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Features of Rumen and Sewage Sludge Strains of *Eubacterium limosum*, a Methanol- and H_2-CO_2-Utilizing Species

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Eubacterium limosum was isolated as the most numerous methanol-utilizing bacterium in the rumen fluid of sheep fed a diet in which molasses was a major component (mean most probable number of 6.3×10^8 viable cells per ml). It was also isolated from sewage sludge at 9.5×10^4 cells per ml. It was not detected in the rumen fluid of a steer on a normal hay-grain diet, although *Methanosarcina*, as expected, was found at 9.5×10^5 cells per ml. The doubling time of *E. limosum* in basal medium (5% rumen fluid) with methanol as the energy source (37°C) was 7 h. Acetate, cysteine, carbon dioxide, and the vitamins biotin, calcium-D-pantothenate, and lipoic acid were required for growth on a chemically defined methanol medium. Acetate, butyrate, and caproate were produced from methanol. Ammonia or each of several amino acids served as the main nitrogen source. Other energy sources included adonitol, arabitol, erythritol, fructose, glucose, isoleucine, lactate, mannitol, ribose, valine, and H_2-CO_2. The doubling time for growth on H_2-CO_2 (5% rumen fluid, 37°C) was 14 h as compared with 5.2 h for isoleucine and 3.5 h for glucose. The vitamin requirements for growth on H_2-CO_2 were the same as those for methanol; however, acetate was not required for growth on H_2-CO_2, although it was necessary for growth on valine, isoleucine, and lactate and was stimulatory to growth on glucose. Acetate and butyrate were formed during growth on H_2-CO_2, whereas branched-chain fatty acids and ammonia were fermentation products from the amino acids. Heat tolerance was detected, but spores were not observed. The type strain of *E. limosum* (ATCC 8486) and strain L34, which was isolated from the rumen of a young calf, grew on methanol, H_2-CO_2, valine, and isoleucine and showed the same requirements for acetate as the freshly isolated strains.

In a recent study Rowe et al. (29) found that acetate was degraded to CO_2 and methane in the rumen of sheep fed a molasses-based diet; and direct microscopic counts of *Methanosarcina*, the probable acetate-cleaving bacterium, were unusually high (6×10^9/ml). This organism also uses methanol.

In an attempt to repeat these results using the most probable number (MPN) technique (28) and an anaerobic culture medium with methanol as the main energy source, we did not find *Methanosarcina* in similarly fed sheep. However, we found large numbers of a methanol-utilizing anaerobe which produced acetate, butyrate, and caproate from methanol.

As one control, we enumerated methanol-utilizing anaerobes in anaerobic sewage digestor sludge using the MPN technique and expected to find *Methanosarcina* as the most numerous organism. However, we again found a fatty acid-producing organism similar to that found in the sheep rumen. Using the same methods, but culturing rumen contents of a steer fed a usual hay-grain diet, *Methanosarcina* was found to be the dominant methanol-utilizing bacterium, as expected (7, 24).

This paper documents the isolation of the fatty acid-producing bacterium from the sheep rumen and digestor sludge and shows it to belong to the species *Eubacterium limosum*. Some other features not previously reported for this species are also indicated, including some nutritional requirements and the ability to utilize H_2-CO_2 as an energy source.

(A brief report of this work appeared previously [B. R. Sharak Genthner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980 I64, p. 95].)

MATERIALS AND METHODS

Source of strains. The type strain of *E. limosum* (ATCC 8486, VPI 0260) was obtained from the Anaerobe Laboratory, Virginia Polytechnic Institute and

State University (VPI), Blacksburg, Va. Strain L34 was a lactate-fermenting strain isolated from the rumen of a young calf (12) and was later identified as *E. limosum* (personal communication from W. E. C. Moore and L. Holdeman, VPI). Strains RF and S of *E. limosum* were isolated from the rumen of a sheep fed a molasses-based diet and from the effluent of the primary anaerobic sewage sludge digestor in Urbana, Ill., respectively.

The daily diet of the sheep contained 1 kg of a molasses mixture, 0.08 kg of soybean oil meal (40% crude protein) and 0.1 kg of wheat straw. The molasses mixture contained 41.3 kg of blackstrap molasses, 1.36 kg of urea, 0.23 kg of trace mineral salts, 0.34 kg of dicalcium phosphate, 0.23 kg of NaCl, and 1.0 kg of water.

Anaerobic methods and media. The anaerobic techniques employed were those of Hungate (16) as modified by Bryant (9), except for the studies on growth of *E. limosum* with H_2-CO_2 for which we employed culture tubes pressurized to 2 atm (203 kPa) with 80% H_2–20% CO_2 and incubated on a shaker (3, 8).

The basal medium contained 5% rumen fluid, B vitamins, minerals, $NaHCO_3$, cysteine-Na_2S reducing solution, and an 80% N_2–20% CO_2 gas phase having a final pH of 7.2. The medium was prepared as previously described (20), except that 10 mg of Na_2SeO_3 was added to the trace mineral solution, and the vitamin solution contained (per liter) 20 mg each of biotin and folic acid, 10 mg of pyridoxal hydrochloride, 60 mg of lipoic acid, and 50 mg each of riboflavin, thiamine hydrochloride, calcium-D-pantothenate, cyanocobalamin, *p*-aminobenzoic acid, and nicotinic acid. The chemically defined medium was the same as the basal medium, except the rumen fluid was replaced by sodium acetate·$3H_2O$ (0.2 or 0.3%). The anaerobic dilution solution had the same composition as the basal medium except the rumen fluid and vitamin solution were deleted.

Methanol was placed in sterile rubber-stoppered tubes and shaken vigorously after flushing under the N_2-CO_2 gas phase (three times) before adding it to the cooled, sterile medium.

Incubations were at 35°C unless otherwise indicated.

Pure cultures were maintained in stabbed slants (9) of basal medium with 0.2% methanol and 1.0% agar (Difco Laboratories) added. They were incubated until growth occurred and stored at 4°C until needed.

Oxygen sensitivity was determined in methanol stab slants as described previously (10), and by inoculating a glucose (0.3%) medium similar to the basal medium, except that it was prepared aerobically with a potassium phosphate buffer (0.05 M, pH 7.4).

MPN of methanol-utilizing bacteria. Anaerobic digestor sludge was collected for MPN determination in a 1-liter Erlenmeyer flask with a one-way gas valve. Similar samples of rumen fluid were collected 7 h after feeding from two fistulated sheep on the molasses-based diet and from a fistulated steer on a 70% hay–30% grain mixture diet.

Samples were diluted in 10-fold serial dilutions in anaerobic dilution solution. Aliquots (1 ml) of the diluted sample containing between 1 μl and 1 pl of the original rumen fluid were inoculated into triplicate tubes of the basal medium with 0.3% (vol/vol) methanol and 0.1% (wt/vol) sodium acetate·$3H_2O$ (enrichment medium). Growth was estimated daily by optical density. The gas phases of the cultures were then analyzed for methane (20). The MPN was estimated from the number of tubes showing increased growth compared with control cultures without methanol (28).

Isolation of methanol-utilizing *E. limosum*. MPN cultures which contained the least amount of inoculum but indicated growth on methanol were diluted with anaerobic dilution solution, and the appropriate dilution was used to inoculate roll tubes containing the same MPN medium with 2% (wt/vol) agar. After incubation, well-isolated colonies were picked and stabbed (9) into methanol slants similar to those used for the maintenance of strains.

Morphology. Morphology, motility, and culture purity were determined by phase-contrast microscopic examinations of wet mounts from the water of syneresis of methanol slant cultures.

Temperature of growth. Temperature range and optimum temperature for growth (optical density) on methanol was determined for the temperatures from 10 to 49°C at intervals of 2 to 3°C. Triplicate tubes of basal medium with 0.3% (vol/vol) methanol were inoculated and incubated at each temperature.

Heat resistance and spore formation. Heat resistance and spore formation were determined in 10-day- and 1-month-old cultures in the basal medium with 0.3% (vol/vol) methanol and in 1-month-old cultures in the sporulation medium of Duncan and Strong (14). Triplicate tubes of the methanol medium were inoculated with 0.1 ml of the cultures under study. The tubes were heated in a water bath to 70°C for 10 min. A second set of tubes was heated to 80°C for 15 min. Tubes were quickly cooled in an ice bath and incubated. Growth was monitored for 2 weeks. Detection of spores was attempted with the above cultures by examining wet mounts with the phase-contrast microscope as well as smears of the cultures stained with the Schaeffer and Fulton stain (23).

Energy sources. Utilization of energy sources was determined in the basal medium containing a 20 mM concentration of the compound to be tested and appropriate controls were included.

Growth on 80% H_2–20% CO_2. The basal medium was used for growth on H_2-CO_2, except that sodium bicarbonate was doubled. Samples (5 ml) were placed in serum-capped tubes (18 by 150 mm), autoclaved, and stored under a 60% N_2–40% CO_2 gas phase at atmospheric pressure. Tubes were inoculated via syringe with 0.1 ml of culture. After inoculation the gas phase was changed to 80% H_2–20% CO_2 at 2 atm of pressure (3). Cultures were maintained on H_2-CO_2 by weekly transfers of 0.1 ml of culture and were repressurized daily.

Nutritional requirements. The requirement for acetate during growth on a number of substrates was determined by comparing growth in the basal medium supplemented with the energy source with and without rumen fluid and with sodium acetate·$3H_2O$ (0.3%) replacing the rumen fluid (chemically defined medium). Acetate requirements for growth on H_2-CO_2 were investigated in an H_2-CO_2 medium lacking rumen

fluid with inocula which had been washed three times in anaerobic dilution solution.

To determine the effect of CO_2 on methanol utilization, the basal medium was used with 0.3% (vol/vol) methanol, except that the bicarbonate buffer was replaced by a potassium phosphate buffer (0.05 M), and the gas phase contained 100% N_2. The final pH was 7.4.

Vitamin requirements for growth on methanol were investigated by deleting single vitamins, or both folic acid and *p*-aminobenzoic acid, from the chemically defined 0.2% methanol medium containing 0.2% sodium acetate·$3H_2O$. Vitamins which affected growth during the single deletion experiment were added to the chemically defined methanol (or H_2-CO_2) medium in various combinations at the concentrations described for the basal medium with no other vitamins present. Cultures were serially passed through these minimal vitamin media five times.

Nitrogen sources were studied by using a chemically defined methanol medium containing 1.0 mM NH_4Cl supplemented with 2.0 mM of the nitrogen compound to be studied. All nitrogen compounds were added to the medium before autoclaving, except urea, biuret, and allantoin, which were filter sterilized before being added to sterile medium.

Casein, gelatin, and esculin hydrolysis were investigated by the procedures described in the *Anaerobe Laboratory Manual* (VPI [14a]), except that the media used contained the vitamin and mineral solutions described for the basal medium and were buffered with sodium bicarbonate and an 80% N_2–20% CO_2 gas phase to a pH of 7.2.

Analytical methods. Growth was determined by measuring the optical density at 600 nm in (13- by 100-mm) tubes with a Bausch & Lomb Spectronic 70, except for growth on H_2-CO_2 for which 18- by 150-mm tubes were used. Generation times were calculated and gas analysis was done as previously described (20).

Volatile fatty acids and other organic acids in the culture supernatant were determined as described by Salanitro and Muirhead (30), or volatile fatty acids were determined by acidification to free fatty acids in which 0.2 ml of 30% phosphoric acid was added to 1.8 ml of culture supernatant in acid-cleaned 13- by 100-mm screw cap tubes. A 0.2-ml amount of the acidified samples was transferred to conical chromatograph vials and sealed with a serum cap. Free fatty acids and methanol were analyzed by using an SP-1000 column (10% SP-1000, 1% H_3PO_4; 100/120 Chromosorb W, 6 ft; Supelco, Inc., Bellefonte, Pa.) at 150 and 80°C, respectively, in a Hewlett Packard 5830A Gas Chromatograph with a hydrogen-flame detector and 18850A GC terminal. Ammonia was determined by the method of Chaney and Marbach (13).

To determine the percentage of guanine plus cytosine of the deoxyribonucleic acid, a 3% inoculum was added to a serum-stoppered bottle containing 2 liters of basal medium with 0.3% (vol/vol) methanol. Cells were harvested during logarithmic growth by centrifuging at 10,400 × *g* for 20 min. Deoxyribonucleic acid was isolated and purified by the method of Marmur (19). The guanine-plus-cytosine content was determined by the buoyant density method of Schildkraut et al. (31).

RESULTS

Numbers and kinds of methanol-utilizing bacteria. Direct microscopic observations of rumen fluid from the two sheep fed the molasses diet showed a very large proportion of relatively large, nonmotile, gram-positive bacteria, many of which showed club shapes and branching. No bacteria similar to *Methanosarcina* were evident.

The MPN methanol cultures inoculated with rumen fluid from these sheep showed a predominance of similar bacteria and gave an MPN of 1.1×10^9 and 1.5×10^8 bacteria per ml of rumen fluid. Methane, even in tubes containing large inocula, was never found in more than trace amounts, whereas qualitative analysis of the culture supernatant indicated that large amounts of acetate and butyrate and very little propionate were produced from methanol. Maximum growth occurred within 10 days in all cultures showing growth, including those cultures which received the most dilute inoculum.

The MPN methanol cultures of sewage sludge gave similar results, except that numbers were lower (9.5×10^4 bacteria per ml), and some of the cultures receiving more dilute inocula did not reach maximum growth until 11 days of incubation. In addition, methane analysis revealed that from 5 to 9% of the theoretical methane expected from methanol was produced ($4CH_4OH \rightleftharpoons 3CH_4 + CO_2 + 2H_2O$).

In contrast, observations on the MPN methanol cultures inoculated with rumen fluid from a steer receiving a more normal hay-grain diet suggested, as expected (7, 24), that *Methanosarcina* was the dominant methanol-degrading bacterium. Growth did not reach maximum until after 28 days of incubation. A granular sediment which was difficult to disperse was formed in these cultures as compared to an even turbidity produced in the cultures from molasses-fed sheep or sewage sludge. Microscopic examination and methane production, which was approximately equal to the methane expected if all of the methanol was converted to methane and CO_2 (74 μmol of methanol converted to 56 μmol of methane per ml of culture), indicated that an organism similar to or identical with *Methanosarcina barkeri* was dominant (9.5×10^5 bacteria per ml). Since these MPN cultures from the steer rumen served as a control to show that the methanol medium was satisfactory for enrichment and enumeration of *Methanosarcina* from an ecosystem, if present in greater numbers than other methanol-utilizing anaerobes, no further work was done with them.

Isolation and some features of the methanol-degrading, fatty acid-producing bac-

teria. MPN methanol cultures inoculated with the smallest amount of sheep rumen fluid and sewage sludge were diluted and used to inoculate methanol agar roll tubes. After 6 days, well-isolated colonies were evident in tubes inoculated with the highest dilution. Most colonies were observed to be methanol-utilizing rods. Surface colonies were off-white, opaque, entire, convex, and 1 to 2 mm in diameter. Deep colonies were lenticular. Colonies were stabbed into methanol agar slants, and good growth was obtained after 2 days. Microscopic examination revealed nonmotile, regularly shaped, cylindrical cells with rounded ends (average size, 1.2 μm by 3.3 μm) that occurred mainly as single cells. After 4 to 5 days of incubation cells became more pleomorphic, forming elongated cells that often showed knobs, usually centrally located, and branching forms. The colonial and cellular morphologies of isolates from the sheep rumen or sewage sludge were identical, except that the rumen isolates tended to become more pleomorphic than the sewage isolates. All isolates were gram positive. One culture from sheep (strain RF) and one from sludge (strain S) were maintained for further study and were shown to be identical in all other characteristics.

Both strains were anaerobic. They failed to grow above the line of oxidation of resazurin in methanol agar and did not grow in aerobic glucose medium.

The optimum temperature for most rapid growth in methanol medium was 39°C, and the temperature range was 17 to 47°C.

Heat resistance and spore formation. Some methanol-grown cultures survived exposure to 70°C for 10 min, but none survived 80°C for 15 min. All cultures grown in the Duncan-Strong sporulation medium survived 70°C for 10 min, but only one-third of them survived 80°C for 15 min.

In no case were spores observed with the phase contrast microscope or in smears stained to detect spores.

Energy sources. Adonitol, arabitol, erythritol, fructose, glucose, mannitol, and ribose were excellent energy sources, whereas isoleucine, valine, lactate, and methanol supported growth fairly well. Other compounds tested, excluding H_2-CO_2, supported little or no growth. These included pyruvate, xylose, glycerate, glycerol, formate, and sorbitol, which supported only slight growth, and arabinose, galactose, galacturonic acid, gluconate, mannose, rhamnose, cellobiose, lactose, maltose, melebiose, sucrose, trehalose, melezitose, raffinose, dextrin, pectin, starch, dulcitol, salicin, xylitol, xylose, ethanol, propanol, butanol, inositol, acetate, fumarate, malate, succinate, other amino acids, adenine, thymine, cytosine, uracil, allantoin, urea, and methylamine, which supported no growth. Esculin did not support growth, but it was hydrolyzed. Neither gelatin nor casein was hydrolyzed. *E. limosum* ATCC 8486 and strain L34 also grew on methanol, valine, isoleucine, and lactate. Neither strain ATCC 8486 nor L34 grew on esculin, but both hydrolyzed it.

All four strains of *E. limosum* grew with H_2-CO_2 as the energy source, although strains RF and S grew much better than did strains ATCC 8486 and L34. After inoculating the H_2-CO_2 medium with a methanol-grown culture of strains RF and S, 270 h of incubation elapsed before growth was evident; however, growth was relatively good once it was evident. Subsequent transfers into fresh H_2-CO_2 medium resulted in more rapid initiation of growth (Fig. 1). Cultures of strains ATCC 8486 and L34 reached optical densities of approximately 0.2 in this medium. When growth stopped due to lack of H_2, it could be restarted by adding more H_2-CO_2 to the culture. Growth did not occur when H_2-CO_2 was replaced with 80% N_2–20% CO_2.

The generation time of strain RF at 37°C in basal medium (5% rumen fluid) for H_2-CO_2 was 14 h as compared with 7 h for methanol, 5.2 h for isoleucine, and 3.5 h for glucose.

Acetate, butyrate, and caproate were the products of methanol fermentation for both strain RF and strain S. The fermentation balance for growth in chemically defined methanol medium containing 0.2% methanol and 0.2% sodium acetate·$3H_2O$ showed a ratio of acetate/butyrate/caproate of 10.35:8.92:0.13 mmol/liter (1:1:0.1). H_2, propionate, lactate, and ethanol

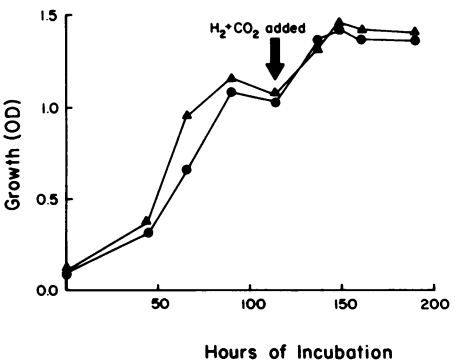

FIG. 1. *Growth of E. limosum (strains RF and S) on 80% H_2–20% CO_2 medium (basal containing 5% rumen fluid). Arrow indicates repressurizing cultures to 2 atm of 80% H_2–20% CO_2. Each point is the mean of triplicate tubes. Symbols: ●, strain RF; ▲, strain S.*

were not detected. Approximately 1 mol of CO_2 was utilized for every 5 mol of methanol (actual values, 49.38 mmol of methanol and 11.88 mmol of CO_2 per liter). Recovery of carbon was 93.3%, and recovery of hydrogen (calculated by the method of Barker [4]) was 89.6%. The above values represent the mean of triplicate cultures after 150 h of incubation and corrected for concentrations in uninoculated medium. When rumen fluid was a component of the methanol medium, propionate levels decreased, and valerate appeared as a product of fermentation.

In the chemically defined H_2-CO_2 medium, 87.8 mM acetate and 2.75 mM butyrate were detected when strain RF was grown to maximum. In chemically defined isoleucine medium (0.2% isoleucine, 0.2% sodium acetate·$3H_2O$) 3.7 mM acetate was taken up, and 15.3 mM isoleucine was utilized. Fermentation products detected were butyrate, 2.0 mM; 2-methyl-*n*-butyrate, 12.1 mM; carbon dioxide, 17.8 mM; and ammonia, 16.9 mM. Acetate, butyrate, caproate, CO_2, and a small amount of H_2 were detected as products of glucose fermentation. Propionate, succinate, lactate, and ethanol were not detected as fermentation products.

Nutritional requirements. Strain S did not grow in methanol medium lacking rumen fluid, but the addition of sodium acetate (chemically defined medium) restored growth (Fig. 2). Sodium propionate could not replace the rumen fluid requirements. Growth in the chemically defined medium was slow as compared with growth in the rumen fluid-based medium, but higher levels of acetate shortened the time required for good growth. The generation time at all levels of acetate was approximately 9 h. Acetate was also required for growth on lactate, valine, and isoleucine and was stimulatory to growth on glucose. Similar results were obtained with strain RF, strain L34, and *E. limosum* ATCC 8486.

In contrast to methanol, cultures grown on H_2-CO_2 could be maintained without rumen fluid or sodium acetate. The doubling time did not increase in the absence of rumen fluid, but the lag phase lengthened.

Carbon dioxide was apparently required for utilization of methanol as growth did not occur in the methanol medium containing 100% N_2. Addition of sodium formate (0.2%) to this medium restored growth (data not shown).

Neither strain RF nor strain S grew in a chemically defined methanol medium when either biotin or calcium-D-pantothenate was deleted, whereas the single deletion of lipoic acid resulted in a decreased growth rate (data not shown). The minimal vitamin requirements for growth on methanol were biotin, calcium-D-pantothenate and lipoic acid as growth was normal for five passages through the methanol medium containing these three vitamins. In the methanol medium containing only biotin and calcium-D-pantothenate the growth rate decreased and the lag phase increased with each subsequent transfer. After the third transfer growth was not evident until 200 h. Results were the same for the chemically defined H_2-CO_2 medium.

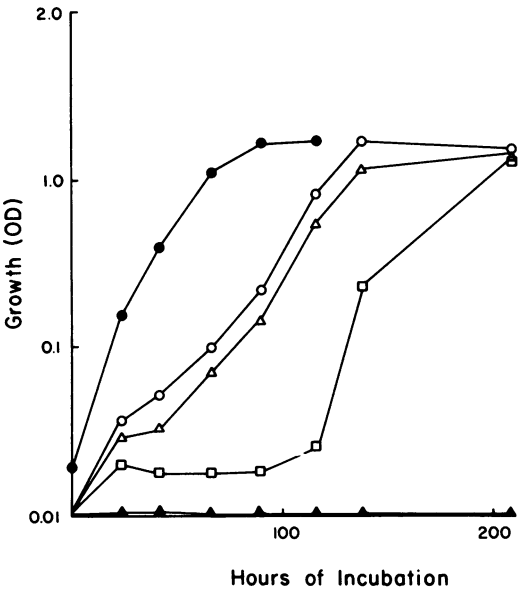

FIG. 2. *Rumen fluid requirement by E. limosum (strain S) for growth in methanol (0.3%) medium replaced by sodium acetate resulting in a chemically defined methanol medium. Each point is the mean of triplicate tubes. Symbols: ●, 5% rumen fluid; ○, 0.3% sodium acetate; △, 0.1% sodium acetate; □, 0.01% sodium acetate; ▲, no additions.*

Cysteine was essential in the methanol medium, as growth did not occur when it was deleted.

The growth response of *E. limosum* to NH_4Cl as nitrogen source in chemically defined methanol medium (Fig. 3) showed that strain S reached higher maximum growth than strain RF, perhaps due to the difference in cellular morphology noted above. Maximum growth was observed at 5.0 mM NH_4Cl.

Only a few other nitrogen sources supported the growth of *E. limosum* (strain RF) as well as NH_4Cl. These included Casamino Acids, cysteine, casitone, isoleucine, and valine. Arginine, aspartic acid, glutamic acid, tyrosine, and leucine were also relatively good nitrogen sources. Histidine, serine, methionine, and phenylalanine were poor nitrogen sources, and tryptophan, threonine, adenine, alanine, allantoin, urea, cytosine, glycine, lysine, proline, thymine, and urea

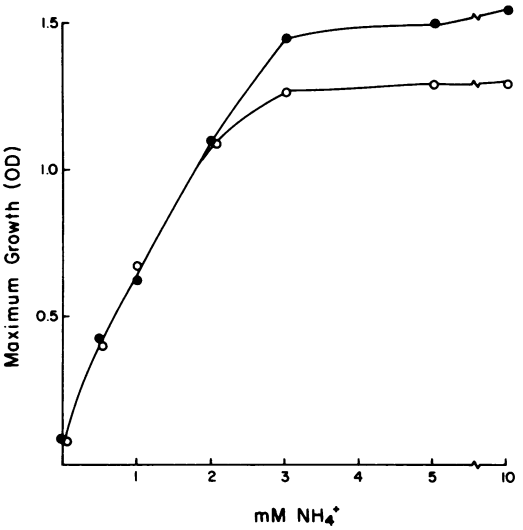

FIG. 3. *Growth of E. limosum (strains RF and S) in chemically defined methanol medium (0.3% methanol, 0.3% sodium acetate·3H₂O) containing ammonium chloride as the sole nitrogen source. Each point is the mean maximum value of triplicate tubes. Symbols: ●, strain S; ○, strain RF.*

were not effective as sole nitrogen sources. Results obtained with strain S were similar.

Guanine-plus-cytosine determination. The buoyant density of the deoxyribonucleic acid isolated from strain RF growth on methanol was 1.708 g cm^{-3}, and the calculated guanine-plus-cytosine content was 49.0 mol%.

DISCUSSION

Results obtained in the present study indicate that strain RF and strain S, obtained from the rumen and anaerobic sewage sludge, respectively, are typical members of the species *E. limosum* (*Butyribacterium rettgeri*) (22). Features that our strains have in common with *E. limosum* include morphology, temperature range, energy sources utilized, fermentation products produced, (5, 15, 22) and percentage of guanine plus cytosine (33a). In addition, they share some relatively unique characteristics such as a requirement for lipoate (17) and the ability to utilize CO_2 in the formation of acetate (6, 25), particularly in the total synthesis of acetate from CO_2 (33).

However, *E. limosum* was not generally known to utilize the one-carbon compounds, methanol, or H_2-CO_2, as energy sources. A strain was first shown to produce acetate from methanol and H_2-CO_2 by Hamlett and Baylock (Bacteriol. Proc., p. 149, 1969), but a full paper was not published by these workers. The present work shows that, in addition to strains RF and S, the type strain of the species (ATCC 8486) and strain L34, isolated from the rumen of a young calf (12), also utilize these energy sources. Thus, it is evident that the ability to utilize methanol and H_2-CO_2 is a general feature of *E. limosum*. Fermentation of isoleucine and valine, which has not been reported previously for *E. limosum*, is also characteristic of all four strains and supports our identification.

The ability to use a variety of energy sources, including sugars, polyhydroxyl alcohols, isoleucine, valine, and lactate, in addition to methanol and H_2-CO_2 indicates the versatility of this organism. Although growth on formate was poor, increasing the level of formate in the medium increased growth. It is possible that cells adapted to H_2-CO_2 would be better able to ferment formate than methanol-adapted cells.

The minimal nutrient requirements for organic compounds during growth of *E. limosum* were also determined. Strains RF and S grew well under lithotrophic conditions with H_2-CO_2 as the energy source, and cysteine, biotin, pantothenate, and lipoate as the only added organic compounds. Acetate was required for fermentation of lactate (5), methanol, valine, and isoleucine. Interestingly, acetate was not required for growth on H_2-CO_2. CO_2 was also required for growth on methanol, but formate could replace the CO_2 requirement in methanol medium. Lipoate was previously known to be required during growth of *E. limosum* on lactate, but not for growth on glucose (17). The present work shows that it was also necessary for growth on methanol and H_2-CO_2.

Our work indicated that *E. limosum* was very versatile in utilization of nitrogen sources, except for a cysteine requirement. Thus, peptides, ammonia, and single amino acids could serve as main nitrogen sources. The ability of *E. limosum* to use valine and isoleucine as both nitrogen and energy sources is also interesting.

The ability to form longer chain fatty acids, i.e., butyrate and caproate, from C_1 compounds seems unique to *E. limosum*. A few anaerobes form acetate from CO_2. *Clostridium thermoaceticum* (32) and *Clostridium formicoaceticum* (1) are able to synthesize acetate from CO_2 in the presence of certain carbohydrates. However, neither organism forms or utilizes molecular hydrogen. *Acetobacterium woodii* forms acetate from H_2-CO_2, but does not utilize methanol and cannot form fatty acids longer than acetate (2). *Clostridium aceticum* also forms acetate from H_2-CO_2 (35).

Zeikus et al. (38) recently described a new genus and species of methanol-utilizing anaerobe and named it *"Butyribacterium methylotrophicum."* However, it was not directly compared to

strains of *E. limosum*, and the data presented strongly suggest that it is a strain of *E. limosum*, especially in view of the present work on one-carbon metabolism in *E. limosum*. Sporelike structures were found in *B. methylotrophicum* (38), and recent work by Tanner et al. (33a) indicates that *E. limosum* is closely related to certain species of *Clostridium*.

Earlier work indicates that *Methanosarcina* is the main bacterium involved in degradation of methanol in the rumen (7, 24). This was confirmed in the present study by using MPN cultures of rumen contents from a steer fed a normal diet. Rowe et al. (29) found large numbers of *Methanosarcina* in the rumen of sheep fed a molasses diet similar to that used in the present experiments. They also found that a large amount of acetate was being degraded in the rumen of their sheep. They concluded that *Methanosarcina* was probably responsible for the acetate degradation in these sheep, and they suggested that this was possible because of the exceedingly long retention time of rumen contents in sheep fed a similar diet.

In view of these results, finding *E. limosum* rather than *Methanosarcina* as the predominant methanol-utilizing organism in our molasses-fed sheep was surprising. However, characteristics of the rumen fluid in these sheep support the presence of high numbers of a methanol-degrading fatty acid-producing organism. First, methanol was readily detected in the rumen during the time molasses was being consumed (C. L. Davis, unpublished data). Methanol has previously been found in the rumen (3 to 60 μg/ml) of animals on hay-grain diets (34). The source of rumen methanol is apparently pectin. Molasses contains 2 to 7% pectin, and 0.5% methoxyl uronic acids have been detected (21). Organisms that contain pectin methyl esterase (EC 3.1.1.11) and ferment polygalacturonic acid derived from pectin are numerous in the rumen (11, 37). Second, large amounts of acetate, butyrate (40 mol%), and *n*-caproate were detected; only very low levels of propionate were present (C. L. Davis, unpublished data). It is interesting to note that high levels of butyrate (40 to 50 mol%) are commonly found in the rumen of animals fed a molasses-based diet (26). Finally, after the molasses had been consumed the level of isovalerate or 2-methyl-butyrate also became unusually high (C. L. Davis, unpublished data).

The above data, in addition to the very high numbers of *E. limosum* found, suggest that other compounds in addition to methanol were being fermented by *E. limosum*. These could include sugars, lactate, and H_2-CO_2, derived from the molasses, and branched-chain amino acids derived from the soybean oil meal, or possibly from the lysis of other rumen organisms.

Further experiments are warranted to determine whether *E. limosum* is an important catabolizer of sugars and lactate, as well as methanol and H_2-CO_2, in the rumen under certain conditions. It is also important to determine whether conditions exist in which *E. limosum* competes with methanogens for the H_2-CO_2 produced by fermentative bacteria and in doing so makes fatty acids, rather than methane, a major product of H_2-CO_2 catabolism in the rumen.

Rode et al. (27) showed that cultures of *Lachnospira multiparus* (11) maintained on 0.2% (wt/vol) pectin medium with daily 50% transfers (2-day retention time) for about 2 weeks produced as final products 4.24 mM methanol, 9.1 mM acetate, no butyrate, 0.9 mM ethanol, 0.8 mM lactate, 0.4 mM formate, 29 mM CO_2, and 1.43 mM H_2. When combined with *E. limosum* (which catabolizes methanol, lactate, and H_2-CO_2 but not pectin, polygalacturonic acid, uronic acids, or ethanol), no methanol, 11.9 mM acetate, 1.4 mM butyrate, no ethanol, lactate, or formate, 31 mM CO_2, and 0.18 mM H_2 (0.6 μM soluble H_2) were the final products. The fact that ethanol was not produced in the coculture indicates that *E. limosum* keeps the concentration of H_2 low enough in interspecies H_2 transfer (37) so that ethanol is then not a product of *Lachnospira*. This suggests that *E. limosum* has the potential to utilize H_2 and maintain it in very low concentrations under some rumen conditions. Further model experiments are warranted to attempt to document the environmental conditions under which *E. limosum* would be favored over methanogens in use of H_2-CO_2 or methanol (or both) in anaerobic ecosystems.

The experiments of Lettinga et al. (18) indicate than an organism such as *E. limosum* may be active under some conditions during the anaerobic treatment of methanolic wastes. Large amounts of acetate and butyrate and smaller amounts of other volatile acids such as isobutyrate and branch-chained valerate were produced.

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