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Cellulose Fermentation by a Rumen Anaerobic Fungus in Both the Absence and the Presence of Rumen Methanogens

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The fermentation of cellulose by an ovine rumen anaerobic fungus in the absence and presence of rumen methanogens is described. In the monoculture, moles of product as a percentage of the moles of hexose fermented were: acetate, 72.7; carbon dioxide, 37.6; formate, 83.1; ethanol, 37.4; lactate, 67.0; and hydrogen, 35.3. In the coculture, acetate was the major product (134.7%), and carbon dioxide increased (88.7%). Lactate and ethanol production decreased to 2.9 and 19%, respectively, little formate was detected (1%), and hydrogen did not accumulate. Substantial amounts of methane were produced in the coculture (58.7%). Studies with [2-^{14}C]acetate indicated that acetate was not a precursor of methane. The demonstration of cellulose fermentation by a fungus extends the range of known rumen organisms capable of participating in cellulose digestion and provides further support for a role of anaerobic fungi in rumen fiber digestion. The effect of the methanogens on the pattern of fermentation is interpreted as a shift in flow of electrons away from electron sink products to methane via hydrogen. The study provides a new example of intermicrobial hydrogen transfer and the first demonstration of hydrogen formation by a fungus.

Cellulose digestion is a common property of many fungi, but until recently virtually all fungi were found to require oxygen for growth. As a result a role for fungi in rumen fermentation had not been considered previously. However, obligately anaerobic fungi were recently discovered in the rumen (18), and the finding that large populations were present attached to fibrous plant fragments (3) led to the suggestion that there should be re-examination of accepted concepts of fiber digestion. The subsequent demonstration of cellulose digestion by rumen anaerobic fungi (4, 5, 19) was additional evidence for a role in fiber digestion. However, cellulose fermentation has not been described and forms part of the present investigation. Our discovery that rumen methanogens formed a stable coculture with the rumen fungus led to the investigation of their effects on the fermentation of cellulose.

Recent studies involving intermicrobial H_2 transfer to methanogens (8, 16) have indicated that in complex ecosystems, such as the rumen, microbial interactions may have profound effects on the pattern of fermentation products. Coculture of H_2-utilizing methanogens with rumen carbohydrate-fermenting bacteria produced a shift in the flow of electrons generated in glycolysis, away from the formation of electron sink products (lactate and succinate) to methane via hydrogen. In each case this was accompanied by an increase in acetate. The pattern of fermentation products in the cocultures could not account for the regeneration of oxidized pyridine nucleotide required in glycolysis. It has been assumed, therefore, that the carbohydrate fermenters were able to produce H_2 directly from reduced pyridine nucleotide. However, calculations have shown that the reaction reduced nicotinamide adenine dinucleotide (NADH) + $H^+ \rightarrow$ oxidized nicotinamide adenine dinucleotide (NAD^+) + H_2 is thermodynamically unfavorable at partial pressures of H_2 above 10^{-3} atm (0.1 kPa) (26). Thus only under conditions of low partial pressures of H_2 maintained by the presence of methanogens could the production of H_2 occur from reduced pyridine nucleotide. The coculture studies (8, 16) provided useful insight into the intermicrobial H_2 transfer mechanisms that could occur in the rumen where the steady-state hydrogen concentration is low (12), and the results were consistent with Hungate's hypothesis (11) that methanogenesis may provide an alternative electron sink to the production of reduced products from pyruvate.

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The present results show that the rumen anaerobic fungi can be grouped physiologically with the few species of bacteria capable of participating in the primary fermentation of plant fiber in the rumen. Coculture of the fungus with methanogens resulted in an enhanced cellulose fermentation rate and an elevated acetate production, an indication of the importance of intermicrobial H_2 transfer in the rumen fermentation.

MATERIALS AND METHODS

Isolation procedures. Sheep rumen contents were strained through cheesecloth, and a 5% inoculum was transferred into 7 ml of standard medium (3) containing an antibiotic mixture (benzylpenicillin, 10^3 IU/ml; streptomycin sulfate, 10^2 IU/ml). The culture was incubated at 38°C, and growth of the fungus was monitored by direct examination with a dissecting microscope (Olympus). After three subcultures a small inoculum from an actively growing fungal culture was transferred to anaerobic broth (standard medium without agar). A single thallus was then removed from the culture with a 1-ml syringe with a 1.5-in (ca. 3.8 cm) 22-gauge needle and washed by transferring it four times through successive tubes to remove contaminating zoospores. The thallus was then transferred to standard medium and incubated. Microscopic examination of the resultant culture indicated a single fungus, based on the morphology of the thallus and zoospores. However, further examination by light and ultraviolet epifluorescence microscopy revealed that the fungus was in coculture with methanogenic bacteria. The coculture was maintained and kept to inoculate experimental media. Pure culture of the fungus, free from methanogens, was obtained by the treatment of the coculture with chloramphenicol (30 μg/ml) and subsequent passage through three subcultures. The fungus was deposited in the culture collection at the Department of Scientific and Industrial Research, Palmerston North, New Zealand and designated as strain PN1. Taxonomy of the organism has yet to be completed.

Culture conditions. Cultures were grown anaerobically at 38°C in tubes of medium prepared by the method of Hungate (13). The gas phase was 70% N_2 and 30% CO_2.

Salt solutions (11) were used in medium preparation. Solution A contained (wt/vol): KH_2PO_4, 0.3%; NaCl, 0.6%; $(NH_4)_2SO_4$, 0.3%; CaCl, 0.03%; and $MgSO_4$, 0.03%. Solution B contained 0.3% (wt/vol) K_2HPO_4.

The medium used was modified from Bauchop (3) and had the following composition: solution A, 165 ml; solution B, 165 ml; cell-free ovine rumen fluid, 170 ml; distilled water, 500 ml; $NaHCO_3$, 5 g; yeast extract (BBL Microbiology Systems, Cockeysville, Md.), 1 g; peptone (Difco Laboratories, Detroit, Mich.), 1 g; cysteine-HCl, 0.2 g; $Na_2S \cdot 9H_2O$, 0.1 g; and resazurin, 0.001 g. Medium (12 ml) was dispensed anaerobically into anaerobic culture tubes (140 by 16 mm; A. H. Thomas) each containing 100 mg of Whatman no. 1 filter paper strips (about 15 by 2 mm). Tubes were sealed with butyl rubber stoppers, and the medium was sterilized by autoclaving. Sodium sulfide was autoclaved separately and added to culture media when cooled to room temperature. The final pH of the culture medium was 6.9.

Culture techniques. The techniques described by Hungate (13) were adapted for the maintenance and subculturing of the fungus alone and fungus-plus-methanogen cultures. Experimental media were inoculated by transferring 1/20 the volume of a 6-day-old culture of fungus alone or fungus plus methanogens. For the former inoculation, this was equivalent to approximately 2.5×10^3 colony-forming units (CFU) of fungus per ml of inoculating culture, and for the latter, it was equivalent to 3×10^3 CFU of the fungus and 1.4×10^7 CFU of methanogens per ml of inoculum. Colony-forming units of the fungus in mono- or coculture were determined after 4 days of incubation in roll tubes inoculated with 5×10^{-2} to 5×10^{-4} ml of culture. Colony-forming units of the methanogens were determined after 21 days of incubation in roll tubes inoculated with 5×10^{-5} to 5×10^{-7} ml of coculture. Glucose (0.1%; wt/vol) was used as the growth substrate for the fungus and H_2-CO_2 (80:20 [vol/vol] in the gas phase) for the methanogens in the roll tubes.

For studies on the purity of the cultures, they were roll-tubed in media described above, but with 0.2% glucose replacing cellulose as the substrate, or in AC medium. The agar concentration was 2% (wt/vol).

Quantitative analysis of substrate and products in culture media. Cellulose was determined by the method of Updegraff (21). Whatman no. 1 filter paper contained 99.7% cellulose. Soluble carbohydrates were determined by a modification of the anthrone method (1), in which glucose was used as the standard.

For the determination of methane, hydrogen, and CO_2 (headspace), 1-ml gas samples were removed from culture tubes and analyzed on a Fisher-Hamilton gas partitioner equipped with a thermal conductivity detector. For methane and CO_2 analyses, gas was fractionated at 25°C on dual columns; column 1 was packed with 2-ethylhexylsebacate on Columnpak, and column 2 was packed with molecular sieve 13×. Helium was the carrier gas. For hydrogen analysis, gas was fractionated on column 2, and argon was the carrier gas. Gases were quantified by comparison with the peak heights of known standards.

Total CO_2 in culture tubes was determined gravimetrically as $BaCO_3$. Culture medium in the sealed culture tubes was acidified to pH 2 by injection of 0.4 ml of 50% (vol/vol) H_2SO_4 to ensure that all of the CO_2 was liberated from the medium. The stopper was pierced with two syringe needles (19 gauge), and CO_2 was flushed from the culture tubes by slowly bubbling nitrogen through the medium for 1 h; CO_2 in the effluent gas was trapped in 3 M NaOH (10 ml). Alkali was then quantitatively removed from the trap and made up to 20 ml with CO_2-free water. Trapped CO_2 was precipitated as $BaCO_3$ by the addition of 1 ml of 12% (wt/vol) $BaCl_2$. The precipitate was collected by centrifugation at 5,000 × *g* for 10 min at 0 to 2°C, suspended in 10 ml of CO_2-free ethanol-H_2O (50:50 [vol/vol]), and centrifuged as before. The $BaCO_3$ pellet

was suspended in 2 ml of ethanol-H_2O mixture and collected on tared glass fiber filter disks (Millipore Corp., Bedford, Mass.). The disks plus $BaCO_3$ were dried to constant weight in a desiccator at 60°C. In culture tubes where gas samples were removed for analysis by gas chromatography, CO_2 determined in these samples was added to the CO_2 determined as $BaCO_3$ to give total CO_2.

For measurement of volatile fatty acids and ethanol, cells were removed by centrifugation at 5,000 × *g* for 20 min at 0 to 2°C. A 2-ml portion of the supernatant was acidified with 0.1 ml of 6 N HCl, and the volatile fatty acids (except formic acid) and ethanol were determined by gas chromatography. A glass column (2 m by 4 mm) packed with 3% (wt) Carbowax 20M-0.5% (wt) H_3PO_4 on 60/80 mesh Carbopak B (Supelco, Inc., Bellefonte, Pa.) was used in a Tracor 560 gas chromatograph equipped with a flame ionization detector. The column temperature was 160°C, and nitrogen was the carrier gas. Fatty acids and ethanol were identified and quantitated by comparison of the retention times and peak height with those of known standards. Formate was determined as described previously (10).

Lactate was converted to methyl-lactate and determined by gas-liquid chromatography. A 2-ml sample of culture medium supernatant or lactic acid standard, 1 ml of 0.2% (wt/vol) malonic acid as internal standard, 1 ml of 50% (vol/vol) sulfuric acid, and 2 ml of distilled water were added to the chamber of a liquid-liquid extraction apparatus. Carboxylic acids were extracted with diethylether for 6 h, and the extract was then reduced to a volume of ≤1 ml by rotary evaporation. Anhydrous sodium sulfate was added (approximately 20 mg), and methylation was achieved by the addition of diazomethane in ether. The methyl esters were separated at 120°C on a glass column (2 m by 4 mm) packed with 10% diethylene glycol succinate on 100- to 120-mesh GasChrom Q, connected to a flame ionization detector. Nitrogen was the carrier gas. Methyl-lactate in samples was determined from the ratios of the peak heights of dimethylmalonate standards to those of methyl-lactate samples and standards.

Radioactive incubations with [2-^{14}C]acetate and analyses. To determine acetate as a precursor of methane during cellulose fermentation by the cocultures, 2 μCi of [2-^{14}C]acetate (56 mCi/mmol) was added to culture tubes at zero incubation time. Fermentation was terminated at various stages of incubation between 0 and 168 h by the injection of 1 ml of 3 N NaOH into culture media, and the CO_2 in the gas phase was absorbed. After complete absorption of CO_2, as ascertained by gas chromatographic procedures, the resulting vacuum in the tubes was relieved by the injection of nitrogen with a glass piston syringe to allow quantitative gas sampling. Tubes were maintained at 2°C, and the total volume of gas was determined by recording the excess gas forced into the syringe. For analysis of radioactive methane, 1-ml volumes of gas were injected into scintillation vials sealed with butyl septum stoppers and counted in 20 ml of toluene-based scintillant as described by Zehnder et al. (27). To ensure that a maximal amount of methane was dissolved, vials were agitated vigorously. Assay of the gas atmosphere above the scintillation fluid revealed that 80 ± 3% of the methane added was absorbed and therefore counted. Corrections were made for methane not absorbed and for quenching. Counting efficiency was 86 ± 2%.

For analysis of radioactive acetate, media were centrifuged at 5,000 × *g* for 15 min at 0 to 2°C. The procedures for the isolation of radioactive acetate from the supernatant and the determination of radioactivity were based on previously described methods (17).

Quantitative measurements of methane and acetate for specific radioactivity determinations were as in the preceding section.

Microscopy. Examination of the monoculture of the fungus and coculture with methanogens was by light microscopy and scanning electron microscopy. Details of sample preparation for scanning electron microscopy were as previously described (3). Because of their characteristic fluorescence at 420 nm, the methanogens were also examined by ultraviolet, epifluorescence microscopy.

Chemicals. All chemicals were obtained from commercial sources and were of reagent grade. The radioisotope, [2-^{14}C]acetate (specific activity, 56 mCi/mmol), was obtained from the Radiochemical Center, Amersham, England.

RESULTS

Description of anaerobic fungus. The anaerobic fungus strain PN1 appears closely related to the rumen phycomycete *Neocallimastix frontalis* (18). The phylogenetic position of these fungi remains to be determined, although the mode of zoosporogenesis from the monocentric, rhizoid-bearing thallus is comparable with that of many chytrids. Figure 1A shows a thallus of the fungus consisting of a sporangium with a highly branched rhizoid. The size of the sporangia varied depending on the stage of development of the thalli, but in glucose medium (3) it was up to 200 μm in length and 140 μm in width. With cellulose as substrate, sporangia up to 180 μm long were obtained. Figure 1B shows a single zoospore. The zoospores were oval to bean shaped (length, 14 to 18 μm; width, 12 to 14 μm) and contained between 9 and 12 flagella. The body of the zoospore in Fig. 1B is slightly out of focus, which makes it appear larger. In older cultures zoospores characteristically round off.

Colonies in roll tubes inoculated with actively fermenting fungal culture probably developed from zoospores, since these were numerous in the culture fluid (>10^3 per ml of 6-day-old culture), whereas free sporangia (detached from solid cellulose substrate) were either absent, or in low numbers, and cellulose was not usually transferred in the inoculum.

Stability of fungus-methanogen coculture. The fungus-methanogen coculture isolated was stable and was maintained in liquid

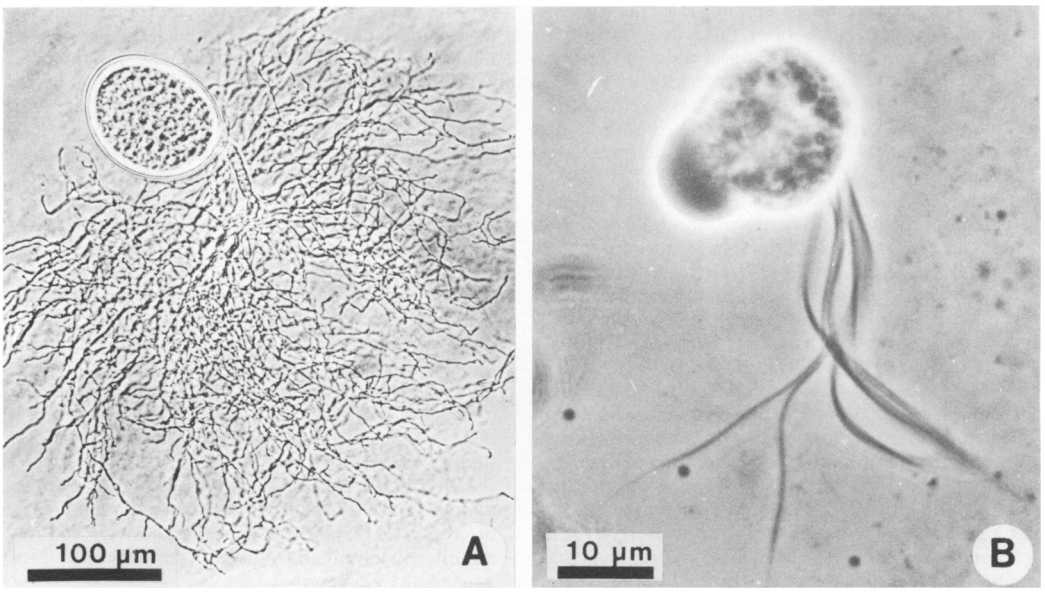

FIG. 1. *(A) Light micrograph of rumen anaerobic fungus. Thallus showing sporangium and highly branched rhizoid. (B) Multiflagellated zoospore of the rumen anaerobic fungus.*

medium containing cellulose for over 1 year by routine transfer every 6 days. During this time there was no loss of cellulolytic or methanogenic activity.

Methanogenic population in the coculture. Colonies in roll tubes with H_2-CO_2 (80:20 in the gas phase), inoculated with 5×10^{-5} to 5×10^{-7} ml of stable fungus-methanogen coculture (6 days old) were of one type and became visible after 1 week of incubation. Surface colonies were translucent, circular with entire margins, off-white to yellow in color, and reached a diameter of approximately 2 mm after 21 days of incubation. Deep colonies were lenticular and did not increase in size beyond 1 mm in diameter. When colonies were repeatedly picked and examined by ultraviolet epifluorescence microscopy, the bacteria exhibited characteristic blue-green fluorescence of methanogens at 420 nm. The cells were gram positive, nonmotile oval rods or cocci, usually 0.7 μm in width, with some cells reaching 1 μm, and 0.8 to 1.6 μm in length, occurring predominantly in pairs but occasionally in chains. These bacteria can clearly be identified in the scanning electron micrograph of the coculture (Fig. 2). Isolated colonies grew well on H_2-CO_2 (80:20 in the gas phase), slowly on formate, but not on acetate, although acetate stimulated growth on H_2-CO_2. The colony-type, morphology, and substrates utilized indicated that the methanogens were most likely *Methanobrevibacter ruminantium*. Also present in the mixed culture but not shown in Fig. 2 were large

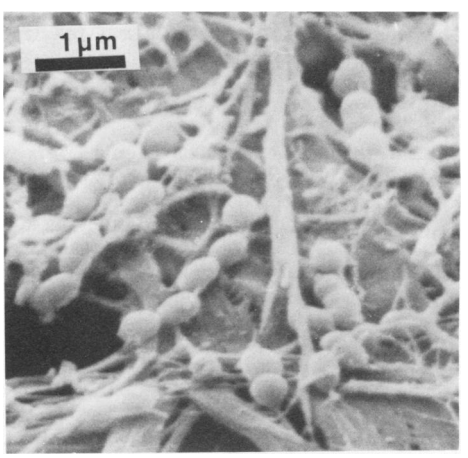

FIG. 2. *Scanning electron micrograph of coculture of anaerobic fungus with rumen methanogens showing methanogens and fungal hyphae grown on paper.*

cocci (about 1.5 μm in size), occurring singly, in pairs, and in clusters, and exhibiting blue-green fluorescence at 420 nm. We were unsuccessful in isolating these organisms, but their morphological features and fluorescence at 420 nm suggested they were methanogens resembling *Methanococcus*. Direct counts of these organisms by epifluorescence microscopy indicated that they accounted for <1% of the total methanogenic population. The organism resembling *M. ruminantium* designated as *Methanobrevibacter* sp. strain RA1, accounted for >99% of the

total methanogens in the coculture, consistent with the presence of only one colony type in the roll tubes.

Fermentation of cellulose. The fermentation of cellulose by the rumen anaerobic fungus alone resulted in the formation of six products, acetate, lactate, formate, ethanol, CO_2, and H_2 (Fig. 3). The lag time for lactate production was longer than for the other products. Fermentation terminated about 300 h after inoculation. In the coculture, the major products were acetate, carbon dioxide, and methane (Fig. 4). Ethanol and lactate production decreased. Hydrogen was not detected during fermentation (detection limit, 5 $\times 10^{-4}$ atm), and formate occurred in only trace amounts. The coculture fermentation terminated after about 200 h of incubation. For both the mono- and cocultures, the pH of the medium decreased from 6.9 at the beginning of incubation to 6.0 at the end of fermentation. The rate and extent of cellulose degradation were greater in the coculture than in the monoculture (Fig. 3 and 4). In the coculture, after 100 h, more than 70% of the initial cellulose had been degraded, and in the monoculture, less than 10% had been degraded. At the end of fermentation, 82% of the initial cellulose had been degraded in the coculture, and 53% had been degraded in the monoculture.

Table 1 compares the fermentation products of cellulose in the mono- and cocultures. In the coculture, carbon dioxide and acetate increased by 51 and 62 mol/100 of hexose, respectively. Lactate and ethanol decreased by 64 and 18.4 mol/100 mol of hexose, respectively. The decrease in hydrogen and formate in the coculture reflected their utilization in methane production. To determine whether acetate, the only other likely methane precursor, contributed to methane in the coculture, fermentation was carried out in the presence of [2-^{14}C]acetate. Data on specific radioactivity ratios (Table 2) indicated that acetate contributed less than 1% of the methane formed during cellulose fermentation and was therefore not a significant precursor. Thus the amount of H_2 required for the formation of methane (58.7 mol of CH_4/100 mol of hexose), including H_2 produced via formate cleavage, would be 235 mol ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$).

Other possible products of cellulose fermentation by the fungus, propionate, butyrate, or succinate were not detected (<0.1 mol/100 mol of hexose fermented), and there was no accumulation of soluble carbohydrates in the mono- or cocultures during the fermentations.

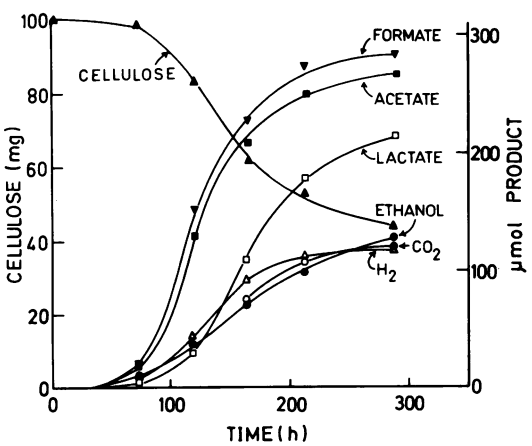

FIG. 3. *Fermentation of cellulose by rumen anaerobic fungus. Cellulose is expressed as total milligrams, and products are expressed as total micromoles in the growth medium (12 ml). Media were inoculated with 0.6 ml of fungal culture (2.5 × 10^3 CFU per ml). Values for acetate, formate, ethanol, and lactate were corrected for amounts transferred in the inoculum, and with the exception of ethanol and lactate, the amounts present in uninoculated media.*

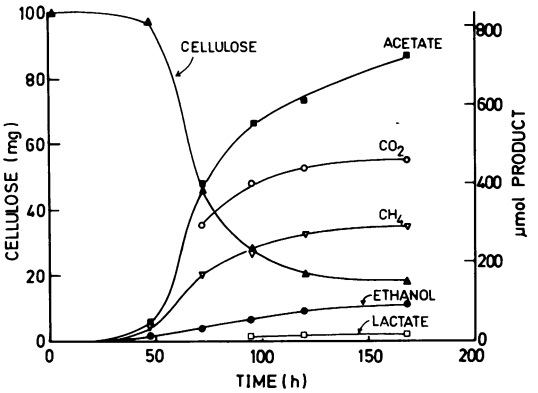

FIG. 4. *Fermentation of cellulose by coculture of anaerobic fungus with rumen methanogens. Cellulose is expressed as total milligrams, and products are expressed as total micromoles in the growth medium (12 ml). Media were inoculated with 0.6 ml of fungus-methanogen coculture (3 × 10^3 CFU of fungus per ml; 1.4 × 10^7 CFU of methanogens per ml). Values for acetate were corrected for the amounts present in the inoculum and in uninoculated media.*

Recombined culture experiments. Isolated colonies of *Methanobrevibacter* sp. strain RA1, were transferred to culture tubes inoculated with the fungus alone. After completion of growth (approximately 170 h), as ascertained by methane production, the recombined cultures were inoculated into fresh media. The inoculum contained approximately 3 × 10^3 CFU of fungus per ml and 1.2 × 10^7 CFU of methanogen per

ml. At the completion of methanogenesis (168 h), products in the culture media were analyzed, and unutilized cellulose was determined. The formation of products was similar to the stable coculture (Table 1), except that ethanol decreased to 9.6 mol/100 mol of hexose. CO_2 was not determined. Formate and hydrogen were absent, and lactate was low (3.5 mol/mol of hexose). Mean values for acetate and methane were 138.5 and 59 mol/100 mol of hexose, respectively. At the end of fermentation, 87% of the initial cellulose had been degraded.

DISCUSSION

The gut anaerobic fungi possess several properties not found in other fungi that are undoubtedly related to their obligately anaerobic lifestyle, perhaps itself the most unusual of their properties. Much of the interest in these fungi so far has concerned the extent of their role in ruminant digestion. But since these are the only fungi known to be obligate anaerobes, the complete spectrum of their properties is of considerable intrinsic interest to microbiologists.

The degradation of cellulose by the monoculture provides the first example of a mixed-acid type of fermentation in fungi, and the products were similar to those that are produced by coliform bacteria during anaerobic growth on glucose. Hydrogen formation has not been reported previously in fungi, and the underlying mechanism of hydrogen formation needs to be studied in detail. In addition, the pathways for lactate, acetate, and ethanol also require study to determine whether or not they are similar to known pathways for the formation of these products. All of the fungal fermentation products are known to be formed by rumen bacteria also, and in the rumen they may be utilized by either the host animal or further fermented by other rumen microbes.

The present work confirms reports (4, 5, 19) of cellulose digestion by the rumen anaerobic fungi and thus extends the known range of cellulolytic microbes capable of participating in this primary fermentative process central to ruminant digestion. Glucose did not accumulate in the medium during growth on cellulose, which may indicate that the cellulase is associated closely with the fungal hyphae and is not released into the medium. This agrees with results of microscopic examination of strips of paper undergoing fermentation, which showed that digestion occurred only in the areas in close proximity to fungal rhizoid (4). Until recently the major cellulolytic microbes found in the rumen have been anaerobic bacteria, and they have long been accepted as the main agents of cellulose digestion. The close association of fungi with fibrous plant fragments suggested that fungi had a role in fiber digestion in the rumen (3). The extent of this role required demonstration of the appropriate enzymatic activities, as well as determination of the mass of fungal vegetative tissues within the tissues of digested plant fragments. The first of these requirements,

TABLE 1. *Fermentation of cellulose by anaerobic fungus in the absence and presence of rumen H_2-formate-utilizing methanogens*[a]

Product	mol/100 mol of hexose units[b]	
	Fungus alone	Plus rumen methanogens
Acetate	72.7 ± 4.9	134.7 ± 8.5
Lactate	67.0 ± 2.8	2.9 ± 0.1
Ethanol	37.4 ± 1.5	19.0 ± 1.0
Methane	0.0	58.7 ± 0.9
Carbon dioxide	37.6 ± 7.0	88.7 ± 5.0
Hydrogen	35.3 ± 2.5	<0.05
Formate	83.1 ± 2.8	1.0 ± 0.07
Carbon recovery (%)	90.3	77.5
Hydrogen recovery (%)[c]	87.0	75.6
Oxidation-reduction index	1.44	1.14

[a] Determined at the completion of fermentation, as ascertained by no further increase in hydrogen (monoculture) or methane (coculture). The percentages of cellulose degraded in the mono- and coculture were 53 and 82%, respectively.

[b] Values are means of duplicate determinations, and where the error is presented, ±1 standard deviation.

[c] Determined according to Barker (2).

TABLE 2. *Comparison of specific radioactivities of methane with [2-^{14}C]acetate during the fermentation of cellulose by coculture of anaerobic fungus with rumen H_2-formate-utilizing methanogens*[a]

Incubation time (h)	Acetate (μmol)	CH_4 (μmol)	$^{14}CH_4$ (dpm)	[2-^{14}C]acetate (dpm)	Sp act of CH_4 (dpm/μmol)	Sp act of acetate (dpm/μmol)	Sp act ratio CH_4/acetate
0	10.8	—[b]	—	4.30×10^6	—	3.9×10^5	—
72	395	178	1.37×10^4	3.72×10^6	76.9	9.1×10^3	8.4×10^{-3}
168	537	335	1.83×10^4	3.37×10^6	54.6	6.3×10^3	8.7×10^{-3}

[a] Tubes contained 12 ml of medium with 100 mg of Whatman no. 1 filter paper strips as substrate (cellulose). A 2-μCi amount of [2-^{14}C]acetate (56 mCi/mmol) was added at zero time. Results are mean values of duplicate tubes.

[b] —, Not done.

the ability to ferment cellulose, has now been demonstrated. It may be important also that fungi, by nature of their mode of growth involving hyphal extension, possess the ability to penetrate deeply into tissues normally inaccessible to bacteria, and this suggests a special role for anaerobic fungi in rumen fiber digestion.

The coculture consisting of an anaerobic fungus and methanogenic bacteria presumably resulted from the insensitivity of the methanogens to the antibiotics used in the isolation of the fungus. However, in the rumen it seems likely that the methanogens would be metabolically associated with the anaerobic fungi as well as other H_2-producing organisms. The coculture interaction provides the first example of H_2 transfer between a fungus and a bacterium. Previous studies on H_2 transfer have involved interbacterial systems (7, 8, 15, 16, 20, 23), although the association of methanogenic bacteria with rumen ciliates suggests the possibility of H_2 transfer between protozoa and bacteria (22).

Production of hydrogen is the key to the cometabolism of the fungus with methanogens, resulting in a decrease in electron sink products, lactate and ethanol. To explain this decrease, it is necessary to postulate the presence of a hydrogenase which catalyzes the production of hydrogen from reduced pyridine nucleotide at low partial pressure of the gas (26). Thus the methanogens, by removing H_2 and maintaining low partial pressures, would facilitate the production of H_2 from reduced pyridine nucleotide by the fungus. Reducing equivalents which would otherwise be used in the formation of lactate and ethanol would be diverted to methane via hydrogen in the coculture. The proportion of methane expected from the electron shift via reduced pyridine nucleotide may be calculated by assuming that the only other source of hydrogen was from reactions leading to the formation of ethanol and acetate and that this was equivalent to the sum of these products (153.7 mol/100 mol of hexose). The assumption was based on monoculture data (Table 1), from which the sum of formate and hydrogen was almost equal to the sum of acetate and ethanol), suggesting a common intermediate for the formation of the 2-carbon products, perhaps acetyl coenzyme A. Thus, out of the total hydrogen required for methane production (235 mol for 58.7 mol of CH_4), about 35% would be expected to be provided from reduced pyridine nucleotide from electron shift.

The carbon and hydrogen recoveries in the coculture were less than in the monoculture. One possible explanation is that one or more products from the fungus were assimilated into cell biomass of the methanogens. Previous studies have shown that both CO_2 and acetate may contribute to cell carbon of methanogens (6, 9, 24, 25) and that as much as 60% of the cell carbon in *M. ruminantium* may be derived from acetate (6). The oxidation-reduction index for both culture fermentations was higher than the theoretical value of 1.0. The deficiency in reduced products may have been due to the formation of cells with an oxidation-reduction state more reduced than that of cellulose (14).

In the coculture, cellulose degradation commenced earlier, the rate was faster, and the quantity digested was greater than in the monoculture (82 versus 53%). These effects might be explained by increased growth resulting from higher energy yields via the "acetate" pathway. In addition, coculture with the methanogen resulted in the removal of a number of the fungal fermentation products, some of which might have been inhibitory to growth of the fungus. Most of the cellulose degradation in the coculture occurred in the period between 50 and 100 h; perhaps application of this system to bioconversion processes, including cellulase production, may warrant future investigation.

In the rumen many aspects of cellulose digestion are still inadequately understood, and the present work now raises questions on accepted concepts. The demonstration of cellulose fermentation is further support for a role of anaerobic fungi in fiber digestion in the rumen and extends the known range of cellulose-fermenting microbes there. The knowledge that similar fungi are present in the foregut and hindgut in a wide range of herbivorous animals (4) likewise raises the question of the extent of the role of these fungi in fiber degradation. The discovery of a fungus-methanogen combination in stable coculture and the resultant altered fermentation pattern obtained bring additional information to understanding some of the complexities of the rumen fermentation.

ACKNOWLEDGMENTS

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