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Isolation of a Butyrate-Utilizing Bacterium in Coculture with *Methanobacterium thermoautotrophicum* from a Thermophilic Digester†

J. MICHAEL HENSON‡* AND P. H. SMITH

Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611

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Sludge from a thermophilic, 55°C digester produced methane without a lag period when enriched with butyrate. The sludge was found by most-probable-number enumeration to have ca. 5×10^6 butyrate-utilizing bacteria per ml. A thermophilic butyrate-utilizing bacterium was isolated in coculture with *Methanobacterium thermoautotrophicum*. This bacterium was a gram-negative, slightly curved rod, occurred singly, was nonmotile, and did not appear to produce spores. When this coculture was incubated with *Methanospirillum hungatei* at 37°C, the quantity of methane produced was less than 5% of the methane produced when the coculture was incubated at 55°C, the routine incubation temperature. The coculture required clarified digester fluid. The addition of yeast extract to medium containing 5% clarified digester fluid stimulated methane production when a *Methanosarcina* sp. was present. Hydrogen in the gas phase prevented butyrate utilization. However, when the hydrogen was removed, butyrate utilization began. Penicillin G and D-cycloserine caused the complete inhibition of butyrate utilization by the coculture. The ability of various ecosystems to convert butyrate to methane was studied. Marine sediments enriched with butyrate required a 2-week incubation period before methanogenesis began. Hypersaline sediments did not produce methane after 3 months when enriched with butyrate.

Methane-producing ecosystems are complex microbial communities composed of many populations of bacteria that interact as three metabolically distinct groups. These groups are the fermentative bacteria, the obligate-proton reducing or hydrogenogenic bacteria, and the methane-producing bacteria (3, 9, 12, 18). Of these three groups, the hydrogenogenic bacteria are the least understood.

In the methanogenic fermentation of organic matter, the hydrogenogenic bacteria utilize the intermediate fermentation products propionate and butyrate producing the methanogenic substrates acetate, hydrogen, and carbon dioxide. Shuba (P. J. Shuba, Ph.D. thesis, University of Florida, Gainesville, Fla., 1973) provided the initial evidence for this physiological group when he rapidly sparged propionate enrichments and detected hydrogen in the gas phase. Methane production decreases under these conditions. In nonsparged propionate enrichments, hydrogen is not detected and the addition of hydrogen causes propionate utilization to cease. Subsequent studies by Boone (D. R. Boone, Ph.D. thesis, University of Florida, Gainesville, Fla., 1977), using rapidly sparged propionate or butyrate enrichments, show that hydrogen is an end product of the degradation of each of these volatile fatty acids (VFAs). The addition of hydrogen to active propionate or butyrate enrichments causes an inhibition of VFA utilization.

The first report of a fatty acid-utilizing hydrogenogenic bacterium is that of an anaerobic bacterium that utilizes butyrate as well as other fatty acids (15). This organism, which has subsequently been named *Syntrophomonas wolfei* (14), β-oxidizes saturated fatty acids (butyrate through octanoate) to acetate and H_2 or to acetate, propionate, and H_2. *Syntrophomonas wolfei* requires the presence of a hydrogenotrophic (hydrogen-utilizing) bacterium, has a doubling time of 54 h with a hydrogenotrophic *Desulfovibrio* sp., and is inhibited when hydrogen is present in the gas phase (13). A second hydrogenogenic species, *Syntrophobacter wolinii* (2), degrades propionate, but not other fatty acids, to acetate and, presumably, H_2 and CO_2 only in the presence of a hydrogenotrophic bacterium. When grown with a hydrogenotrophic *Desulfovibrio* sp., the doubling time is 87 h (2). A sporeforming, butyrate-oxidizing bacterium has recently been isolated in coculture (17). This bacterium oxidized isobutyrate in addition to butyrate, valerate, and caproate (17).

Descriptions of the above three hydrogenogenic bacteria provide the only information concerning the bacteria involved in the utilization of fatty acids in the fermentation of organic matter to methane. The utilization of fatty acids may be the rate-restricting step in this fermentation process (10, 12). Therefore this study was undertaken to increase the understanding of the fatty acid-utilizing hydrogenogenic bacteria and their role in the methane fermentation.

MATERIALS AND METHODS

Organisms, media, and growth conditions. *Methanobacterium thermoautotrophicum* strain ΔH and *Methanospirillum hungatei* strain JF-1 were from our culture collection. *Methanobacterium thermoautotrophicum* was incubated at 55°C in medium number 2 of Balch et al. (1). *Methanospirillum hungatei* was incubated at 37°C in medium number 1 of Balch et al. (1). To prepare these media, stock solutions of trace minerals, trace vitamins, and various salt solutions were prepared and frozen until used.

Medium for butyrate enrichments and butyrate studies was a modification of medium number 1 of Balch et al. (1). This medium was prepared by deleting yeast extract, acetate, and Trypticase (BBL Microbiology Systems, Cockeys-

* Corresponding author.

† Florida Agricultural Experiment Station Journal Series no. 5979.

‡ Present address: Robert S. Kerr Environmental Research Laboratory, U.S. Environmental Protection Agency, Ada, OK 74820.

ville, Md.) from the medium and adding 50 ml of clarified digester fluid per liter of medium. Digester fluid from the digester being studied was clarified by centrifugation at 12,000 × *g* (5°C) for 30 min. The clarified digester fluid (CDF) was frozen until used. After medium was dispensed to several serum tubes, sodium-*n*-butyrate (Pfaltz and Bauer, Stamford, Conn.) was added to the remaining medium to yield a final concentration of 0.3% (wt/vol). The gas phase was 80% N_2–20% CO_2. Final pH was 7.2 to 7.4. This medium was then dispensed, stoppered, and autoclaved for 20 min at 121°C. Noble agar (Difco Laboratories, Detroit, Mich.) was added to a final concentration of 1.5% (wt/vol) when a solid medium was required.

Principles of anaerobic techniques, as described by Hungate (7), were used for media preparation and during experimental procedures. To prepare media, all components except trace vitamins, $NaHCO_3$, cysteine hydrochloride · H_2O, CDF, and sodium *n*-butyrate were added to distilled water in a round-bottom flask. These components were boiled for several minutes and then cooled to room temperature in an ice bath while being sparged with 80% N_2–20% CO_2. The components listed above were added in accordance to the medium being prepared. After the pH was adjusted (if necessary), the medium was dispensed into serum tubes (Bellco Glass, Inc., Vineland, N.J.) or serum bottles (Wheaton Scientific, Millville, N.J.) in which the gas phase had been replaced with 80% H_2–20% CO_2 or 80% N_2–20% CO_2 (1). The serum tubes or serum bottles were closed with butyl rubber stoppers (no. 2048-11800; Bellco), which were held in place by crimped aluminum seals (no. 224193; Wheaton).

Gas mixtures were purchased from Matheson Scientific, Inc., Morrow, Ga., and trace oxygen was removed by passing these gases over heated (350°C) copper turnings.

Transfers of broth cultures were made with sterile needle and syringe units that had been made anoxic by aspirating sterile reduced medium or anoxic gas. Colonies were transferred by sterile bent Pasteur pipettes. Agar slants were prepared by using serum tubes or culture tubes (no. 2044-16150; Bellco).

Before use of all media a volume of 1.25% $Na_2S \cdot 9H_2O$ was injected so that it was diluted 1:50.

Descriptions of ecosystems studied. A thermophilic (55°C), stirred digester served as the source of inoculum. The digester was a 4-liter aspirator bottle with a liquid volume of 3.6 liters. Each day it received 16 g of feed consisting of 75% Bermuda grass and 25% Universal cattle feed (Seminole Brands). The cattle feed provided a high grain and protein supplement. The hydraulic detention time was 20 days.

Freshwater sediment samples were taken at Bivens Arm, a eutrophic lake located near the University of Florida, Gainesville. Marine sediment samples were taken from seagrass beds at Seahorse Key, located near Cedar Key, Fla. Hypersaline sediment samples were taken from Great Salt Lake, Utah, and from salterns of San Francisco Bay, Calif.

Descriptions of butyrate enrichments. Enrichments were begun by placing sludge from the thermophilic digester or sediments from Bivens Arm into butyrate medium. Enrichments with marine sediments were begun with sulfate-free artificial seawater instead of distilled water as given in the description for butyrate medium. The sulfate-free artificial seawater was composed, in grams per liter of distilled water, of the following: NaCl, 21.15; $MgCl_2 \cdot 6H_2O$, 9.65; $CaCl_2$, 1.0; NH_4Cl, 0.25; KCl, 0.5; KBr, 0.086; $SrCl \cdot 6H_2O$, 0.022; H_3BO_4, 0.023. Digester, freshwater, and marine enrichments were begun with a 10% inoculum. Enrichments from hypersaline environments were begun by adding sodium *n*-butyrate to final concentration of 0.3% (wt/vol) to sediment slurries prepared with water collected from the hypersaline environment sampled.

Thermophilic coculture isolation. A stable thermophilic enrichment was used as a source of inoculum for coculture isolation attempts. The enrichment was serially diluted in control medium without butyrate, and roll tubes were prepared from these dilutions. Each dilution was a source of inoculum for two butyrate control roll tubes as well as three butyrate experimental roll tubes containing 0.3% butyrate. Before these tubes were rolled out, they received 0.5 ml of turbid, active *Methanobacterium thermoautotrophicum*, which served as the hydrogenotroph. These roll tubes were incubated at 55°C.

Gas chromatography methods. Methane concentrations were measured with a Hewlett-Packard model 5880A gas chromatograph. Gases were separated in a stainless steel column (1.8 m by 1.0 mm) packed with 80/100 mesh Carbosphere (Alltech Associates, Inc., Deerfield, Ill.) and measured with a thermal conductivity detecter. Helium was the carrier gas. Column and detector temperatures were maintained at 130 and 145°C, respectively. Methane concentrations were determined by comparison to standards prepared from ultrahigh-purity methane (Matheson). Gas pressures in stoppered vessels were determined by using a pressure transducer (Setra Systems, Inc., Acton, Mass.).

VFAs were measured with a Hewlett Packard 5880A gas chromatograph. VFAs were separated in a glass column (1.8 m by 1.0 mm) packed with 8% SP-1000–2% SP-1200–1.5% H_3PO_4 on 80/100 mesh Chromosorb W AW 8100 (Supelco, Bellefonte, Pa.) and measured with a flame ionization detector. Helium was the carrier gas. Injector, oven, and detector temperatures were 145, 130, and 175°C, respectively. Samples were mixed with an equal volume of 4% *o*-phosphoric acid and centrifuged at 12,800 × *g* for 2 min (22°C), and the supernatant was frozen until VFA determinations were made. Individual VFA concentration was determined by comparison to standards.

Microscopy and photomicroscopy. A Carl Zeiss Standard WL microscope equipped for epifluorescence was used for observation of wet mounts and colonies as described (16). A Leica camera back was attached to the microscope for photomicroscopy. Kodak Technical Pan film 2415 was exposed for times determined with previously exposed test rolls. Film was developed as described in Kodak instructions and printed on Kodak F5 RC or polycontrast RC paper.

RESULTS

Production of methane when various ecosystems were enriched with butyrate. When various ecosystems were enriched with butyrate, not all produced methane (Table 1). Anaerobic digesters and freshwater sediments produced methane with little or no lag, whereas marine sediments in sulfate-free artificial seawater required about 2 weeks for methane production to begin. Methane was not produced after several months of incubation when hypersaline sediments were enriched with butyrate.

Description of thermophilic butyrate enrichments. The population of butyrate-utilizing bacteria in a 55°C digester was enumerated by the five-tube most-probable-number (MPN) method. After a 4-week incubation period, 4.5×10^6 butyrate-utilizing bacteria per ml of sludge were found. The lower-dilution MPN tubes produced significantly more methane than did the higher-dilution MPN tubes. Examination

TABLE 1. Examination of various ecosystems for methane production from butyrate enrichments

Source of enrichment	Methane produced[a]	Lag period (days) before onset of methane production
Thermophilic digester	Yes	None
Mesophilic digester	Yes	None
Bivens Arm	Yes	3
Halodule sp. seagrass bed	Yes	14
Thalassia sp. seagrass bed	Yes	14
Great Salt Lake	No	
San Francisco Bay saltern	No	

[a] Indicates production of methane in medium with butyrate minus methane production in medium without butyrate.

revealed that the lower-dilution MPN tubes contained a *Methanosarcina* sp. Butyrate enrichments were established by using each of these distinct dilutions as an inoculum. The greater methane production by the enrichment containing the *Methanosarcina* sp. is shown in Fig. 1. Acetate accumulated in the enrichment without the *Methanosarcina* sp. but did not accumulate in the enrichment with the *Methanosarcina* sp. Butyrate was utilized by both enrichments. The *Methanosarcina* sp. was isolated and did not grow at 55°C after a 4-week incubation period when H_2-CO_2 was the only methanogenic substrate present; the organism grew only in the presence of acetate. This organism was similar to *Methanosarcina* sp. strain TM-1 (20). Both enrichments contained rod-shaped bacteria that autofluoresced when examined by epifluorescence microscopy at 420 nm, indicating the presence of factor F_{420} found in methanogens (5, 15). This methanogenic rod-shaped bacterium, probably a strain of *Methanobacterium thermoautotrophicum*, grew at 55°C and utilized H_2-CO_2 for growth. There were several other rod-shaped nonfluorescing bacteria present. Therefore, it was difficult to determine which bacterium utilized butyrate. The enrichments were transferred every 7 to 10 days after being analyzed and found positive for methane production. After 4 months, a stable enrichment was obtained. A stable enrichment was defined as an enrichment having only several consistently present morphotypes. *Methanobacterium thermoautotrophicum* was always present as the largest population of bacteria, generally constituting 90% of the bacteria in each microscopic field.

Isolation of thermophilic butyrate-utilizing cocultures. The stable thermophilic enrichment was used as a source of inoculum for attempts to isolate butyrate-utilizing cocultures. *Methanobacterium thermoautotrophicum* was used as a hydrogenotrophic partner. Anaerobic roll tubes were incubated for 3 to 4 weeks at 55°C until methane was detected in the gas phase and colonies appeared. Basically, two colony types were present. One colony type was brownish-orange, granular, and irregular in shape, averaged 2 mm in diameter, and autofluoresced when exposed to 420-nm light. This colony resembled *Methanosarcina* sp. strain TM-1, but when a wet mount of the colony was examined, it was found to be composed of the *Methanosarcina* sp., *Methanobacterium thermoautotrophicum*, and a nonfluorescing, slightly curved bacterium. The second colony type was white and circular with an entire margin and averaged 1 mm in diameter. The colony autofluoresced when exposed to 420-nm light. Upon microscopic examination, the colony was composed of *Methanobacterium thermoautotrophicum* and a nonfluorescing, slightly curved bacterium. The two colony types were predominantly composed of *Methanobacterium thermoautotrophicum*. The colonies only appeared in the medium when butyrate was present.

A photomicrograph of the two-membered coculture is shown in Fig. 2. *Methanobacterium thermoautotrophicum* was rod shaped and autofluoresced upon exposure to 420-nm light. Several examples are indicated by the single arrows. The butyrate-utilizing bacterium was a slightly curved gram-negative rod that averaged 2 to 3 μm in length, occurred singly, was nonmotile, and did not contain spores. Several examples are indicated by the double arrows (Fig. 2).

Colonies were picked, placed into fresh medium, and rolled out. Once colonies appeared again, they were picked and placed onto slants prepared in serum tubes or culture tubes. Growth occurred only on slants prepared with the culture tubes.

Studies on the coculture. The butyrate-utilizing coculture was examined to determine the temperatures for growth (Table 2). When *Methanospirillum hungatei*, a mesophilic hydrogen-utilizing methanogen (6), was added, only trace amounts of methane were formed at 37°C. The amount of methane formed was less than 5% of the methane formed when the coculture was incubated at 55°C. Methane was not produced when the coculture was incubated at 45 or 70°C.

The coculture was examined to determine whether CDF was required or could be replaced by rumen fluid (RF). When neither CDF nor RF was a component of the medium, methane production was greatly diminished (Table 3). The addition of RF did not stimulate methane production and was inhibitory at concentrations of 20% and above on day 17. CDF addition resulted in consistent methane production at all concentrations tested.

The addition of yeast extract resulted in a 142% increase in methane produced after 18 days of incubation by the coculture containing the acetate-utilizing *Methanosarcina* sp. (Table 4). At day 22, the increase in methane production by the coculture with the *Methanosarcina* sp. was 28%. Yeast extract did not stimulate methane production and showed a slight inhibition of methane production in the coculture when the *Methanosarcina* sp. was absent.

Antibiotics known to affect cell wall synthesis of eubacteria but not of archaebacteria were added to the butyrate-utilizing coculture. Penicillin G (3,000 U/ml) and D-cyclo-

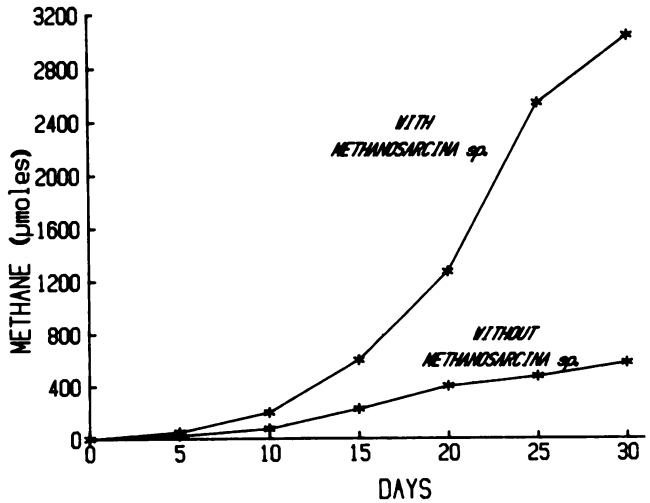

FIG. 1. Methane production by thermophilic butyrate-utilizing enrichments with and without *Methanosarcina* sp. present.

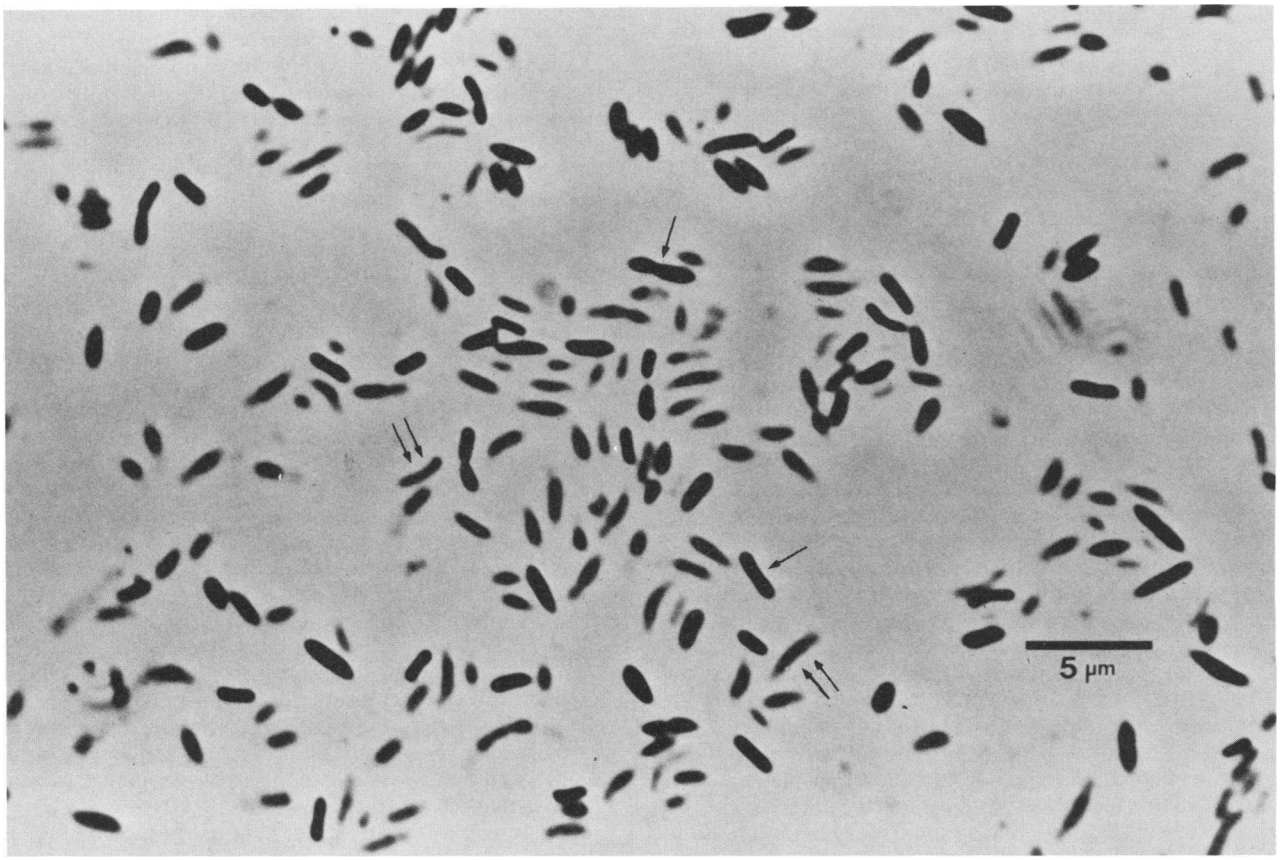

FIG. 2. Photomicrograph showing *Methanobacterium thermoautotrophicum*, indicated by a single arrow, and the butyrate-utilizing bacterium, indicated by the double arrows.

serine (0.1 mg/ml) caused the complete inhibition of methane production by the coculture (Fig. 3).

The presence of hydrogen in the gas phase (80% H_2–20% CO_2) inhibited the utilization of butyrate by the coculture (Fig. 4). The gas phase was replaced every day until day 8. At that time (indicated by the arrow in Fig. 4), hydrogen was not detected in the gas phase because of its removal by *Methanobacterium thermoautotrophicum*. The gas phase was replaced with 80% N_2–20%CO_2. The culture was allowed to continue incubation in the absence of hydrogen to determine whether the butyrate utilizers had been killed by the hydrogen or merely inhibited. After a lag period, rapid butyrate utilization began (Fig. 4).

In general, the enrichments and cocultures used butyrate faster and produced methane quicker when they were incubated without shaking. When the enrichments and cocultures were shaken, methane production showed a longer lag

TABLE 2. Effects of various temperatures on the methane production of a thermophilic butyrate-utilizing coculture

Temp (°C)	Total methane production (μmol)[a]		
	Day 8	Day 13	Day 26
37[b]			
A	5 ± 3	8 ± 5	14 ± 5
B	MND[c]	MND	MND
C	2	1	0.96
45	MND	MND	MND
55	52 ± 10	305 ± 19	299 ± 15
70	MND	MND	MND

[a] Values represent a mean of triplicate determinations plus or minus standard deviation, except for *Methanospirillum hungatei* alone (C), where they are single determinations.

[b] A, Coculture with *Methanospirillum hungatei;* B, coculture without *Methanospirillum hungatei*; C, *Methanospirillum hungatei* alone.

[c] MND, Methane not detected.

TABLE 3. Effects of various concentrations of RF or CDF on methane production by thermophilic coculture

Substance added (%)	% Methane in the gas phase[a]	
	Day 9	Day 17
No addition (control)	1 ± 0.02	6 ± 0.42
RF		
5	4 ± 2	38 ± 11
10	LTC	41 ± 0.5
20	LTC	24 ± 5
30	LTC	LTC
CDF		
5	7 ± 1	43 ± 2
10	8 ± 1	41 ± 2
20	9 ± 1	40 ± 3
30	8 ± 3	37 ± 3

[a] Percentages of methane given for the various concentrations of RF and CDF are for medium with butyrate minus medium without butyrate. Each value represents the mean of three tubes plus or minus standard deviation.

[b] LTC, Less than control value.

TABLE 4. Effects of the addition of 0.1% yeast extract to thermophilic, butyrate-utilizing cocultures

Day of expt	Total methane production (μmol)[a]			
	Without *Methanosarcina* sp.		With *Methanosarcina* sp.	
	Without YE	With YE	Without YE	With YE
11	21	2	1	0
18	37	55	99	241
22	62	54	244	313

[a] Values represent means of duplicate tubes. YE, Yeast extract.

period, but if the shaking was stopped, methane production increased.

DISCUSSION

Organic matter is degraded exclusively to methane and carbon dioxide in nongastrointestinal ecosystems in which light, nitrate, oxygen, and sulfate are absent. This degradation requires at least three groups of bacteria (12, 17): fermentative, hydrogenogenic, and methanogenic. The hydrogenogenic bacteria are the least understood of the three groups: only three species are known (2, 13, 14, 17).

When butyrate was added to several ecosystems, only digesters and freshwater sediments produced methane with a short lag period. These ecosystems continually receive organic matter which undergoes degradation to methane and, therefore, have a population of butyrate-utilizing bacteria. When seagrass beds were enriched with butyrate in sulfate-free artificial seawater, methane was not produced until after a 2-week lag period. The lag in methane production may have resulted from butyrate being utilized by fatty acid-utilizing, sulfate-reducing bacteria which were reducing the sulfate that remained in the sediments. These enrichments smelled strongly of H_2S before and after methane production began. *Desulfovibrio* spp. will degrade lactate (4, 11) or ethanol (4) in the absence of sulfate when methanogens are present to remove hydrogen, the electron sink product. When sulfate was depleted in these marine sediments, the methanogens may have participated in the degradation of butyrate by removing H_2 produced by sulfate-reducing bacteria. The inability of the microflora of hypersaline sediments to produce methane when enriched with butyrate indicated that butyrate may not be a typical substrate in the ecosystems examined.

The thermophilic digesters were chosen for more detailed study. Thermophilic digestion may have several advantages over mesophilic digestion. Varel et al. (19) reported the advantage of being able to reduce retention times to less than 6 days at thermophilic (45°C) temperatures. The thermophilic digester had ca. 5×10^6 butyrate-utilizing bacteria per ml of sludge, a finding similar to that of another study (8). When a thermophilic *Methanosarcina* sp. that utilized acetate but not H_2-CO_2 was present in butyrate enrichments, greater quantities of methane were produced and the enrichments seemed more stable. The enhanced methane production resulted from the decarboxylation of acetate with the concomitant production of methane by the *Methanosarcina* sp. The *Methanosarcina* sp. may also provide an increased surface area where the hydrogenogenic and hydrogenotrophic bacteria may grow in close proximity, therefore allowing interspecies hydrogen transfer to occur more efficiently.

Thermophilic methanogenic butyrate enrichments were composed primarily of the H_2-utilizing *Methanobacterium thermoautotrophicum* with other morphotypes present in smaller numbers. Therefore, *Methanobacterium thermoautotrophicum* was the bacterium of choice to use as a hydrogenotrophic partner for coculture isolation attempts. When butyrate enrichments were cultured with *Methanobacterium thermoautotrophicum*, two colony types were observed. One colony type was composed of *Methanobacterium thermoautotrophicum* and a butyrate-utilizing bacterium. The second colony was composed of these two bacteria plus a *Methanosarcina* sp.

The previously described hydrogenogenic bacteria are mesophilic (2, 13, 14, 17). To determine whether the butyrate-utilizing bacterium isolated from the thermophilic digester was in fact a thermophile, the coculture was incubated at several temperatures. When the coculture was

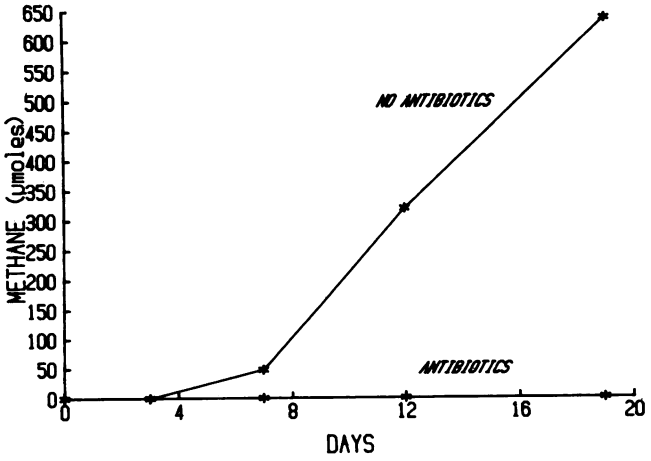

FIG. 3. The effect of eubacterial antibiotics on methane production by a thermophilic butyrate-utilizing coculture. The antibiotics were penicillin G (3,000 U/ml) and D-cycloserine (0.1 mg/ml).

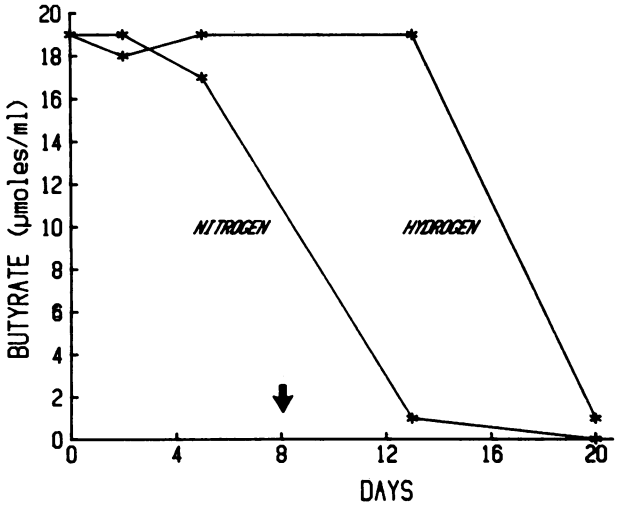

FIG. 4. The effect of hydrogen on butyrate utilization by a thermophilic butyrate-utilizing coculture. The gas phase for the data labeled nitrogen was 80%N_2–20%CO_2 for the entire experiment. The gas phase for the data labeled hydrogen was 80%H_2–20%CO_2 and was replaced daily until day 8, when 80%N_2–20%CO_2 was used to replace the gas phase.

incubated at 37°C (Table 2), methane was not detected. The lack of methane production could have resulted from the inability of *Methanobacterium thermoautotrophicum* to grow. Therefore, a mesophilic H_2-CO_2-utilizing methanogen, *Methanospirillum hungatei*, used in the study of the reported hydrogenogens (2, 13, 14, 17), was added to the coculture. If the butyrate-utilizing bacterium could produce H_2 from butyrate at 37°C, then *Methanospirillum hungatei* would oxidize it and methane should be detected. A small amount, ca. 14 μmol, of methane was produced after 26 days of incubation (Table 2). When the coculture was incubated at 55°C, the temperature of isolation, ca. 300 μmol of methane was produced by day 13, half the time required for production of 14 μmol of methane by the coculture with *Methanospirillum hungatei* at 37°C. This indicated that this butyrate utilizer was a thermophilic bacterium and, hence, was different from *Syntrophomonas wolfei*, a mesophile. In addition, the thermophilic butyrate utilizer was a nonmotile, slightly curved rod of 2 to 3 μm in length, whereas *Syntrophomonas wolfei* exhibits sluggish motility and is 7 μm in length (13). Therefore, it appears that this bacterium is a new species of anaerobic hydrogenogens. It required a growth factor (or factors) that was present in CDF or in RF (Table 3). The addition of 0.1% yeast extract to the *Methanosarcina* sp. culture containing enrichments resulted in a 142% increase in methane production after 18 days of incubation (Table 4). Yeast extract can replace CDF for growth of *Methanosarcina* sp. strain TM-1 (P. A. Murray and S. H. Zinder, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, I8, p. 141). The addition of yeast extract to medium with CDF had no effect on the methane production when the *Methanosarcina* sp. was not present in the enrichment.

The addition of penicillin and D-cycloserine completely inhibited methane production by the thermophilic coculture (Fig. 3). *Syntrophomonas wolfei* is inhibited by the addition of penicillin and possesses a peptidoglycan cell wall (14). Since the thermophilic coculture was inhibited by antibiotics that inhibit eubacteria, it appears that the thermophilic butyrate-utilizing bacterium was a eubacterium and possessed a peptidoglycan cell wall like that of *Syntrophomonas wolfei* (14).

The presence of 80% hydrogen in the gas phase inhibited butyrate utilization by *Syntrophomonas wolfei* (14). When the thermophilic coculture was placed under an 80% hydrogen gas phase, butyrate was not utilized (Fig. 4). However, when hydrogen was removed from the thermophilic coculture, butyrate was utilized (Fig. 4). This result indicated that hydrogen inhibited but did not kill the thermophilic butyrate-utilizing bacteria.

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