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Characterization of *Methanobacterium mobilis*, sp. n., Isolated from the Bovine Rumen

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A methanogenic bacterium, present in bovine rumen contents at concentrations of approximately 2×10^8 cells per ml, has been isolated in pure culture. The organism is a strictly anaerobic, weakly motile, nonsporeforming, gram-negative rod ($0.7\ \mu \times 1.5$ to $2.0\ \mu$) with rounded ends. There is a single polar flagellum. The organism grows at temperatures between 30 and 45 C, with an optimum at 40 C, and at *p*H values between 5.9 and 7.7, with optimal growth between *p*H 6.1 and 6.9. Of the 17 substrates tested, only formate and H_2 plus CO_2 supported growth. An unidentified, heat-stable factor(s) was required by the organism. The factor, which was not one of the common ones, was present in rumen fluid, mixed rumen bacteria, and yeast extract. On the basis of colony morphology, Gram reaction, and motility, the organism is classified as a new species of methanogenic bacterium, and the name *Methanobacterium mobilis* sp. n. is proposed.

Large quantities of methane are produced by the rumen fermentation. In cattle, methane can constitute 18.8% (molar basis) of the fermentation products (6), and calculations have indicated a value of as much as 16.8% for sheep (5). Until now, *Methanobacterium ruminantium* was the only methanogenic organism isolated in pure culture from rumen contents and shown to occur in the rumen in sufficient numbers to play a significant role in methanogenesis (2, 11). The occurrence of *Methanosarcina* in goat rumen contents was reported by Beijer (1), and *Methanobacterium formicicum* and a methanogenic acetate utilizer were demonstrated in cattle by Opperman et al. (10). The numbers of these latter organisms were not determined, and their importance in the rumen is questionable.

During attempts to isolate *M. ruminantium* from the bovine rumen, a new and seemingly important species of *Methanobacterium* was encountered.

MATERIALS AND METHODS

Cultural procedures. The organism was grown on a mixture of 80% H_2 and 20% CO_2 in a medium containing the following ingredients, at the indicated final percentage compositions (w/v): clarified rumen fluid (rumen contents centrifuged at $25,000 \times g$ for 15 min), 30; KH_2PO_4, 0.05; K_2HPO_4, 0.05; NaCl, 0.1; $(NH_4)_2SO_4$, 0.05; $MgSO_4$, 0.01; $CaCl_2$, 0.01; resazurin, 0.0001; $NaHCO_3$, 0.5 or 0.2; cysteine·HCl, 0.03; and Na_2S, 0.03. The first eight ingredients were prepared and dispensed in 4.1- or 4.5-ml amounts into narrow-necked culture tubes (16 × 150 mm; Belco Glass, Inc., Vineland, N.J.). The 80% H_2-20% CO_2 gas mixture was used to prevent access of oxygen. Extreme anaerobic procedures (4, 6) were used.

Tubes were sealed with #00 recessed butyl rubber stoppers (9) and sterilized at 120 C for 15 min. The appropriate volumes of sterile solutions of sodium sulfide [3% Na_2S (w/v), prepared under hydrogen], cysteine hydrochloride [3% (w/v), prepared under carbon dioxide], and sodium bicarbonate [10% (w/v), prepared under carbon dioxide and sterilized by filtration] were injected through the stoppers, by use of 1-ml syringes fitted with 1-inch, 21-gauge, Huber-point needles (the dead space previously filled with the appropriate solution). All subsequent additions to or removals from the tubes were made with syringes, except when an inoculum was inserted into an initial tube of a dilution series.

The medium had a final *p*H of 6.95 [0.5% (w/v) sodium bicarbonate] or 6.65 [0.2% (w/v) sodium bicarbonate]. Solid medium for roll tube cultures was prepared by including Oxoid Ionagar No. 2 [0.7% (w/v) final concentration] in the medium. Cultures were incubated at 39 C. Tubes containing broth cultures were tilted approximately 30° to the horizontal and shaken rapidly throughout the incubation period.

Every 1 to 2 days, cultures were equlibrated to room temperature and fed with the 80% hydrogen-20% carbon dioxide gas mixture as follows. A sterile, cotton-plugged, Pasteur pipette was attached to the hydrogen-carbon dioxide gas supply and held horizontal. The capillary of the pipette was broken off to give a wider opening. While gas was flowing through the pipette (approximately 300 ml per min), the needle (1-inch, 21-gauge) of a sterile, greased 10-ml syringe was inserted, and during a 20- to 30-sec period the syringe was flushed out with gas three or four times

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and finally filled. Next, with the stopper of the culture tube held directly beneath the Pasteur pipette and needle, the syringe needle was rapidly withdrawn from the pipette and inserted a short way into the stopper. The gas in the syringe was allowed to come to atmospheric pressure, the volume was noted, and then the needle was thrust completely through the stopper. If methane had been formed by the culture, the gas pressure in the tube was below atmospheric, and gas was taken into the tube from the syringe until the pressure in the tube was equivalent to that of the atmosphere. The volume of gas taken up was noted.

Assay of methane. Gas samples were assayed for methane chromatographically by use of a Perkin-Elmer Vapor Fractometer model 154 B fitted with thermistor detectors and a silica gel column. The column was operated at 22 to 25 C, with N_2 as the carrier gas, at a pressure of 8 psi.

Since the volume of gas taken up by a culture, as measured with the syringe technique described above, was proportional to the amount of methane produced by the culture (Fig. 1), the gas uptake of a culture was usually used as the index of the amount of methane produced. Normally, volumes were not corrected to standard temperature and pressure, because throughout any one experiment room temperature did not vary by more than 2 C, nor atmospheric pressure by more than 3 mm of Hg.

Source and sampling of rumen contents. Samples of rumen contents, for use either as inocula or as a source of clarified rumen fluid for growth medium, were taken from a fistulated, Holstein heifer which was fed alfalfa hay. The animal was fed twice daily, and samples were taken 2 to 4 hr after feeding.

Fractionation of rumen contents into bacterial and protozoal components. One liter of rumen contents was incubated at 37 C for 30 min, in an Erlenmeyer flask. Three layers formed: a top layer consisting mainly of plant debris, a middle layer containing mainly bacteria, and a bottom layer which was greatly enriched with protozoa.

The top layer was sucked off and discarded. The middle layer was sucked off and centrifuged at 650 $\times$ *g* for 10 min to remove protozoa and large plant material. The bacteria were then sedimented at 13,000 $\times$ *g* for 30 min, washed once with anaerobic salts solution [consisting of 16.5% (w/v) each of salt solutions A and B (*see* Table 5), 0.5% (w/v) $NaHCO_3$, and 0.03% (w/v) each of Na_2S and cysteine·HCl, equilibrated with CO_2], recentrifuged, and suspended in water (5%, w/v). Microscopic examination revealed no contamination with protozoa or plant debris.

The bottom layer was transferred to a 100-ml Erlenmeyer flask, and the volume was made up to approximately 100 ml with anaerobic salts solution containing 0.05% (w/v) glucose. After incubation at 37 C for 10 min, when most of the protozoa had settled to the bottom of the flask and contaminating plant debris had risen to the surface, the material above the protozoal layer was sucked off and discarded. The procedure was repeated two more times, with the use of a chromatographic column (20 $\times$ 1 cm) in place of the flask. The protozoa were finally collected by running them off from the bottom of the column. They were sedimented at 500 $\times$ *g* for 2 min, and an 18% (w/v) suspension was made in water. Microscopic examination showed mainly holotrichs and little contamination with bacteria or plant tissue, except for that inside the protozoa.

Preparation of Escherichia coli, mixed rumen bacteria, protozoal, and alfalfa hay extracts. Alfalfa hay was finely chopped in a Waring Blendor, and a 10% (w/v) slurry was made with water.

E. coli (C 6001) was grown aerobically on 2.8% (w/v) *Brucella* broth (Albimi Laboratories, Inc., Flushing, N.Y.) for 13 hr at 37 C. Cells were harvested, and a 7% (wet w/v) suspension was made in water.

The above suspensions, and those of mixed rumen bacteria and of protozoa, were sealed under air and extracted by heating to 120 C for 15 min. Precipitates were centrifuged off at 35,000 $\times$ *g* for 20 min, and the supernatant fractions were tubed anaerobically under 80% H_2-20% CO_2, and then sterilized at 120 C for 15 min.

RESULTS

Isolation and enumeration. Rumen contents were serially diluted directly in agar medium, and roll tube cultures were prepared. After 10 days of incubation, negative pressures and methane were detected in high dilutions. After feeding with gas mixture, cultures were incubated for a further 6 days, and methanogenic colonies were tentatively identified as those which had increased in size during the second incubation period. These included colonies assumed to be *M. ruminantium* and others, usually about equal in number, which

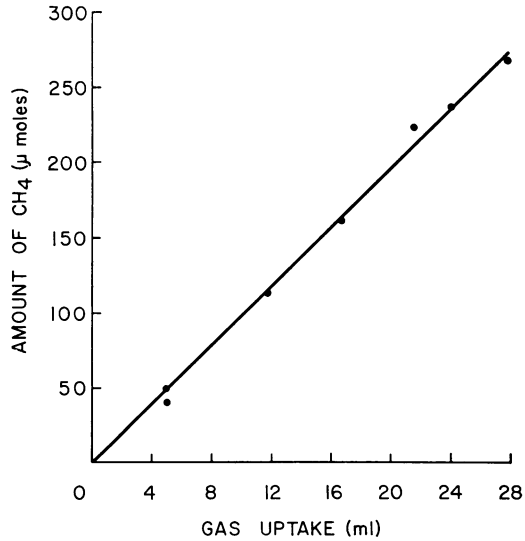

FIG. 1. *Relationship between the total gas uptake of a culture, as measured with the syringe technique, and the total amount of methane produced. Each point represents the mean of duplicate determinations.*

were more convex and more translucent. These were serially diluted into agar roll cultures and were incubated. If a picked colony was methanogenic, the respective dilution series showed negative gas pressures and methane at high dilutions. In this way, a pure methanogenic culture was obtained, which was designated as strain 1.

Criteria of purity were as follows: only one colony type was evident in serial dilutions, colony counts followed the serial dilution, and microscopic examination disclosed only one type of cell morphology.

To determine the approximate numbers of the organism in rumen contents, serial dilutions were inoculated into the rumen fluid agar culture medium supplied with the H_2-CO_2 mixture. Colonies of the organism were identified on the basis of characteristic colony morphology, ability to produce methane when transferred to broth medium, cell morphology, motility, and Gram stain. Concentrations of 2×10^8 and 3×10^8 cells per ml were obtained in replicates on two samples of rumen contents taken on different days from the Holstein heifer.

Four additional pure cultures resembling strain 1 were isolated at different times from the same animal, but only strain 1 was maintained.

Colony and cell morphology. Colonies were always small. They were barely visible to the naked eye after 4 days of incubation. After 15 days, surface colonies reached a maximal diameter of 0.7 to 1.0 mm. They had entire edges, were translucent, colorless to pale yellow, smooth, and convex. Deep colonies were lenticular and 0.5 to 0.7 mm in diameter after 15 days of incubation.

Phase microscopy showed the organism to be a straight to slightly curved rod with rounded ends, 0.7 μ wide and 1.5 to 2.0 μ long, never in chains. Spores were never observed. Cells from a 4-day broth culture, absorbed on the surface of a film of 3% washed agar, are shown in Fig. 2. Cells from broth cultures exhibited weak tumbling motility. Examination with an electron microscope showed that the organism has a single polar flagellum (Fig. 3) ca. 81 A wide and up to 12.5 μ long, with a mean wavelength of 1.8 μ.

Both young and old cells were examined for their Gram reaction, by use of Burke's (3) modification and with *Escherichia coli* and *Streptococcus bovis* as controls. The organism was gram-negative.

Effect of oxygen. To demonstrate the strict anaerobic nature of the organism, the following experiment was performed. Gas uptake was measured for a series of broth cultures (5 ml), and after 4.5 days replicate cultures were injected with 0, 0.02, 0.04, 0.08, 0.12, 0.2, 0.3, and 0.5 ml of sterile water, previously equilibrated with pure oxygen at atmospheric pressure. Incubation was continued with daily measurement of gas uptake. The amounts of O_2 injected were calculated from the solubility of O_2 in water at 20 C and 760 mm of Hg. Figure 4 shows that gas uptake was not affected by 0.38 μmole or less of O_2 [standard temperature and pressure (STP)], but was completely terminated by 0.63 μ mole, corresponding to an oxygen concentration of 0.1% of the gas phase. Initially, the medium contained sodium sulfide and cysteine more than sufficient for complete reaction with the maximal amount of O_2 introduced. After addition of the O_2, the amounts of oxygen in the cultures diminished, and ultimately they were probably zero. If so, the results suggest that relatively short exposures to low oxygen concentrations can kill the organism.

Effect of temperature. Replicate broth cultures (5 ml) were shaken at various temperatures, and the maximal rates of gas uptake were determined. Table 1 shows that gas uptake occurred between 30 and 45 C, with an optimum in the region of 40 C. No growth occurred at 28 or 50 C (28 days of incubation).

Effects of pH. Broths were prepared with *p*H values ranging from 5.1 to 8.0. In one series, the final bicarbonate concentration varied from 0.1 to 1.6% (w/v); in others, the bicarbonate was re-

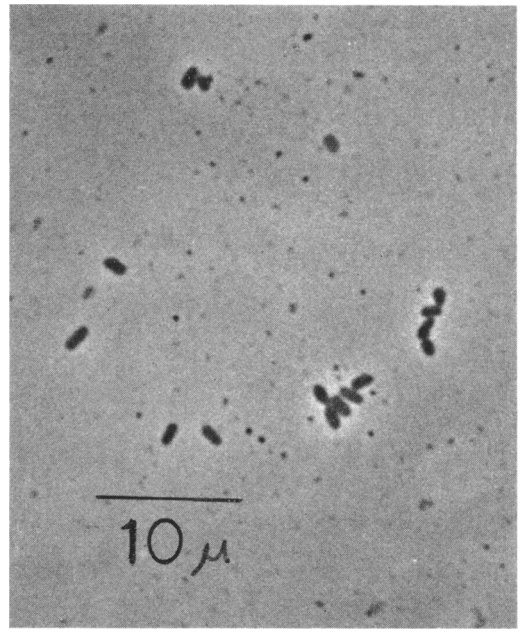

FIG. 2. *Phase-contrast photomicrograph of the methanogenic bacterium isolated from bovine rumen contents,* × *1,900.*

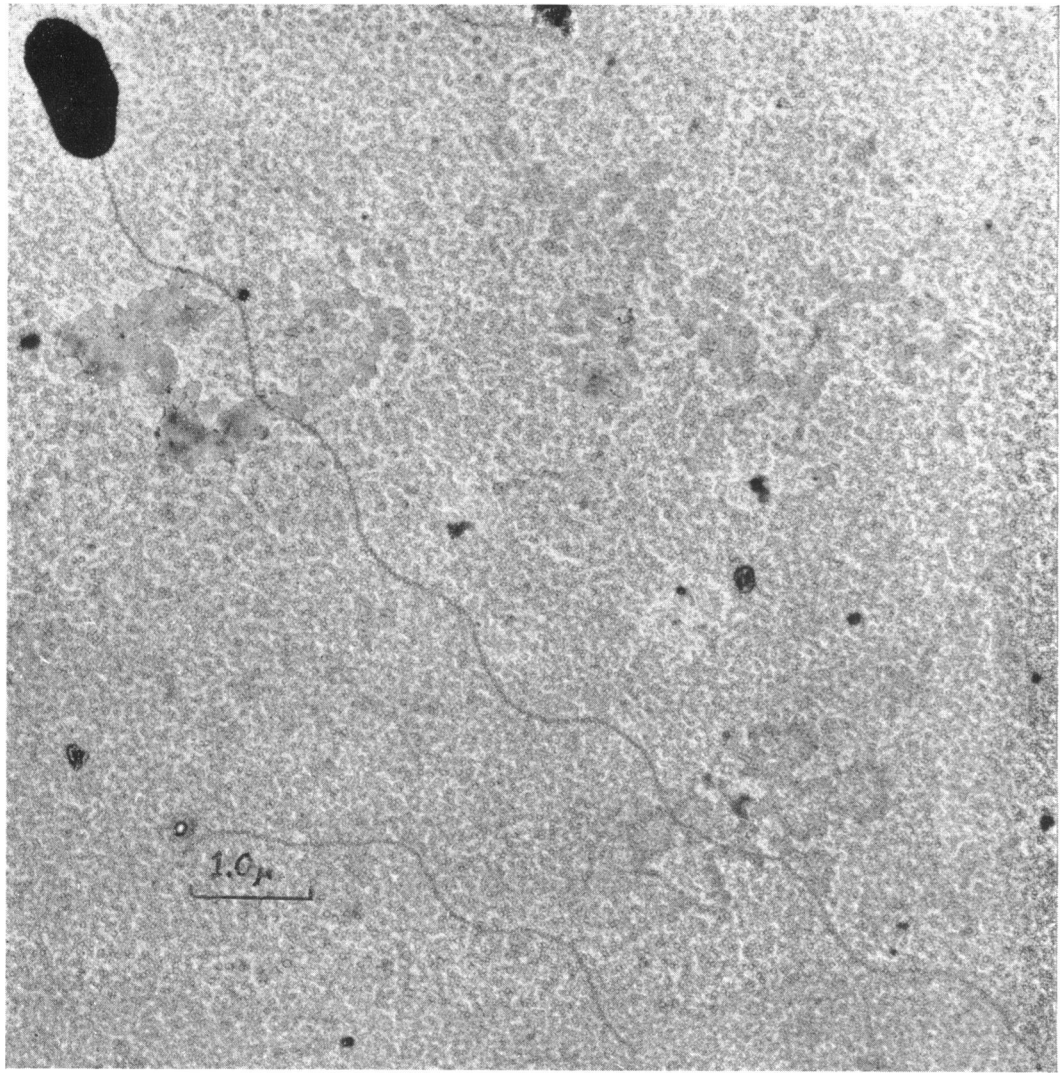

FIG. 3. *Electron micrograph of the methanogenic bacterium isolated from bovine rumen contents, showing the single polar flagellum. Fixed with osmic acid and stained with phosphotungstic acid.* × *15,000.*

placed with acetate, phosphate (KH_2PO_4-NaOH), or tris(hydroxymethyl)aminomethane (Tris-chloride) buffers at final concentrations of 0.1 M. Replicate tubes (5 ml of broth) for each *p*H value were inoculated and incubated at 39 C with shaking; the maximal rate of gas uptake was then measured (Fig. 5). Growth occurred between *p*H 5.9 and 7.7 and was optimal between *p*H 6.1 and 6.9. Tris-chloride buffer caused complete inhibition of gas uptake, and phosphate buffer caused a 45 to 55% inhibition, compared with acetate or bicarbonate buffers at the same *p*H.

Substrate utilization. Duplicate tubes of broth (final volume, 5 ml), containing a final concentration of 1% (w/v) sodium bicarbonate under a CO_2 gas-phase (final *p*H 6.8), were injected with the following substrates, anaerobically prepared: 250 μmoles of glucose or valerate, or 500 μmoles of any one of the remaining compounds listed in Table 2. Acids were previously adjusted to *p*H 6.8 with NaOH. Pyruvate was sterilized by filtration (under CO_2), the alcohols were unsterilized, and the remaining compounds were sterilized at 120 C for 15 min. After inoculation, each tube was injected with 41 μmoles of hydrogen (STP) and incubated for 12 days. The following controls

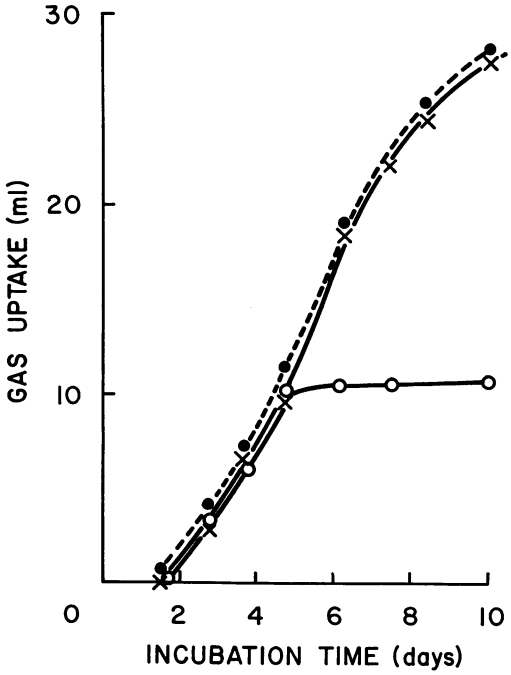

FIG. 4. *Effect of oxygen on gas uptake of cultures. Symbols: ×, no O_2 injected; ●, 0.38 μmoles (STP), or less, of O_2 injected after 4.5 days of incubation; ○, 0.63 μmoles (STP) of O_2 injected after 4.5 days of incubation. Each point represents the mean of duplicate determinations.*

TABLE 1. *Effect of temperature on the maximal rate of gas uptake by the methanogenic organism*

Temp	Maximal rate of gas uptake (mean of duplicates)
C	*ml/day*
28	0
30	1.4
35	2.4
40	5.7
45	0.7
50	0

were used: (i) no added substrate other than 41 μmoles of H_2, (ii) the same but uninoculated, (iii) no added substrate or hydrogen, and (iv) the standard rumen-fluid broth medium prepared under 80% H_2-20% CO_2.

After cultures were equilibrated to room temperature and atmospheric pressure (by inserting a needle attached to a well-lubricated horizontal syringe containing a measured volume of CO_2), the concentration of methane in the gas was measured. The volume of gas in each tube was estimated by adding enough water to occupy the gas space and measuring the increase in weight. The total amounts of methane produced by each culture are presented in Table 2. No methane was formed by uninoculated cultures or in the presence of CO_2 alone; 41.1 μmoles of H_2 gave rise to 10.1 and 10.3 μmoles of methane in replicate cul-

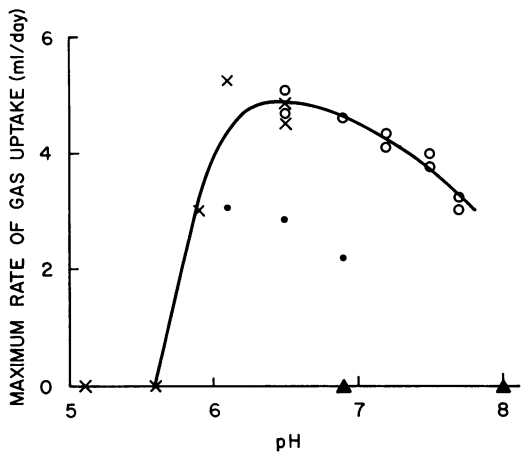

FIG. 5. *Effect of pH on rate of gas uptake by methanogenic culture. Symbols: ×, acetate buffer; ○, bicarbonate buffer; ●, phosphate buffer; ▲, Tris-chloride buffer.*

TABLE 2. *Effect of added substrates on methane production*

Substrates tested	Total amt of CH_4 per culture after 12 days (μmoles at STP)	
	Replicate 1	Replicate 2
1. 100% CO_2 gas phase	0	0
2. 80% H_2 20% CO_2 gas phase	124	123
3. 100% CO_2 gas phase + 41 μmoles (STP) of H_2	10.3	10.1
4. As 3, uninoculated	0	0
5. As 3 + formate	123	130
6. As 3 + acetate	10.2	11.3
7. As 3 + propionate	10.3	9.8
8. As 3 + butyrate	10.5	9.9
9. As 3 + isobutyrate	10.1	9.7
10. As 3 + valerate	10.2	9.8
11. As 3 + isovalerate	10.2	10.3
12. As 3 + caproate	5.0	10.3
13. As 3 + succinate	10.7	10.7
14. As 3 + glucose	10.4	10.3
15. As 3 + pyruvate	10.2	9.9
16. As 3 + methanol	10.5	10.5
17. As 3 + ethyl alcohol	10.1	10.1
18. As 3 + propanol	10.3	10.3
19. As 3 + isopropanol	10.8	10.3
20. As 3 + *n*-butyl alcohol	7.0	0

tures, in approximate agreement with the stoichiometry expected from the equation $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$.

With one exception, all other tests yielded methane approximately equal to or slightly less than the amount expected from the quantity of hydrogen injected. The only other compound metabolized to methane was formate. On the assumption that all the formate was utilized, and with a correction for the amount of methane (average of 10.2 μmoles) arising from the added hydrogen, 500 μmoles of formate gave rise to 112.8 and 119.8 μmoles of methane, respectively, in replicate cultures. This is in satisfactory agreement with the reaction $4HCOOH \rightarrow CH_4 + 3CO_2 + 2H_2O$.

Failure to produce methane from the other compounds tested cannot be ascribed to unsatisfactory growth conditions, because in all but one instance (one of the cultures with *n*-butyl alcohol) methane was produced in amounts approximately equivalent to that expected from the quantity of injected hydrogen. Thus, the organism grew in the presence of all of the substrates tested.

An experiment was performed to determine whether hydrogen could be an intermediate in formate utilization. Formate broth (5 ml per tube) was prepared as before, except that no hydrogen was injected. It was inoculated with 0.05 ml of a formate-grown culture, and the gas in the tube was analyzed for both methane and hydrogen after various times of incubation. The results (Table 3) indicate that hydrogen may be an intermediate in formate utilization. For at least 7 days of the incubation period, hydrogen was more abundant than methane. The hydrogen-methane ratios were similar after both 4 and 7 days of incubation, i.e., 1.4 and 1.5, respectively.

Attempts to demonstrate formation of formate when the organism was grown on 80% H_2-20% CO_2 were inconclusive.

Requirements for rumen fluid. An actively growing broth culture was serially diluted in solid medium containing 30% (v/v) preheated, clarified rumen fluid (*see below*), and also in media in which the rumen fluid had been replaced with: (i) 0.5% (w/v) yeast extract plus 1% (w/v) Casamino Acids (Vitamin Free), (ii) 0.5% (w/v) yeast extract plus 1% (w/v) tryptone, or (iii) tissue culture medium 199 (Flow Laboratories, Inglewood, Calif.). Since the same syringe was used through the dilution procedure, the dilution of the bacteria did not correspond with the dilution of the liquid; i.e., the dilutions were not quantitative.

After 15 days of incubation, each series was examined for total gas uptake and for the highest dilution at which methane could be detected. The results are shown in experiment 1 of Table 4. Medium 199 did not support growth. The small amount of gas taken up is presumably due to the small quantity of rumen fluid introduced with the inoculum. This is supported by the fact that methane was detected only in the second tube. The yeast extract-Casamino Acids mixture supported limited growth, approximately 16% of that supported by the 30% (v/v) preheated, clarified rumen fluid medium. This small amount of growth was not due to carry-over of rumen fluid with the inoculum, because methane was detected in the seventh tube. Replacing Casamino Acids with tryptone slightly diminished the growth.

In a second experiment, the control rumen fluid agar medium was compared with the follow-

TABLE 3. *Amounts of methane aud hydrogen detected in a culture at various times during the fermentation of 500 μmoles of formate*

Incubation time	Total CH_4	Total H_2
days	*μmoles*	*μmoles*
4	2.90	3.94
7	53	80
14	120	4.0

TABLE 4. *Total amounts of gas taken up per dilution series with various media, and the highest dilution tube containing methane*

Standard growth medium with clarified rumen fluid replaced as indicated	Total gas uptake by dilution series	Highest dilution tube containing CH_4
	ml	
Experiment 1		
30% pre-heat-treated clarified rumen fluid	19.6	11th
0.5% yeast extract, 1% Casamino Acids	3.2	7th
0.5% yeast extract, 1% tryptone	2.9	4th
Tissue culture medium 199	2.6	2nd
Experiment 2		
30% pre-heat-treated clarified rumen fluid	79.4	9th
0.5% yeast extract, 1% Casamino Acids	12.3	7th
1% Casamino Acids	4.8	2nd
0.5% yeast extract	14.0	6th
Vitamins, trace minerals	1.7	2nd
Vitamins, trace minerals, 1% Casamino Acids	6.6	2nd
Vitamins, trace minerals, 1% Casamino Acids, 0.5% yeast extract	27.9	8th

ing nutrients, substituted for rumen fluid: (i) 0.5% (w/v) yeast extract plus 1% (w/v) Casamino Acids; (ii) 1% (w/v) Casamino Acids; (iii) 0.5% (w/v) yeast extract; (iv) 2% (v/v) vitamins solution (see Table 5) plus 2% (v/v) trace minerals solution (see Table 5); (v) 2% (v/v) vitamins solution, 2% (v/v) trace minerals solution, plus 1% (w/v) Casamino Acids; and (vi) 2% (v/v) vitamins solution, 2% (v/v) trace minerals solution, 1% (w/v) Casamino Acids, plus 0.5% (w/v) yeast extract. In this experiment, the total capability of the control rumen fluid-agar medium to support methanogenesis was measured by flushing out the accumulated methane. The customary 10-ml syringe full of 80% H_2-20% CO_2 was first used to replenish any vacuum. It was then removed, refilled, and reinjected, and the barrel of the syringe was repeatedly forced partially down and then allowed to be forced back. This equilibrated the spent gas within the tube with that in the syringe, and subsequent withdrawal of the syringe removed about half of the accumulated methane, replacing it with the H_2-CO_2 mixture.

The results (experiment 2 of Table 4) show that yeast extract supports limited methanogenesis, but without rumen fluid does not support the development of colonies from cells in the highest dilutions. The vitamins, trace minerals, and Casamino Acids did not support much growth, but when added to yeast extract caused an approximate twofold increase in growth compared with yeast extract alone. These experiments suggest that the organism requires a growth factor (or factors) in rumen fluid and also (to a lesser extent) in yeast extract. The factor is not one of the common ones, such as amino acid, vitamin, purine, or pyrimidine.

Since Bryant and Nalbandov (Bacteriol. Proc., p. 90, 1966) found that *M. ruminantium* required acetate, 2-methylbutyrate, and an unidentified factor in rumen fluid, liquid cultures were tested, by use of the medium without rumen fluid shown in Table 5. The total amount of gas uptake was never greater than 18% of that obtained with 30% (v/v) preheated, clarified rumen fluid medium. Lack of growth was not due to inhibition, since, in cultures on this medium supplemented with limiting amounts of rumen fluid, the total gas uptake, in excess of the amount taken up with no added rumen fluid, was proportional to the amount of rumen fluid added (Fig. 6). These experiments do not eliminate the possibility that one or more volatile fatty acids are required by the organism, but they do indicate that some other factor(s) in rumen fluid is needed.

It was observed that the organism produced larger colonies when whole, unclarified rumen fluid (UCRF) replaced clarified rumen fluid (CRF) in growth media. This suggested that essential nutrients were provided by the solids (chiefly bacterial cells) of UCRF. To investigate this further, three portions of a sample of rumen fluid were prepared in the following ways before they were used as the 30% (v/v) rumen fluid component in three rumen fluid broth media: (i) rumen fluid with no treatment (UCRF); (ii) clarified by centrifugation (CRF), i.e., freed of cells, before heating to sterilize; and (iii) heated, sealed under air, to 120 C for 15 min before centrifugation (preheated CRF), i.e., heated prior to removal of cells. Replicates (5-ml) of each broth were inoculated and incubated with shaking; the gas uptakes were measured daily. The results (Fig. 7) show that almost three times as much gas was taken up by the UCRF culture as by the CRF

TABLE 5. *Nonrumen fluid medium*[a]

Ingredient	Final concn
	%
Trypticase	2.0 (w/v)
Yeast extract	0.5 (w/v)
Volatile fatty acids mixture[b]	10.0 (v/v)
Trace metals solution[c]	1.0 (v/v)
Hemin	0.0098 (w/v)
Vitamins solution[d]	1.0 (v/v)
Salt solution A[e]	16.5 (v/v)
Salt solution B[f]	16.5 (v/v)
Resazurin	0.0001 (w/v)
$NaHCO_3$	0.2 (w/v)
Cysteine·HCl	0.03 (w/v)
Na_2S	0.03 (w/v)

[a] Gas phase 80% H_2–20% CO^2. Final *p*H, 6.7.

[b] Volatile fatty acids mixture contained the following (mmoles per 100 ml): acetate, 91; propionate, 30; butyrate, 15; isobutyrate; 3; *n*-valerate, 3; isovalerate, 3; DL-α-methyl butyrate, 3; and *n*-caproate, 1.5. The *p*H was adjusted to 6.7.

[c] Trace metals solution contained the following (g per liter): nitrilotriacetic acid, 1.5; $MgSO_4 \cdot 7H_2O$, 6.2; $MnSO_4 \cdot H_2O$, 0.5; NaCl, 1.0; $FeSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.13; $CoCl_2 \cdot 6H_2O$, 0.17; $ZnSO_4 \cdot 7H_2O$, 0.18; $CuSO_4 \cdot 5H_2O$, 0.02; $AlK(SO_4)_2$ $12H_2O$, 0.018; H_3BO_3, 0.01; and $Na_2MoO_4 \cdot 2H_2O$, 0.011.

[d] Vitamins solution contained the following (mg per liter): biotin, 2; folic acid, 2; pyridoxine·HCl, 10; riboflavine, 5; thiamine·HCl, 5; nicotinic acid, 5; D-calcium pantothenate, 5; vitamin B_{12} (crystalline), 0.1; DL-thioctic acid, 5; and *p*-aminobenzoic acid, 5.

[e] Salt solution A contained the following ingredients at the percentage concentrations (w/v) indicated: KH_2PO_4, 0.3; NaCl, 0.6; $(NH_4)_2$ SO_4, 0.3; $MgSO_4 \cdot 7H_2O$, 0.06; and $CaCl_2 \cdot 2H_2O$, 0.06.

[f] Salt solution B contained 0.3% (w/v) K_2HPO_4.

culture. The preheated, clarified rumen fluid showed a total gas uptake only slightly less than that of the unclarified rumen fluid culture. This experiment indicates that heat releases some required nutrient(s) from the solids component (presumably the microorganisms) of rumen contents.

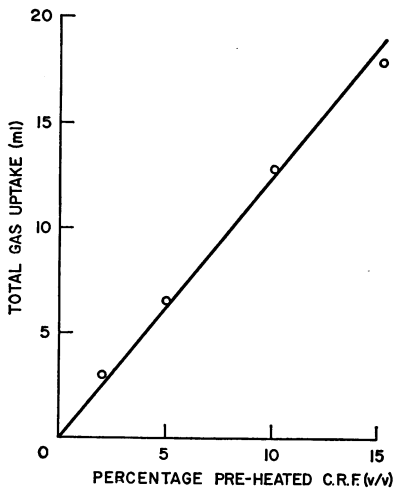

FIG. 6. *Effect of concentration of preheated, clarified rumen fluid on total gas uptake of culture. Preheated, clarified rumen fluid was added to nonrumen fluid medium shown in Table 5. Total gas uptake was corrected for gas uptake with no added rumen fluid.*

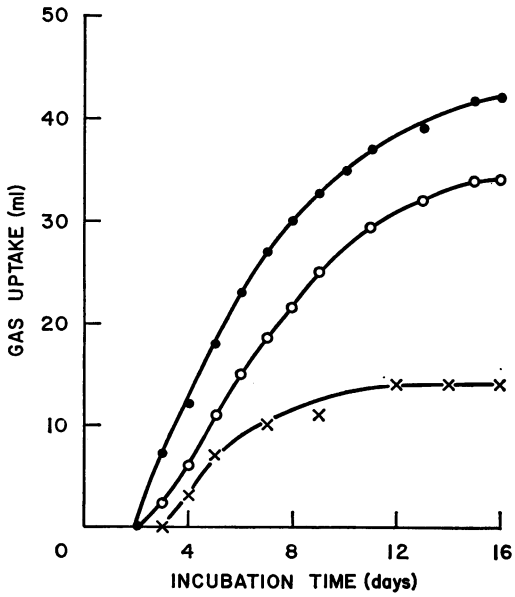

FIG. 7. *Gas uptakes of cultures growing on media containing 30% (v/v) rumen fluid treated in various ways. Symbols: ●, whole, unclarified rumen fluid; ○, pre-heat-treated clarified rumen fluid; ×, clarified rumen fluid.*

Extracts of mixed rumen bacteria, mixed protozoa, and alfalfa hay, respectively, were prepared and added, at two concentrations, to tubes of the nonrumen fluid medium shown in Table 5. These were inoculated and incubated, and gas uptakes were measured daily until no further changes occurred. An extract of *E. coli*, the organism used by Smith and Hungate (9) to aid in the culturing of *M. ruminantium*, was also tested for its ability to stimulate growth. These authors thought that the function of the *E. coli* cells was to remove traces of oxygen in the medium, and also to help develop a low redox potential. An alternative explanation is that *E. coli* supplied some nutrient required by *M. ruminantium*.

The total amounts of gas taken up by the various cultures are shown in Table 6. Stimulation occurred only with the mixed rumen bacterial extract. If the gas uptakes were corrected for that present in the control with no added supplement, the amount of stimulation was approximately proportional to the amount of mixed rumen bacterial extract added. The growth factor appears to be of bacterial origin, but is not present in all bacteria, as shown by the lack of stimulation by the *E. coli* extract.

DISCUSSION

The morphology of the cell and the ability to produce methane place the organism described in the genus *Methanobacterium* Kluyver and van Niel (1936). Of the five species in this genus described in the 7th edition of *Bergey's Manual*, the present strains most closely resemble *M. ruminantium* (11) both in morphology and physiology. There are three important differences: the colony morphology of the organism is distinct from that of *M. ruminantium*; *M. ruminantium* is gram-positive whereas this organism is gram-negative;

TABLE 6. *Effect on gas uptake of various extracts added to nonrumen fluid medium (see Table 5)*

Extract	Vol added	Total gas uptake (mean of duplicates)
	ml	*ml*
None	—	5.5
Alfalfa	0.2	7.7
	0.4	6.7
Escherichia coli	0.2	4.7
	0.4	4.2
Mixed rumen bacteria	0.2	17.0
	0.4	31.9
Rumen protozoa	0.2	5.5
	0.4	6.0

and the present bacterium is motile, whereas *M. ruminantium* is nonmotile. On the basis of these three differences, it is proposed that the isolated strains constitute a new species. The name *Methanobacterium mobilis* sp. n. is proposed, the species name being derived from the motile nature of the organism. Strain 1 is the type strain.

The unknown factor required for growth may be the same as the unknown factor required by *M. ruminantium* (M. P. Bryant and O. Nalbandov, Bacteriol. Proc., p. 90, 1966). Both factors are found in rumen fluid, are heat stable, and occur in ruminal bacteria.

Earlier calculations (11) indicated that *M. ruminantium* could account for all the methane formed in the rumen, if its enzyme system were saturated with hydrogen. But hydrogen concentrations in the rumen are not enzyme-saturating (7). The finding of a second type of methanogenic bacterium, in numbers often equal to the numbers of *M. ruminantium*, assists in explaining how the less than saturating concentrations of hydrogen in rumen contents can account for the actual rate of rumen methanogenesis.

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LITERATURE CITED

1. BEIJER, W. H. 1952. Methane fermentation in the rumen of cattle. Nature **170**:576–577.
2. BRYANT, M. P. 1965. Rumen methanogenic bacteria, p. 411–418. *In* R. W. Dougherty [ed.], Physiology of digestion in the reminant. Butterworths, London.
3. BURKE, V. 1922. Notes on the gram stain with description of a new method. J. Bacteriol. **7**:159–182.
4. HUNGATE, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. **14**:1–49.
5. HUNGATE, R. E. 1960. Symposium: selected topics in microbial ecology. I. Microbial ecology of the rumen. Bacteriol. Rev. **24**:353–364.
6. HUNGATE, R. E. 1966. The rumen and its microbes, p. 26–30. Academic Press, Inc., New York.
7. HUNGATE, R. E. 1967. Hydrogen as an intermediate in the rumen fermentation. Arch. Mikrobiol. **59**:158–164.
8. HUNGATE, R. E., G. D. PHILLIPS, D. P. HUNGATE, AND A. MACGREGOR. 1960. A comparison of the rumen fermentation in European and Zebu cattle. J. Agr. Sci. **54**:196–201.
9. HUNGATE, R. E., W. SMITH, AND R. T. J. Clarke. 1966. Suitability of butyl rubber stoppers for closing anaerobic roll culture tubes. J. Bacteriol. **91**:908–909.
10. OPPERMAN, R. A., W. O. NELSON, AND R. E. BROWN. 1957. *In vitro* studies on methanogenic rumen bacteria. J. Dairy Sci. **40**:779–788.
11. SMITH, P. H., AND R. E. HUNGATE. 1958. Isolation and characterization of *Methanobacterium ruminantium* n. sp. J. Bacteriol. **75**:713–718.