic reaction was occurring (Table 2). The product distributions found and similar rates of catabolism of methyl- and carboxyl-labeled acetate are clear evidence that the aceticlastic reaction was the primary mechanism of methanogenesis from acetate in the digestor. About 2% of the label catabolized to gasses was incorporated into particulate material for either position of acetate. No attempt was made to determine whether [^{14}C]acetate was converted into other soluble products.

The relative turnovers of $^{14}CH_3COO^-$ and $^{14}CO_2$ to $^{14}CH_4$ were examined in digestor sludge which had been preincubated in vials for 4 h with feed. $^{14}CH_3COO^-$ was rapidly metabolized to $^{14}CH_4$ (Fig. 4). We chose to use initial rates rather than first-order decay kinetics (3) to calculate rates of methanogenesis from acetate because the acetate pool size

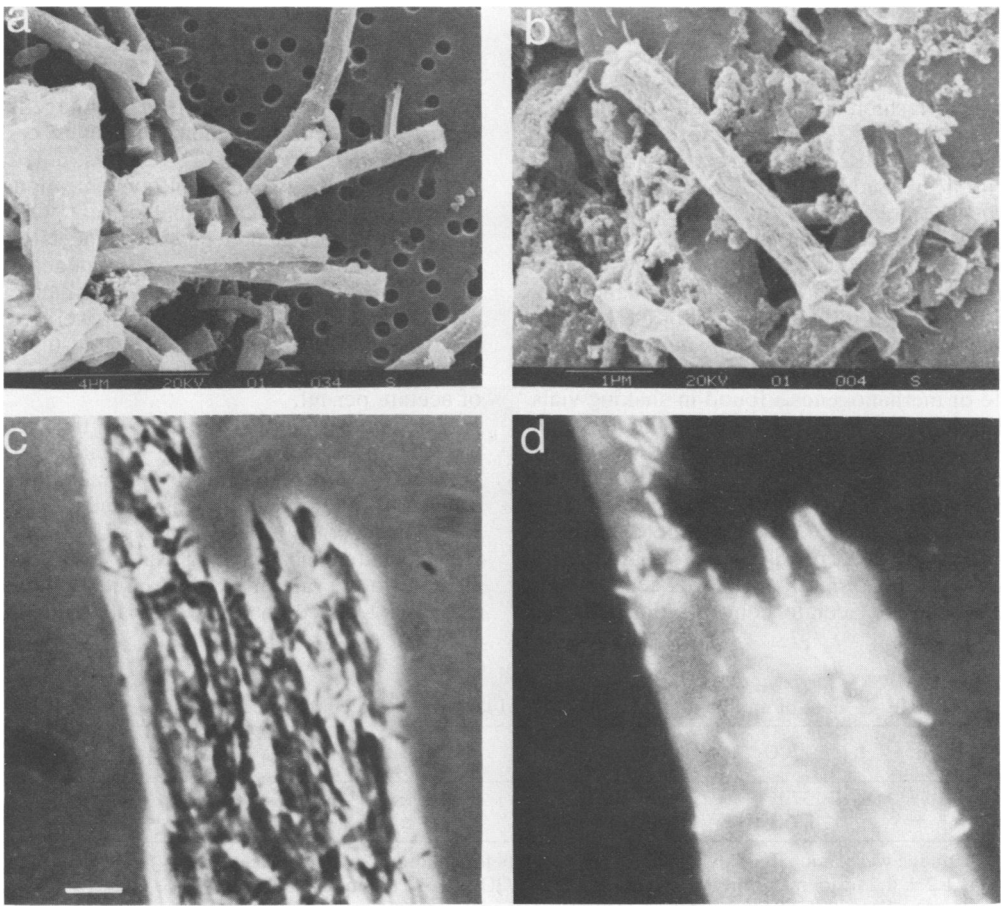

FIG. 2. Photomicrographs of microorganisms found in a 58°C anaerobic digestor. (a) Scanning electron micrograph of a portion of an aggregate of *Methanothrix*-like cells. Marker bar = 4 μm. (b) Scanning electron micrograph of a *Methanothrix*-like cell associated with a particle and with other organisms. (c) Phase-contrast micrograph of a fiber particle taken from the digestor and stained with DNA-specific fluorescent stain, DAPI. Marker bar = 10 μm. (d) Epifluorescence micrograph of the same fiber particle illuminated to stimulate DAPI fluorescence in cells attached to the fiber particle.

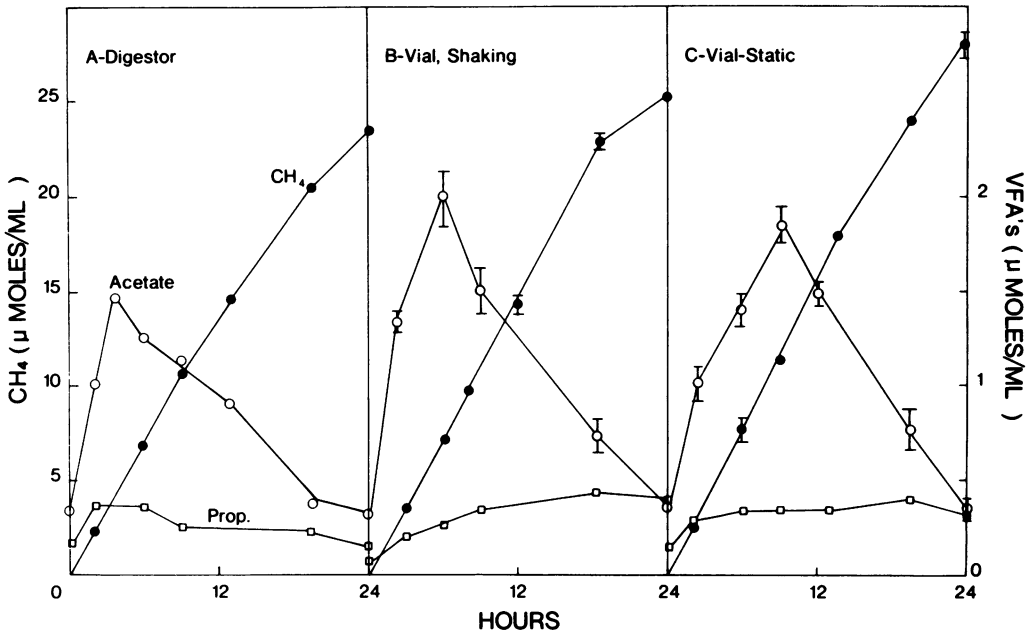

FIG. 3. Diurnal cycle in a 58°C anaerobic digestor after feeding and comparison with the cycle found in sludge samples incubated in vials. (A) Cycle in the digestor; (B) cycle for 10 ml of fed sludge incubated in 37-ml vials in a 58°C shaking water bath (150 rpm); (C) cycle for 10 ml of fed sludge incubated in 37-ml vials in a 58°C static water bath. Symbols: ●, CH_4; ○, acetate; □, propionate. Note 10-fold expansion of scale for fatty acids versus CH_4.

changed in a nonlinear fashion after incubation times greater than 30 min. During the first 20 min, the acetate pool size remained constant at 1.6 ± 0.2 μmol of acetate per ml, and the specific activity decreased less than 20%. The initial rate calculated was 0.73 μmol of CH_4 per ml per h. $^{14}CH_4$ production from $^{14}CO_2$ was essentially linear during the incubation period because only a small fraction of the large CO_2 pool (47 μmol/ml) was used. The rate of methanogenesis from CO_2 was calculated to be 0.43 μmol of CH_4 per ml per h. The sum of the calculated rates of methanogenesis from acetate and CO_2 (1.16 μmol of CH_4 per ml per h) was similar to the rate of methanogenesis found in shaking vials (1.2 μmol of CH_4 per ml per h; Fig. 3B) during the first 12 h of incubation. If acetate and CO_2 were the only major substrates for methanogenesis, then the relative contributions would be 63% from acetate and 37% from CO_2 during this early phase of the diurnal cycle. Another determination yielded a value of 66% for the contribution from acetate.

The nearly constant rates of methanogenesis despite relatively large changes in the acetate concentration (Fig. 3) suggested that the aceticlastic methanogens in the digestor were nearly saturated when the acetate concentration was greater than 0.5 μmol/ml. To test this, unfed sludge samples were incubated with different concentrations of acetate (0.2 μmol/ml to 10.2 μmol/ml) labeled with $^{14}CH_3COO^-$, and the initial rates of methanogenesis from acetate were measured. A clear saturation response was obtained and an apparent K_m value of 0.3 μmol of acetate per ml and a maximum rate of 1.1 μmol of CH_4 per ml per h were estimated from a Lineweaver-Burk plot (19) (r^2 value = 0.98). Several determinations of the K_m value all yielded values below 0.5 μmol of acetate per ml.

Since the partial pressure of H_2 in the digestor was below our limit of detection, we did not attempt to measure the kinetics of H_2 turnover. We did find that methanogenesis from CO_2 was significantly stimulated by addition of H_2 (Fig. 5). Addition of 1.2 atm (121 kPa) of H_2 to the headspace of a vial incubated in a shaking water bath caused a 12.6-fold increase in the rate of $^{14}CH_4$ production (5.7 μmol/ml per h) from $^{14}CO_2$ as compared with vials incubated for 2 h with

TABLE 2. Fate of methyl-labeled and carboxyl-labeled [^{14}C]acetate added to digestor sludge[a]

Label	$^{14}CH_4$[b]	$^{14}CO_2$[b]	($^{14}CH_4$ + $^{14}CO_2$)/ label added	Particulate[c]	Particulate/ ($^{14}CH_4$ + $^{14}CO_2$)	Soluble[b]	Label recovery
$CH_3{}^{14}COO^-$	<1.7[d]	157 ± 7	0.32	3.1 ± 0.3	0.020	266 ± 0	87%
$^{14}CH_3COO^-$	163 ± 3	4.7 ± 0	0.32	2.3 ± 0.3	0.014	346 ± 20	99%

[a] Samples (10 ml) of sludge were incubated in vials with feed for 4 h before addition of labeled acetate. At the time of label addition, the acetate concentration was 1.2 ± 0.2 μmol/ml. Either 490 kdpm of $CH_3{}^{14}COO^-$ or 520 kdpm of $^{14}CH_3COO^-$ was added to each of duplicate vials. Incubation time was 45 min. See the text for a detailed description of vial preparation.

[b] Results expressed as thousands of disintegrations per minute ± standard deviation.

[c] For particulate label, the amount found in the particulate fraction immediately after label addition was subtracted from the amount found after 45 min to account for nonspecific adsorption of acetate. Results are expressed as thousands of disintegrations per minute ± standard deviation.

[d] Less than the limit of detection.

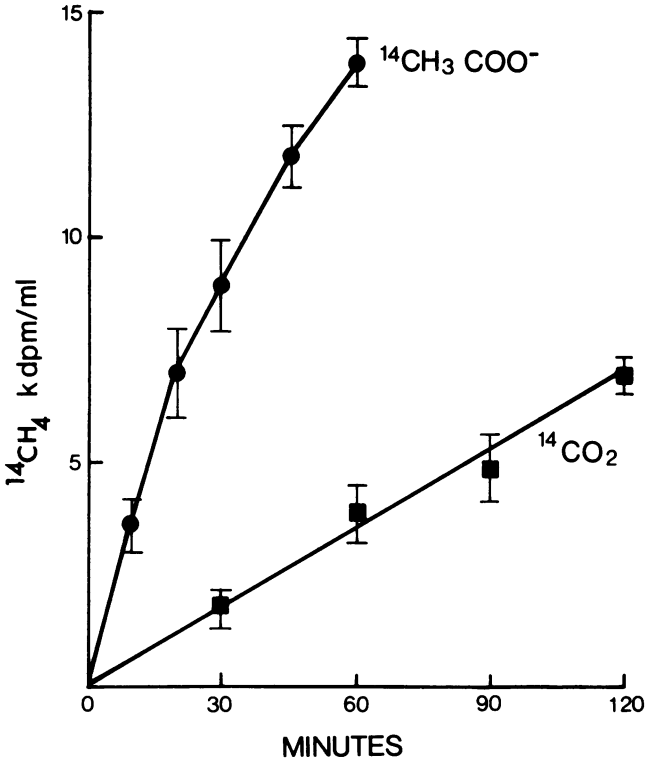

FIG. 4. $^{14}CH_4$ production from $^{14}CH_3COO^-$ (●) or $^{14}CO_2$ (■) added to sludge preincubated for 4 h with feed in a 58°C shaking water bath. $^{14}CH_3COO^-$ (46 kdpm/ml) was added to an acetate pool size of 1.6 ± 0.2 μmol/ml, or 395 kdpm of $NaH^{14}CO_3$ was added to a total CO_2 pool size of 47 μmol/ml.

feed (0.45 μmol/ml per h). Addition of H_2 to a static culture caused a more modest increase (2.4 μmol/ml per h), indicating that agitation was needed for adequate gas transfer. Thus, CO_2-reducing methanogens were capable of rates of methanogenesis at least 12-fold greater than the maximum rates of methanogenesis from CO_2 normally found in the digestor. The actual V_{max} may be even greater than the maximum rate we measured if gas transfer was not adequate (32).

These experiments predict that the aceticlastic methanogens in the digestor would be easily overloaded if the digestors received a shock load of a rapidly degraded substrate. We have found that cellobiose is rapidly utilized by digestor populations. When cellobiose was added to unfed sludge (Fig. 6) at a concentration of 5.8 μmol/ml (theoretical CH_4 yield, 34 μmol/ml), acetic acid built up to ca. 10 μmol/ml and then decreased. Addition of 29 μmol of cellobiose per ml resulted in acetic acid accumulation to 35 μmol/ml, enough to lower the sludge pH to 4.5. No H_2 was detected in the headspace of any of the vials. Propionate accumulated to 1.5 μmol/ml (48 h) in the vials receiving 29 μmol of cellobiose per ml, and no butyrate or other fatty acids were detectable. It is possible that nitrogen limitation also may have contributed to acetate build up.

Metabolism of added short-chain fatty acids. In methanogenic ecosystems, short-chain fatty acids are metabolized by hydrogen-producing acetogenic bacteria coupled with CO_2-reducing methanogens (4, 7, 25, 42). The ability of the digestor populations to metabolize a modest addition (4.5 μmol/ml) of various short-chain fatty acids was examined. Added propionate was slowly metabolized with 3.3 μmol/ml remaining after 48 h, and 96 h was required for complete degradation. Methanogenesis was slightly inhibited (20 to 30%) in vials containing propionate until most of the propionate had disappeared (data not shown).

Short-chain fatty acids longer than propionate were undetectable in the sludge, yet significant numbers of bacteria capable of oxidizing *n*-butyrate (and most likely longer-chain fatty acids [25]) could be enumerated (Table 1). As shown in Fig. 7A, added *n*-butyrate was almost completely metabolized within 12 h, and *n*-butyrate metabolism was accompanied by significant accumulation of acetate and methane, as well as a small accumulation of isobutyrate. It was of interest to determine whether isobutyrate was metabolized by the microbial populations. Figure 7B shows that after a lag of ca. 6 h the isobutyrate was broken down. Isobutyrate degradation was accompanied by acetate and *n*-butyrate accumulation. An enrichment culture converting isobutyrate to methane was established which contained rod-shaped bacteria, an autofluorescent organism resembling *Methanobacterium thermoautotrophicum*, and the *Methanothrix*-like organism. Nearly 1.5 mol of acetate was formed per mol of isobutyrate added by the enrichment, and probably the stoichiometric amount of acetate (2 mol per mol added) would have formed if acetate was not also being concurrently degraded. Small amounts of *n*-butyrate accumulated during the period of active isobutyrate degradation. *n*-Butyrate was not found in control cultures which did not contain isobutyrate. Valerate was degraded within 24 h by the digestor populations (Fig. 7C), and stoichiometric amounts of propionate were formed. Since less methane was formed than expected, it is likely that the propionate formed had inhibited methanogenesis, as previously found.

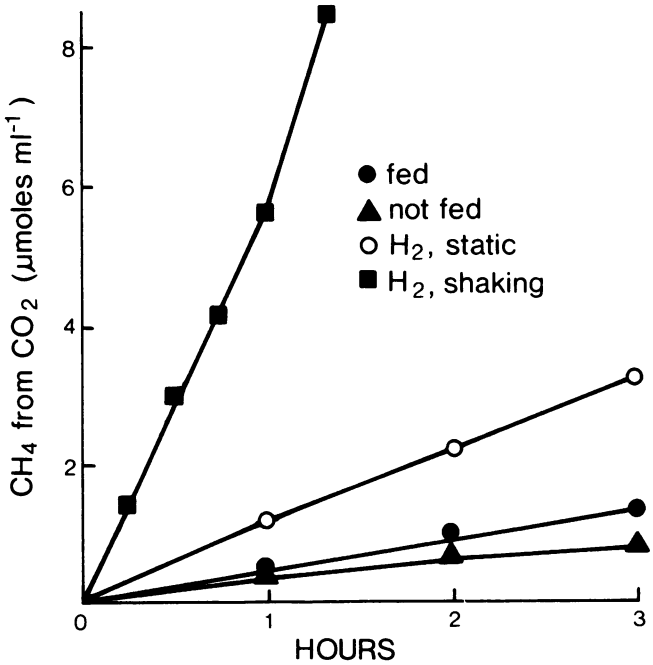

FIG. 5. Effect of added H_2 on $^{14}CO_2$ reduction to $^{14}CH_4$ in sludge samples taken from a 58°C anaerobic digestor. H_2 (121 kPa of H_2 and 30 kPa of CO_2) was added to unfed sludge after 1 h of preincubation. $NaH^{14}CO_3$ (395 kdpm/ml) was added to each vial.

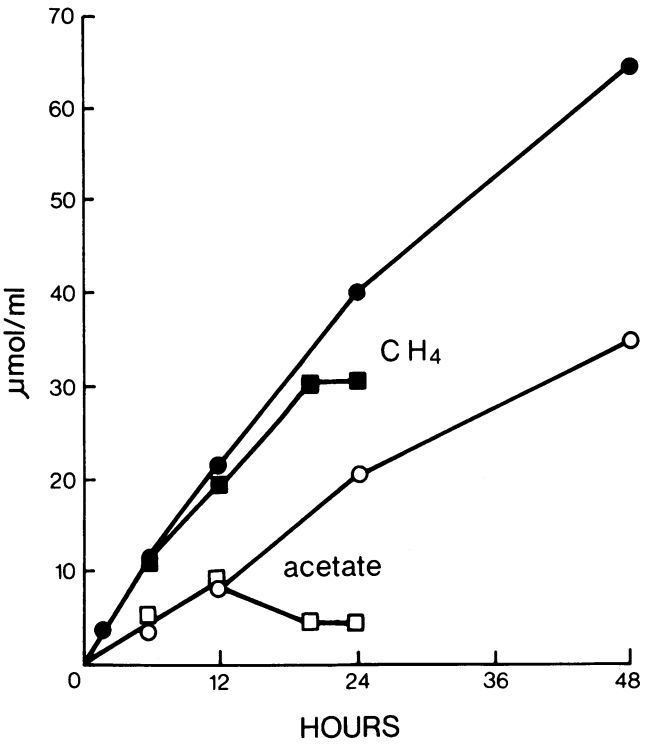

FIG. 6. Methanogenesis and acetate concentration in 10 ml unfed sludge samples taken from a 58°C digestor and incubated at 58°C with added cellobiose. Symbols: ■, CH_4 in vials receiving 5.8 μmol of cellobiose per ml; ●, CH_4 in vials receiving 29 μmol of cellobiose per ml; □, acetate in vials receiving 5.8 μmol of cellobiose per ml; ○, acetate in vials receiving 29 μmol of cellobiose per ml.

DISCUSSION

The ability to grow on acetate, methanogenesis from acetate via the aceticlastic reaction, and a singular morphology (sheathed filament) are all evidence that the organism which we found in our digestor is a thermophile related to the mesophilic *Methanothrix soengenii* (14, 44). The finding of a thermophilic *Methanothrix* sp. was not expected. It had not been described in previous literature, and one of us (S.H.Z.) had previously made numerous thermophilic acetate enrichments from a variety of habitats and had encountered only *Methanosarcina* sp. One exception to this was a rod-shaped thermophilic enrichment culture which was found to require two organisms coupled via interspecies hydrogen transfer for methanogenesis from acetate (S. Zinder and M. Koch, Abst. Annu. Meet. Am. Soc. Microbiol. 1983, I46, 147). Neither organism resembled *Methanothrix* sp.

Our thermophilic *Methanothrix* culture seems similar to the Russian culture (30). It is of interest that both thermophilic cultures apparently contain gas vacuoles, since mesophilic *Methanothrix* cultures have not been reported to contain them. Some mesophilic *Methanosarcina* cultures have been found to contain gas vacuoles (21, 47). The function of the *Methanothrix* gas vacuoles in a stirred digestor, if any, is unknown. The thermophilic *Methanothrix* sp. grew faster (doubling time at 60°C = 32 h [48]) than its mesophilic counterpart (doubling time = 3.5 to 6 days [14, 44]), as was the case for the thermophilic *Methanosarcina* sp. strain TM-1. The faster growth of thermophilic aceticlastic methanogens compared with mesophilic ones may explain why thermophilic digestors can be operated stably at retention times as short as 3 days (40).

We believe that *Methanosarcina* sp. was initially the

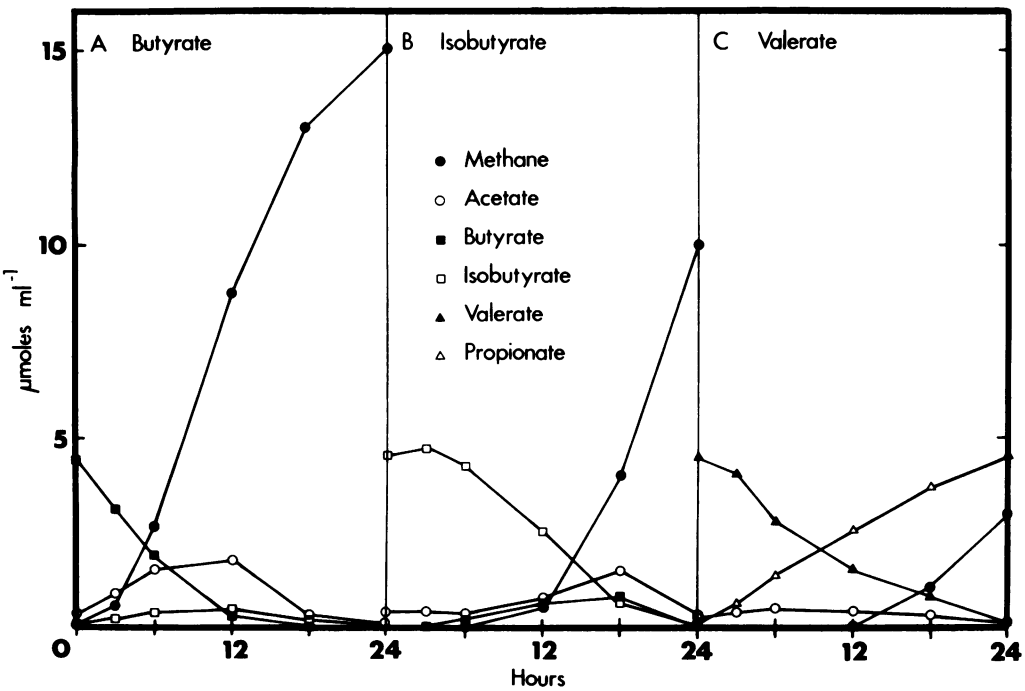

FIG. 7. Metabolism of 4.5 μmol of (A) *n*-butyrate, (B) isobutyrate, or (C) *n*-valerate per ml added to unfed sludge from a 58°C anaerobic digestor. Values for CH_4 represent the extra amount produced as compared with controls receiving no addition. The theoretical CH_4 yield from butyrate and isobutyrate was 11.25 μmol/ml and from valerate, 6.75 μmol/ml, assuming that the propionate produced is not metabolized further.

dominant aceticlastic methanogen in the digestor and that it was eventually displaced by *Methanothrix* sp. Autofluorescent *Methanosarcina*-like clumps were initially numerous in sludge viewed under the microscope, and colony counts were 10^5 to 10^6 CFU/ml. If one conservatively estimates the biovolume of the typical *Methanosarcina* clump in Fig. 1a to be the equivalent to a sphere with a radius of 10 μm, then its volume would be 1,000 times that of a cell of typical procaryote dimensions (radius = 0.5 to 1.0 μm). If each CFU represented a clump this size, then 10^5 CFU/ml would be the equivalent of over 10^8 CFU/ml of a typical procaryote. Thus it was concluded that *Methanosarcina* sp. was present in the digestor in ecologically significant numbers and was probably responsible for methanogenesis from acetate in the digestor during this period. Evidence for this displacement was the nearly 1,000-fold drop in the counts of *Methanosarcina* sp. in the sludge and the significant numbers of *Methanothrix* sp. found after this period. Further evidence is that, as described in another publication (48), the temperature responses (optima and maxima) for methanogenesis from acetate in the digestor sludge during January 1982 resembled those of a *Methanosarcina* culture, whereas in July 1982, they resembled those of a *Methanothrix* culture.

The faster-growing *Methanosarcina* sp. would probably be favored under start-up conditions, which resemble a batch culture, especially since it is capable of even more rapid growth if other substrates such as methanol or methylamines are present (34, 49). Once the digestor was being fed on a semicontinuous basis (10-day retention time), competition might be based on K_m for substrate. The K_m value for methanogenesis from acetate by our digestor was estimated at 0.3 μmol/ml during the period when that *Methanothrix* sp. was apparently dominant. This value is similar to that obtained with the mesophilic *Methanothrix* culture (14). We did not perform a K_m determination for methanogenesis from acetate during the period in which *Methanosarcina* sp. was abundant, but *Methanosarcina* cultures have been found to have values ranging from 3 to 5 μmol of acetate per ml (33, 34, 38) for methanogenesis or growth. This type of competition on the basis of the K_m value is often encountered in chemostats run at low dilution rates (15, 24) and has been compared with *r* versus *K* selection in animals (15). Factors other than K_m value may also play a role in competition.

The low K_m value we obtained for methanogenesis from acetate (0.3 μmol/ml) was similar to the value obtained by Kaspar and Wuhrmann (16) for a mesophilic sludge digestor which they believed was dominated by *Methanothrix* sp. Mackie and Bryant (20) did not directly determine a K_m value for methanogenesis from acetate in the 60°C cattle waste digestor they studied. However, they found that a threefold increase in acetate concentration in the digestor from 2 μmol/ml to 6 μmol/ml led to a threefold increase in the rates of methanogenesis from acetate (Fig. 2 and 3B, from reference 20), suggesting that the aceticlastic methanogens were not nearly saturated for substrate in this concentration range. It is of interest that the predominant organism seen in their cultural counts of aceticlastic methanogens was *Methanosarcina* sp. (M. P. Bryant, personal communication). Brune et al. (6) reported a value of 16 μmol of acetate per ml for a thermophilic 60°C culture converting acetic acid and furfural to CH_4. The dominant organism was *Methanosarcina* sp. Lawrence and McCarty (18) studied mesophilic acetate enrichment cultures in chemostats containing varying proportions of *Methanosarcina* sp. and *Methanothrix* sp. On the basis of classical chemostat kinetics, they calculated K_s values for methanogenesis from acetate ranging from 2.3 to 14.4 μmol/ml. It seems likely that digestor systems dominated by *Methanothrix* sp. will show K_m values for methanogenesis from acetate of less than 1 μmol/ml, whereas those dominated by *Methanosarcina* sp. would have values greater than 3 μmol/ml. Although a low K_m value for the aceticlastic methanogen population may be desirable in terms of digestor efficiency, it also means that the population is easier to overload, as demonstrated when we added a shock load of cellobiose to the sludge.

Similar to the results of Kaspar and Wuhrmann (16), we found that the CO_2-reducing methanogens were quite far from saturation for H_2. The maximum rate of methanogenesis from CO_2 we found (5.7 μmol/ml per h) was much higher than the V_{max}we calculated for methanogenesis from acetate (1.1 μmol/ml per h). Recently, K_m values for methanogenesis from hydrogen were calculated for a variety of mesophilic anaerobic habitats, including digestor sludge, rumen fluid, and lake sediments, and were found to be near 10^{-2} atm (1 kPa; 6 μM). If fatty-acid oxidation is to be thermodynamically feasible (26), then the H_2 partial pressure must be maintained well below 10^{-3} atm. If the CO_2-reducing methanogen population in our digestor had a similar K_m value, then it should have been undersaturated since butyrate was rapidly oxidized in the digestor.

The percentage of methane derived from the methyl group of acetate that we calculated (63 to 66%) was somewhat lower than other values obtained, which ranged from 68 to 86% (3, 20, 27, 37). A likely explanation is that we measured methanogenesis from acetate during a period when acetate concentrations were high and thus the aceticlastic methanogens were saturated for substrate, whereas the CO_2-reducing methanogens were keeping pace with H_2 production. It is likely that the contribution of acetate would be greater later on in the digestion cycle, but the rapid turnover of acetate when the concentrations were low made accurate rate determinations difficult.

Added propionate was only slowly metabolized by the digestor populations, indicating that there was little potential for propionate metabolism in the digestor. One possible explanation is that H_2 partial pressures were too high to support rapid propionate oxidation, but low enough to support butyrate oxidation (26). This may have been especially true for sludge in serum vials when there appeared to be some perturbation of propionate oxidation. Alternatively, the propionate oxidizers may have been present in the low numbers found in our cultural counts (Table 1) and were saturated for their substrate at the concentration found in the digestor (0.2 to 0.3 μmol/ml). In general, the methanogenic dissimilation of propionate seems to be problematical. Boone and Bryant (4) were unable to rid their *Syntrophobacter* populations of sulfate reducers, and mesophilic propionate utilizing cocultures described have doubling times of 5 to 7 days (4, 17). A thermophilic enrichment culture derived from the sludge barely grew faster (estimated doubling time of 4 to 5 days). Propionate apparently also inhibited methanogenesis, as found previously (1). The slow degradation of propionate and its toxicity indicates that a batch of digestor feed with a high propionate concentration could cause problems for the digestor.

In contrast to propionate, added *n*-butyrate, isobutyrate, or valerate were readily metabolized by microbial populations in the digestor. The metabolism of isobutyrate is of interest. McInerney and Bryant (25) found that the *n*-butyrate-oxidizing *Syntrophobacter wolfii* did not use isobutyrate. Isobutyrate, a product of valine fermentation, cannot

be used directly by beta-oxidation (19), and the usual mechanism of isobutyrate metabolism is via propionate (19). The evidence from the digestor and the enrichment culture studies indicates that isobutyrate was metabolized to two molecules of acetate in the same manner as *n*-butyrate. A likely mechanism is for isobutyrate to be isomerized to *n*-butyrate and then subsequently oxidized to acetate. This mechanism is supported by the appearance of *n*-butyrate during isobutyrate degradation in both the digestor sludge and in an enrichment culture. The mechanism would be clarified by isolating an organism responsible for isobutyrate breakdown.

The anaerobic digestor has advantages as a microbial ecosystem for study. It is better defined than many more "natural" systems, and well-known metabolic processes are carried out at high rates, which make their assay relatively easy. One major advantage of thermophilic anaerobic digestors is that less species diversity is often found in high-temperature habitats, making them simpler to study (5). For example, no eucaryotes, which have an upper temperature limit of 60°C (5), seem to be present in significant numbers in the digestor. In the mesophilic sludge digestor studied by Smith (36), several genera of CO_2-reducing methanogens were found. It is possible that knowledge of the physiology of representative strains of only a few organisms may be useful in predicting thermophilic digestor performance.

ACKNOWLEDGMENTS

This research was supported by contract no. DE-AC02-81ER10872 of the U.S. Department of Energy.

We thank W. C. Ghiorse for the use of the epifluorescence microscope and J. Lee for use of the scanning electron microscope. The expert typing of Patti Lisk is acknowledged.

LITERATURE CITED

1. **Andrews, J. F.** 1969. Dynamic model of the anaerobic digestion process. J. Sanit. Eng. Div. Proc. Am. Soc. Civ. Eng. **95**:95–116.
2. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevalution of a unique biological group. Microbiol. Rev. **43**:260–296.
3. **Boone, D. R.** 1982. Terminal reactions in the anaerobic digestion of animal waste. Appl. Environ. Microbiol. **43**:57–64.
4. **Boone, D. R., and M. P. Bryant.** 1980. Propionate-degrading bacterium, *Syntrophobacter wolinii* sp. nov. gen. nov., from methanogenic ecosystems. Appl. Environ. Microbiol. **40**:626–632.
5. **Brock, T. D.** 1978. Thermophilic microorganisms and life at high temperatures. Springer Verlag, New York.
6. **Brune, G., S. M. Shoberth, and H. Sahm.** 1982. Anaerobic treatment of an industrial wastewater containing acetic acid, furfural and sulphite. Proc. Biochem. **17**(3):10–35.
7. **Bryant, M. P.** 1979. Microbial methane production-theoretical aspects. J. Anim. Sci. **48**:193–201.
8. **Buhr, H. O., and J. F. Andrews.** 1977. The thermophilic anaerobic digestion process. Water Res. **11**:129–143.
9. **deMan, J. C.** 1975. The probability of most probable numbers. Eur. J. Appl. Microbiol. **1**:67–78.
10. **Doddema, H. J., and G. D. Vogels.** 1978. Improved identification of methanogenic bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **36**:752–754.
11. **Ferguson, T. J., and R. A. Mah.** 1983. Isolation and characterization of an H_2-oxidizing thermophilic methanogen. Appl. Environ. Microbiol. **45**:265–274.
12. **Goering, H. K., and P. J. van Soest.** 1970. Forage fiber analysis. Agricultural handbook no. 379. U.S. Department of Agriculture, Washington, D.C.
13. **Hobson, P. N., B. Bonsfield, and R. Summers.** 1974. Anaerobic digestion of organic matter. Crit. Rev. Environ. Control **4**:131–190.
14. **Huser, B. A., K. Wuhrmann, and A. J. B. Zehnder.** 1982. *Methanothrix soehngenii* gen. nov. sp. nov., a new acetotrophic non-hydrogen-oxidizing methane bacterium. Arch. Microbiol. **132**:1–9.
15. **Jannasch, H. W.** 1979. Microbial ecology of aquatic low nutrient habitats. p. 321–325. *In* M. Shilo (ed.), Strategies of microbial life in aquatic habitats. Verlag Chemie, New York.
16. **Kaspar, H. F., and K. Wuhrmann.** 1978. Kinetic parameters and relative turnovers of some important catabolic reactions in digesting sludge. Appl. Environ. Microbiol. **36**:1–7.
17. **Koch, M., J. Dolfing, K. Wuhrmann, and A. J. B. Zehnder.** 1983. Pathways of propionate degradation by enriched methanogenic cultures. Appl. Environ. Microbiol. **45**:1411–1414.
18. **Lawrence, A. W., and P. L. McCarty.** 1969. Kinetics of methane fermentation in anaerobic treatment. J. Water Pollut. Control Fed. **45**:R1–R17.
19. **Lehninger, A. L.** 1975. Biochemistry, 2nd ed. Worth, Inc., New York.
20. **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**:1363–1373.
21. **Mah, R. A., D. M. Ward, L. Baresi, and T. L. Glass.** 1977. Biogenesis of methane. Annu. Rev. Microbiol. **31**:309–341.
22. **Mah, R. A., M. R. Smith, and L. Baresi.** 1978. Studies on an acetate-fermenting strain of *Methanosarcina*. Appl. Environ. Microbiol. **35**:1174–1184.
23. **Malick, L. E., and R. B. Wilson.** 1975. Evaluation of a modified technique for SEM examination of vertebrate specimens without evaporated metal layers, p. 258–264. SEM/1975. Illinois Institute of Technology Research Institute, Chicago, Ill.
24. **Matin, A., and H. Veldkamp.** 1978. Physiological basis of the selective advantage of a *Spirillum* sp. in a carbon-limited environment. J. Gen. Microbiol. **105**:187–197.
25. **McInerney, M. J., M. P. Bryant, and N. Pfenning.** 1979. Anaerobic bacterium that degrades fatty acids in association with methanogens. Arch. Microbiol. **122**:129–135.
26. **McInerney, M. J., and M. P. Bryant.** 1981. Review of methane fermentation fundamentals, p. 20–46. *In* D. L. Wise (ed.), Fuel gas production from biomass. Chemical Rubber Co. Press, Inc., West Palm Beach, Fla.
27. **Mountfort, D. O., and R. A. Asher.** 1978. Changes in proportion of acetate and carbon dioxide used as methane precursors during the anaerobic digestion of bovine waste. Appl. Environ. Microbol. **35**:648–654.
28. **Nelson, D. R., and J. G. Zeikus.** 1974. Rapid method for the radioisotopic analysis of gaseous end products of anaerobic metabolism. Appl. Microbiol. **28**:258–261.
29. **Ng, T. K., P. J. Weimer, and J. G. Zeikus.** 1977. Cellulolytic and physiological properties of *Clostridium thermocellum*. Arch. Microbiol. **114**:1–7.
30. **Nozhevnikova, A. N., and T. F. Yagodina.** 1982. A thermophilic methane-producing bacterium. Microbiology **51**:534–541.
31. **Paul, J. H.** 1982. Use of Hoechst dyes 33258 and 33342 for enumeration of attached and planktonic bacteria. Appl. Environ. Microbiol. **43**:939–944.
32. **Robinson, J. A., and J. M. Tiedje.** 1982. Kinetics of hydrogen consumption by rumen fluid, anaerobic digestor sludge, and sediment. Appl. Environ. Microbiol. **44**:1374–1384.
33. **Schonheit, P., J. K. Kristjansson, and R. K. Thauer.** 1982. Kinetic mechanism for the ability of sulfate reducers to outcompete methanogens for acetate. Arch. Microbiol. **132**:285–288.
34. **Smith, M. R., and R. A. Mah.** 1978. Growth and methanogenesis by *Methanosarcina* strain 227 on acetate and methanol. Appl. Environ. Microbiol. **36**:870–879.
35. **Smith, M. R., S. H. Zinder, and R. A. Mah.** 1980. Microbial methanogenesis from acetate. Proc. Biochem. **15**(4):34–39.
36. **Smith, P. H.** 1966. The microbial ecology of sludge methanogenesis. Dev. Ind. Microbiol. **7**:156–161.
37. **Smith, P. H., and R. A. Mah.** 1966. Kinetics of acetate metabolism during sludge digestion. Appl. Microbiol. **14**:368–371.
38. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy

conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41**:100–180.
39. **Umbreit, W. W., R. H. Burris, and J. F. Stauffer.** 1957. Manometric techniques, 3rd ed., Burgess Publishing Co., Minneapolis, Minn.
40. **Varel, V. H., H. R. Isaacson, and M. P. Bryant.** 1977. Thermophilic methane production from cattle waste. Appl. Environ. Microbiol. **33**:298–307.
41. **Walsby, A. E.** 1975. Gas vesicles. Annu. Rev. Plant. Physiol. **26**:427–439.
42. **Wolin, M. J., and T. L. Miller.** 1982. Interspecies hydrogen transfer: 15 years later. ASM News **48**:561–565.
43. **Zehnder, A. J. B., B. Huser, and T. D. Brock.** 1979. Measuring radioactive methane with the liquid scintillation counter. Appl. Environ. Microbiol. **37**:897–899.
44. **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.
45. **Zeikus, J. G.** 1977. The biology of methanogenic bacteria. Bacteriol. Rev. **41**:514–541.
46. **Zeikus, J. B., and R. S. Wolfe.** 1972. *Methanobacterium thermoautotrophicus* sp. n., an anaerobic, autotrophic, extreme thermophile. J. Bacteriol. **109**:707–713.
47. **Zhilina, T. N., and T. A. Zavarzin.** 1979. Comparative cytology of methanosarcinae and description of *Methanosarcina vacuolata* sp. nova. Mikrobiology **48**:279–285.
48. **Zinder, S. H., S. C. Cardwell, and T. Anguish.** 1984. Effects of temperature on methanogenesis in a thermophilic (58°C) anaerobic digestor. Appl. Environ. Microbiol. **47**:808–813.
49. **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. **38**:996–1008.