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Effect of *Methanobrevibacter smithii* on Xylanolytic Activity of Anaerobic Ruminal Fungi

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Three different ruminal anaerobic fungi, *Neocallimastix frontalis* PNK2, *Sphaeromonas communis* B7, and *Piromonas communis* B19, were grown axenically or in coculture with *Methanobrevibacter smithii* on xylan. *N. frontalis* and *S. communis* in monoculture and coculture accumulated xylobiose, xylose, and arabinose in the growth medium; arabinose was not metabolized, but xylobiose and xylose were subsequently used. The transient accumulation of xylose was much less evident in cocultures. Both the rate and extent of xylan utilization were increased by coculturing, and metabolite profiles became acetogenic as a result of interspecies hydrogen transfer; more acetate and less lactate were formed, while formate and hydrogen did not accumulate. For each of the three fungi, there were marked increases in the specific activities of extracellular xylanase (up to fivefold), α-L-arabinofuranosidase (up to fivefold), and β-D-xylosidase (up to sevenfold) upon coculturing. The stimulating effect on fungal enzymes from coculturing with *M. smithii* was independent of the growth substrate, and the magnitude of the stimulation varied according to the enzymes and the incubation time. For an *N. frontalis*-*M. smithii* coculture, the positive stimulation was maintained during an extended (18-day) incubation period, and this affected not only hemicellulolytic enzymes but also polysaccharidase and glycoside hydrolase enzymes that were not involved in xylan breakdown. The specific activity of cell-bound endopeptidase was not increased under the coculture conditions used in this study. The higher enzyme activities in cocultures are discussed in relation to catabolite repression.

Anaerobic chytridiomycete fungi are part of the complex microbial consortium that colonizes and degrades plant material in the ruminal ecosystem (27). The fungi are able to degrade and utilize plant storage and structural polysaccharides (28, 30) by producing a wide range of polysaccharide depolymerase and glycoside hydrolase enzymes (12, 29, 39, 40). It has therefore been proposed that the anaerobic chytrid fungi may have an important role in ruminal fiber digestion (2).

In an ecosystem as complex as the rumen, both synergistic and competitive microbial interactions take place. Many diverse microbial groups closely associate with the plant fragments, and these are thus the site for potentially important interspecies interactions (41). The anaerobic fungi extensively colonize the plant fragments and are thus likely to interact with other particle-associated microbial populations. In the rumen bacteria adhere to the fungal sporangia, and in vitro the chytrids have been shown to interact with both fibrolytic and nonfibrolytic ruminal bacteria (27, 31). Fungal digestion of straw was inhibited by coculture with *Ruminococcus* spp. but was increased in coculture with *Bacteroides* (*Fibrobacter*) *succinogenes*. The cellulolytic activity of *Neocallimastix frontalis* was also enhanced by coculture with lactate-utilizing strains of *Megasphaera elsdenii* or *Veillonella alcalescens* (A. J. Richardson, C. S. Stewart, G. P. Campbell, A. B. Wilson, and K. N. Joblin, Abstr. XIV Int. Congr. Microbiol. 1986, PG2-24, p. 233). The chytrid genera *Neocallimastix* (3, 24), *Piromonas* (15), and *Sphaeromonas* (11) form stable cocultures with methanogens. These cocultures degrade straw and lignocellulosic substrates more effectively than do the fungal monocultures, and the production of cellulose-degrading enzymes by *N. frontalis* increases markedly in the presence of methanogenic bacteria (47).

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The plant cell wall, however, is a macromolecular complex, and effective cellulolysis is dependent on the removal of the closely associated hemicellulose component. In the forages consumed by ruminants, the major hemicellulosic heteropolysaccharide of the plant cell wall is a substituted xylan. Although the ruminal fungi have been shown to utilize xylan and produce an array of active xylanolytic enzymes (23, 39, 40), the consequences of interspecies interactions on fungal xylanolysis have not been determined. The present study was, therefore, undertaken to assess the effect of coculture with the methanogen *Methanobrevibacter smithii* on the utilization of xylan and on the formation of xylanolytic polysaccharidase and glycoside hydrolase enzymes by ruminal anaerobic chytrid fungi.

MATERIALS AND METHODS

Organisms. Anaerobic chytridiomycete fungi belonging to three different genera were investigated. *N. frontalis* PNK2 (previously designated strain K2) was isolated from the rumen of a roughage-fed sheep and has been described previously (14). *Piromonas communis* B19 and *Sphaeromonas communis* B7 were isolated from fresh bovine feces by the roll-tube method of Joblin (14). Strain B19 has a branched filamentous rhizoid and uniflagellated zoospores similar to those of *P. communis* (26). Strain B7 has an extended bulbous rhizoid and multiflagellated zoospores similar to those of *S. communis* (25). When grown on cellobiose, each of the three strains produce H_2, acetate, lactate, and formate. Propionate and butyrate were not detected as metabolites from any of the strains, but ethanol was produced by *N. frontalis* PNK2. Fungi were stored at 39°C by the roll-tube procedure of Joblin (14), as modified by Joblin and Naylor (16). *M. smithii* PS (kindly provided by A. J. Richardson) was stored at −60°C in culture broth containing 3% (vol/vol) dimethyl sulfoxide as a cryoprotectant.

Media and growth conditions. The methods used for the preparation of prereduced medium, salts solution A, salts solution B, and anaerobic culture techniques were those described by Hungate (13). Basal medium, which was modified from that of Joblin (14), consisted of salts solution A (17 ml), salts solution B (17 ml), clarified ruminal fluid (30 ml), $NaHCO_3$ (500 mg), yeast extract (100 mg; Difco Laboratories, Detroit, Mich.), casein hydrolysate (100 mg; GIBCO Diagnostics, Madison, Wis.), distilled water (36 ml), 0.1% (wt/vol) resazurin (0.3 ml), and cysteine hydrochloride (30 mg). Ruminal fluid from a rumen-fistulated cow grazing a ryegrass pasture was clarified by centrifugation at 10,000 × *g* for 30 min and stored at −20°C. After thawing, ruminal fluid was reclarified of precipitates by centrifugation at 1,000 × *g* for 15 min. Carbohydrate growth substrates were added to basal medium before sterilization. Complete media contained oat spelt xylan (Sigma Chemical Co., London, England) or cellobiose (0.5%; wt/vol). For the preparation of media containing solid substrates, pieces of sisal (*Agave sisalone* L) twine (20 mg) or alfalfa (*Meticago sativa*) chaff (30 mg) were added to each tube before the addition of prereduced basal medium (10 ml).

Media under O_2-free CO_2 were dispensed in 10- or 75-ml volumes into CO_2-filled, 16-ml screw-cap Hungate tubes (Bellco Glass Inc., Vineland, N.J.) or 100-ml crimp-sealed serum bottles (Wheaton Scientific, Millville, N.J.) fitted with butyl rubber septa and were sterilized at 120°C for 20 min.

M. smithii was cultured in basal medium under a CO_2-H_2 atmosphere in Hungate tubes that were shaken (100 rpm) horizontally. *M. smithii* readily formed a stable association with each of the three fungi after an inoculum (5%; vol/vol) was added to the fungus growing in sisal medium. Fungus-*M. smithii* cocultures and *N. frontalis* and *P. communis* monocultures were maintained by subculturing on sisal broth at 5-day intervals. *S. communis* grew slowly and could be maintained only by subculturing at 9- to 10-day intervals. All cultures were incubated without shaking.

Inocula for xylan degradation and utilization studies were grown in cellobiose medium in Hungate tubes for 72 h. Washed inocula were used to avoid the cotransfer of extracellular enzymes. Fungi in monoculture or coculture were vortexed to dislodge fungi from the tube walls and were sedimented in unopened culture tubes by centrifugation (100 × *g*, 15 min) at room temperature. Supernatants were removed from culture tubes by the injection of sterile O_2-free CO_2 (10 ml) to displace the supernatant into the syringe. Sterile anaerobic basal medium (10 ml) was added with a syringe, fungal thalli were suspended and centrifuged, and the supernatant was removed as described above. An inoculum (4%; vol/vol) of washed fungal thalli from monocultures or cocultures suspended in sterile anaerobic basal medium was injected into culture vessels. The fungal biomass in the inocula was determined by filtering the contents of representative washed cultures through preweighed filters (pore size, 0.8 μm), washing the filters with basal medium, and drying the filters to constant weight over P_2O_5 in vacuo.

Although the washed fungal biomass from cocultures retained sufficient *M. smithii* to reestablish cocultures, this took several days (as evidenced by the presence of H_2 in the headspaces). To ensure rapid and complete reestablishment of fungus-*M. smithii* associations, inocula (3%; vol/vol) of washed and suspended *M. smithii* in basal medium were added to cultures inoculated with fungal biomass from cocultures. All incubations were done at 39°C.

Xylan utilization studies. Batches of 0.25% (wt/vol) xylan medium (10 ml) in Hungate tubes were inoculated with *N. frontalis* PNK2 or *S. communis* B7 in monoculture and coculture with *M. smithii*. Triplicate tubes from each culture line were harvested at intervals of 1, 2, 3, 5, and 7 days after inoculation. Gas production was measured by gas chromatography by the method of Wallace and Joblin (34). After sedimentation by centrifugation at 4°C (1,000 × *g* for 10 min for fungi; 10,000 × *g* for 20 min for cocultures), portions of cell-free culture supernatants were removed for assay of extracellular enzymes, quantitation of end products, and determination of xylan degradation and utilization. When samples could not be processed immediately, portions for metabolite quantification and enzyme determinations were stored frozen at −20°C. For residual xylan determination, samples (3.0 ml) were heated at 100°C for 10 min to inactivate extracellular xylanolytic enzymes. The heat-treated samples were stored at 4°C, as xylan can be displaced from solution at −20°C; sodium azide (0.02%; wt/vol) was added to inhibit microbial growth during storage.

Fermentation products and the xylan breakdown products arabinose, xylose, and xylobiose were identified and quantified by high-pressure liquid chromatography by using authentic materials as calibrants. Analyses were carried out isocratically on an isocratic analyzer (model 1706; Bio-Rad Laboratories, Richmond, Calif.). Samples (20 μl) were injected via an autosampler (model AS-48; Bio-Rad) onto a cation exchange column (300 by 7.8 mm; HPX 87H; Bio-Rad) that was maintained at 65°C and were eluted with H_2SO_4 (1.3 mM) at a flow rate of 0.8 ml/min. Fermentation products and sugars were monitored in a single elution by using UV absorbance (210 nm) and refractive index, respectively. The xylobiose standard was purchased from Bachem UK, Saffron Waldon, United Kingdom. The residual carbohydrate in the cell-free supernatant and the nonpolymeric degradation products remaining after fractionation with 80% ethanol (7) were quantified colorimetrically by the phenol-sulfuric acid procedure (9), so that the extent of xylan degradation and xylan utilization could be determined.

Enzyme preparation and assay procedures. Extracellular activities were measured in cell-free culture supernatants that were dialyzed at 4°C against 0.025 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.5) for 24 h. Vegetative material from a culture volume of 75 ml was suspended in 5 ml of 0.025 M MES buffer (pH 6.5) by homogenization and treated in an ultrasonic disintegrator at 4°C for four 30-s periods with 60-s intervals (39). After centrifugation (5,000 × *g*, 5 min, 4°C), the supernatant fraction was used to assay the enzymes associated with the vegetative material. The protein content of the extracellular and vegetative enzyme preparations was determined by the dye-binding procedure of Bradford (6).

Polysaccharidase activities were determined by measuring the amount of reducing sugar released from the substrate following incubation at 39°C for 30 min. The reaction was stopped by heating at 100°C for 5 min, and reducing sugars were quantified by the *p*-hydroxybenzoic acid hydrazide reaction (17) with the appropriate monosaccharide standard (glucose or xylose). The polysaccharide substrates oat spelt xylan (xylanase), *Lolium perenne* hemicellulose B (hemicellulase), amorphous cellulose (46) (cellulase), and sodium carboxymethyl cellulose (carboxymethyl cellulase) were dissolved or dispersed in 0.025 M MES buffer (pH 6.5) containing dithiothreitol (0.2 mg/ml) at a concentration of 2.5 mg/ml, with the exception of the low-viscosity carboxymethyl cellulose, which was used at an initial concentration of 5 mg/ml. One unit of polysaccharidase activity released 1 μmol of reducing sugar in 1 min under the assay conditions.

Glycoside hydrolase activities were determined by measuring the rate of p-nitrophenol release from the appropriate p-nitrophenol derivative (5 mM in 0.025 M MES buffer [pH 6.0] containing 0.2 mg of dithiothreitol per ml) after incubation at 39°C for a maximum period of 30 min (40). The p-nitrophenol released was determined spectrophotometrically at 420 nm. One unit of glycoside hydrolase activity released 1 μmol of p-nitrophenol in 1 min under these conditions.

Endopeptidase activity was determined by measuring the rate of p-nitroaniline release from leucine p-nitroanilide after 60 min at 39°C in an assay mixture containing 1 ml of leucine p-nitroanilide substrate, 0.8 ml of 100 mM phosphate buffer (pH 6.5), and 0.2 ml of enzyme preparation. The phosphate buffer was prepared in distilled water that was degassed by boiling and that was cooled under continuous gassing with O_2-free N_2. The reaction was stopped by the addition of trichloroacetic acid (25% [wt/vol]; 0.5 ml). The p-nitroaniline released was determined after centrifugation (11,500 × g, 5 min, 4°C) by diazotization of the supernatant (1). One unit of endopeptidase activity released 1 μmol of p-nitroaniline in 1 min in the assay system described above.

Commercially available substrates were purchased from Sigma Chemical Co. Ltd. (Poole, Dorset). *L. perenne* hemicellulose B was prepared as described by Morrison (19).

RESULTS

Effect of coculture with *M. smithii* on xylan utilization. For the preparation of washed cells for inocula, fungi were grown on cellobiose (0.5%; wt/vol) for 72 h. Cultures of fungi grown with *M. smithii* produced significantly more biomass than did fungi grown in monoculture. Inocula contained the following biomasses, in milligrams per milliliter: *N. frontalis*, 0.68; *N. frontalis-M. smithii*, 0.80; *S. communis*, 0.70; *S. communis-M. smithii*, 0.81; *P. communis*, 0.45; and *P. communis-M. smithii*, 0.60.

N. frontalis and *S. communis* in monoculture and coculture with *M. smithii* degraded similar amounts (in excess of 90%) of xylan over a 7-day incubation period (Fig. 1). Coculturing of fungi with *M. smithii* had little effect on the rates of degradation. However, the rate and extent of utilization of the degradation products by the fungi increased in coculture with *M. smithii* (Fig. 1). After 7 days of culture, *N. frontalis* utilized 81.9% of the xylan and *S. communis* utilized 52.3% of the xylan, whereas the utilization values for the equivalent cocultures were 88.5 and 80.5%, respectively. The corresponding utilization values for *P. communis* in monoculture and coculture were 83.3 and 86.6%, respectively. *M. smithii* was unable to degrade or utilize xylan when it was grown in xylan (0.3%; wt/vol) medium under an H_2-CO_2 atmosphere.

The accumulation profiles of sugars during the 7-day incubations showed that xylan was broken down principally to xylose, arabinose (Fig. 1), and xylobiose. For example, 2 days after inoculation, over 60% of the xylan was degraded and xylose and arabinose made up >70% of the ethanol-soluble carbohydrate in fungal cultures. In the final stages of fermentation, xylose and arabinose accounted for >95% of the ethanol-soluble fraction (Fig. 1). Xylobiose and xylose accumulated transiently during the course of xylan fermentation. Xylobiose occurred in all cultures at low levels (0.3 to 0.4 mM), but only during the first 48 h of the fermentation, and it was not detectable from day 3 on. The xylobiose concentrations, after 24 h in both the *S. communis* monoculture and coculture, were 0.29 mM; after 48 h the values

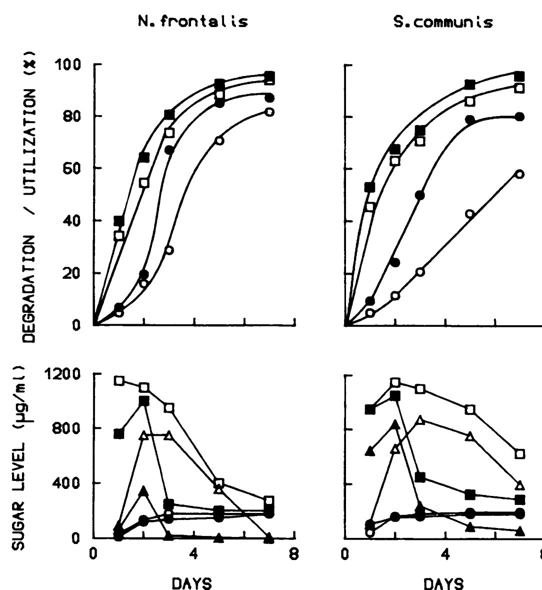

FIG. 1. Xylan degradation (□, ■) and xylan utilization (○, ●) by monocultures (open symbols) of *N. frontalis* and *S. communis* and in cocultures (closed symbols) of the fungi with *M. smithii*. The concentrations of ethanol-soluble carbohydrate (□, ■), xylose (△, ▲), and arabinose (○, ●) in the culture supernatants are also presented. Data are mean values from triplicate cultures.

were 0.43 and <0.1 mM, respectively. The corresponding values for the *N. frontalis* monoculture and coculture were 0.37 and <0.1 mM after 24 h and 0.25 and 0.31 mM after 48 h, respectively. The concentration of xylose was higher in monocultures, indicating less efficient uptake or usage, since the overall polymer degradation was similar in monocultures and cocultures. Arabinose gradually accumulated in all cultures as xylan was degraded. The released arabinose (1.2 to 1.5 mM after 7 days) was similar in both the absence and presence of methanogen (Fig. 1) and, apparently, was not metabolized by the fungi either in monoculture or in coculture. Complete release of arabinose from the xylan (9% arabinose) would have produced an arabinose concentration of 1.5 mM.

Effect of coculture with *M. smithii* on fungal xylanolytic enzymes. The extracellular xylan-degrading activities of *N. frontalis* PNK2 and *S. communis* B7 growing on xylan (0.25%; wt/vol) in monoculture or coculture with *M. smithii* were determined 1, 3, and 7 days after inoculation. For both fungi, the specific activities (milliunits per milligram of protein) of xylanase, α-L-arabinofuranosidase, and β-D-xylosidase increased with increasing culture age (Fig. 2); and the activity of each of the three xylan-degrading enzymes was strongly affected by coculturing with *M. smithii*. In general, in the early stages of growth, differences in specific activities were not marked, but as the extracellular activity increased, the differences between monoculture and coculture became more pronounced. A comparison of enzyme titers from cocultures with those from monocultures indicated that the maximum relative increase in activity (up to ninefold) occurred 3 days after inoculation; with the exception of α-L-arabinofuranosidase in the *S. communis* cultures, the positive effect from coculturing with the methanogenic bacterium decreased somewhat in 7-day cultures. The relative specific activities (RSAs) of α-L-arabinofuranosidase, β-D-glucosidase, β-D-xylosidase, and xylanase in the culture

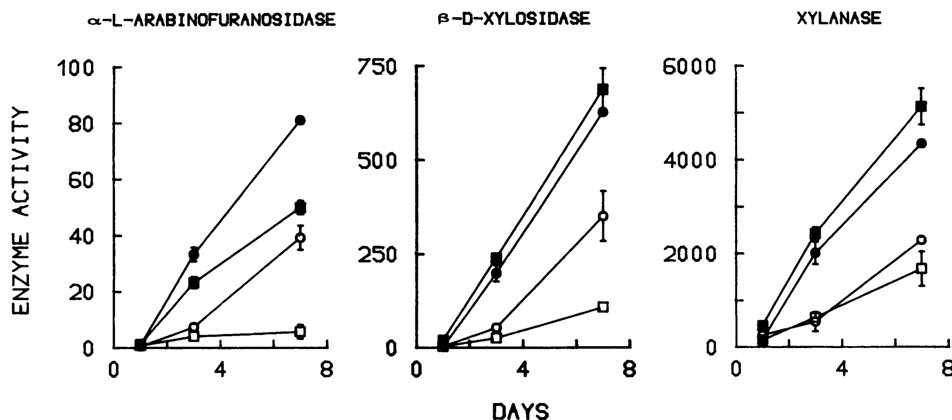

FIG. 2. Specific activities (milliunits per milligram of protein) of extracellular hemicellulose-degrading enzymes in monocultures of *N. frontalis* (○) and *S. communis* (□) and the methanogenic cocultures *N. frontalis-M. smithii* (●) and *S. communis-M. smithii* (■) grown for 7 days on xylan. The data presented are means ± standard deviations for triplicate cultures.

supernatant of *P. communis* after 5 days of growth on xylan were also higher. Xylanolytic enzymes were not detectable in cell-free supernatants of *M. smithii* grown in basal medium containing xylan (0.3%; wt/vol).

The specific activities of the extracellular xylan-degrading enzymes of *N. frontalis*, *S. communis*, and *P. communis* were also increased as a result of coculture with *M. smithii* for 5 days when either a monosaccharide or a solid substrate (plant tissue) was the carbon source (Table 1). The hemicellulolytic enzymes formed constitutively by all three fungi, and the enhanced exocellular activity in the presence of *M. smithii* was not substrate dependent. The specific activities of the extracellular cellulolytic glycoside hydrolases β-D-glucosidase and β-D-cellobiosidase formed by *N. frontalis* grown under similar conditions on filter paper for 5 days were also increased in the presence of the methanogen; the relative specific activities were 3.5 (β-D-glucosidase) and 7.2 (β-D-cellobiosidase).

Effect of coculture with *M. smithii* on metabolite formation from xylan. The principal acidic metabolites produced from the fermentation of xylan by *N. frontalis* and *S. communis* were acetic, lactic, and formic acids. Ethanol (6.1 mM after 7 days) was produced by *N. frontalis*. Coculture with *M. smithii* resulted in a metabolic shift as a consequence of interspecies hydrogen transfer. Methane, not hydrogen gas, accumulated, and the fermentations became acetogenic (e.g., in the *S. communis-M. smithii* coculture the acetate concentration after 7 days was 13.8 mM compared with 5.5 mM in the axenic fungal culture). Formic acid was no longer detectable as an end product, and the amount of lactic acid produced was reduced by approximately 95%, from 17 to 18 to <1 mM, after growth for 7 days. Ethanol was not detected in the culture supernatants of *M. smithii* and *N. frontalis* cocultures.

TABLE 1. RSAs[a] of enzymes in cell-free culture supernatants of ruminal fungi grown in the presence or absence of *M. smithii* on alfalfa stem tissue or a monosaccharide

Fungus	Carbon source[b]	Enzyme			
		α-L-Arabinofuranosidase	β-D-Xylosidase	β-D-Glucosidase	Xylanase
N. frontalis	A	5.0	5.7	3.7	2.7
	M	4.4	7.3	4.0	5.8
S. communis	A	0.8	3.6	3.8	1.7
	M	1.2	5.3	4.6	1.9
P. communis	A	2.8	3.1	3.5	1.8
	M	1.6	4.5	4.1	1.4

[a] RSA = (specific activity in coculture supernatant/specific activity in monoculture supernatant).

[b] A, Alfalfa stem tissue; M, the monosaccharide carbohydrate growth substrate, which was glucose (*N. frontalis*, *S. communis*) or xylose (*P. communis*).

Effect of *M. smithii* on xylanolysis and the formation of polysaccharolytic enzymes by *N. frontalis*. (i) Xylanolysis. To determine whether the specific activities of enzymes in, and xylanolysis by, a fungal monoculture increased to the coculture levels on prolonged incubation, cultures (75 ml) of *N. frontalis* and *N. frontalis-M. smithii* in xylan (0.25%) medium were incubated for periods of up to 18 days.

In the coculture, methane and metabolite production increased for 5 days, with acetate as the principal acidic metabolite; the low lactate levels declined after 5 days (Fig. 3). In the *N. frontalis* monoculture, hydrogen formation, and hence growth, continued for 8 to 12 days, with the formation rate being greatest in the early phase of the incubation (to 5 days); some additional lactate and formate were produced when active growth ceased. The cessation of active growth corresponded with the disappearance of soluble carbohydrates from the culture. The residual 80% ethanol-soluble carbohydrate from day 5 (coculture) and day 12 (monoculture) was predominantly arabinose (>97 and >95.3%, respectively).

Although the fungus degraded xylan in a very similar manner in both monoculture and coculture, the rate of utilization was considerably higher in the coculture. After 5 days, 82% of the xylan was utilized by the coculture, whereas 59% of the xylan was utilized by the fungal monoculture (Fig. 3). At the completion of the incubation (18 days), xylan utilization was somewhat greater in the coculture (84.3%) than it was in the monoculture (78.3%), whereas the extent of xylan degradation was similar in the presence and absence of *M. smithii* (91.0 and 89.0%, respectively). The transient accumulation of ethanol-soluble degradation products was more marked in the monoculture (Fig. 3). The saccharide moieties that accumulated were the monomers xylose and arabinose. The proportion of free pentose

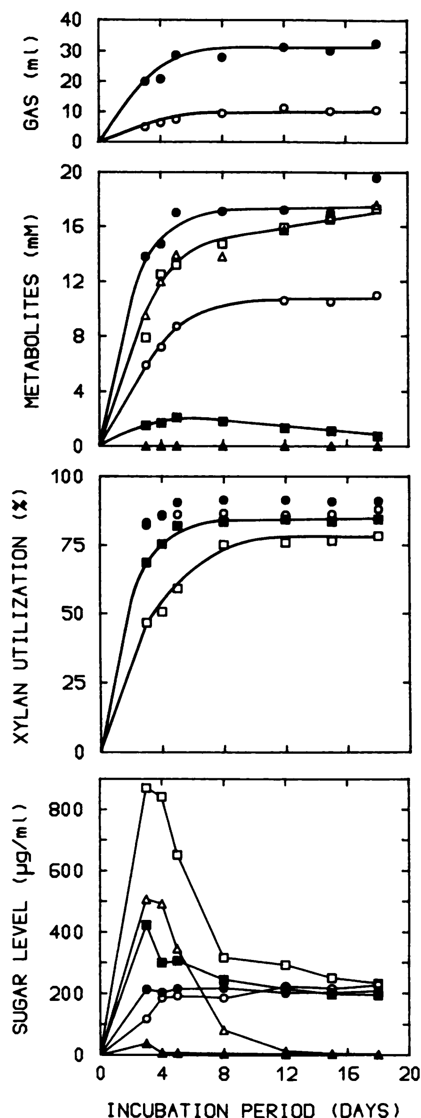

FIG. 3. Fermentation characteristics of *N. frontalis* grown axenically (open symbols) or in coculture with *M. smithii* (closed symbols) for an 18-day period on xylan. The fermentation metabolites are hydrogen (○), methane (●), acetic acid (○, ●), lactic acid (□, ■), and formic acid (△, ▲). Other data are xylan degradation (○, ●), xylan utilization (□, ■), and the extracellular concentrations of ethanol-soluble carbohydrates (□, ■), xylose (△, ▲), and arabinose (○, ●).

monosaccharides in the solubilized degradation products in the coculture increased from 59% at day 3 to >95.0% by day 8 as the xylan was broken down. Monosaccharides also predominated in the xylanolysis products of the *N. frontalis* monoculture (Fig. 3). The proportion of arabinose in these ethanol-soluble degradation products increased from 13.4% at day 3 to >90% by day 15. In the coculture, arabinose represented 50% of the products at day 3, and this value increased to >93% by day 8; xylose did not accumulate (8.7% of products at day 3). In the fungal culture, however, xylose accounted for >50% of the ethanol-soluble material until day 5; the proportion declined during the remainder of the incubation.

(ii) Enzyme formation. The cell-associated and extracellular activities of seven glycoside hydrolase and four polysaccharidase enzymes were measured to monitor changes in the specific activities of enzymes during the extended incubation. In both monoculture and coculture, the specific activities of all the extracellular enzymes (Table 2) increased with increased incubation times and were close to attaining maximal values after 12 days. Coculturing of *N. frontalis* with *M. smithii* markedly increased the specific activities of not only hemicellulolytic enzymes but also the polysaccharolytic enzymes and glycohydrolase enzymes that were not involved in xylan degradation (Table 2). Increases were not attributable to *M. smithii* per se, as the polysaccharidase and glycosidase activities were not detected in *M. smithii* grown in the presence of xylan. The amount of protein present in coculture supernatants was 10 to 30% higher than that in monoculture supernatants, probably as a result of increased fungal growth in the cocultures.

Extracellular activities were generally 2- to 5-fold greater in the coculture, although β-D-cellobiosidase activity was 10- to 16-fold higher in the early stages of fermentation (days 4 to 8). α-D-Galactosidase and cellulase activities were low or undetectable in the *N. frontalis* culture supernatants but were detected in the coculture supernatant (Table 2). The extent of the differences in the RSAs (specific activity of the coculture: specific activity of the monoculture) of the extracellular enzymes decreased during the course of incubation; for example, the RSAs after 3 and 18 days for β-D-glucosidase, β-D-cellobiosidase, α-L-arabinofuranosidase, and hemicellulase were 5.5 and 3.6, 8.2 and 5.7, 1.5 and 1.2, and 4.1 and 2.6, respectively (Table 2).

For cell-associated enzymes, the patterns of change in specific activities with increased incubation times (Table 3) were somewhat different from those of the extracellular enzymes. For example, the specific activities of xylanolytic enzymes in the fungal monoculture were at, or close to, their highest values in the early stages, when the xylan substrate was available, and then declined as the culture aged. This effect was not found for extracellular enzymes (Table 2).

The effect on cell-associated enzymes from coculturing of *N. frontalis* with *M. smithii* was, in general, similar to that found with extracellular enzymes. After 18 days of incubation, the specific activities of enzymes were increased 2- to 10-fold in the cocultures. Cell-bound α-D-glucosidase and endopeptidase specific activities were little affected by coculturing (Table 3). As the activities of cell-associated enzymes increased only in the coculture during incubation, the differences in RSAs were most pronounced at the end of the incubation. RSAs at 3 and 18 days for β-glucosidase, xylanase, and carboxymethyl cellulase were 1.9 and 3.6, 1.1 and 3.3, and 0.8 and 3.2, respectively.

DISCUSSION

Degradation by ruminal fungi of heteropolysaccharides such as the hemicellulose in plant cell walls requires the concerted action of different hydrolytic enzymes (4). In the present study, strains of *N. frontalis*, *P. communis*, and *S. communis* constitutively produced the xylanoltyic enzymes α-L-arabinofuranosidase, xylanase, and β-D-xylosidase. As has been noted by other investigators (12, 39, 40), there were no marked intergeneric differences in the characteristics of the enzymes.

The high level of activity of the xylan depolymerase system of ruminal fungi was demonstrated by the rapid and extensive depolymerization of xylan which occurred early in incubations of *N. frontalis* PNK2 and *S. communis* B7. Xylan degradation products were utilized for growth at a

TABLE 2. Specific activities of fibrolytic enzymes in cell-free culture supernatants of *N. frontalis* grown in the absence and presence of the methanogen *M. smithii*

Enzyme	Methanogen[a]	Sp act after the following periods of growth (days)[b]:						
		3	4	5	8	12	15	18
α-D-Galactosidase	−	ND[c]	ND	ND	ND	0.81	0.54	0.57
	+	0.53	3.9	2.7	4.0	5.6	5.1	5.1
β-D-Galactosidase	−	0.38	0.39	0.59	0.97	0.81	1.3	2.0
	+	3.1	6.6	6.3	7.1	9.9	9.6	10.3
α-D-Glucosidase	−	0.57	0.88	0.78	0.98	2.8	2.4	2.5
	+	1.9	3.1	3.1	3.5	4.1	3.5	4.1
β-D-Glucosidase	−	14.8	25.2	27.8	41.5	62.2	59.8	64.8
	+	81.7	128	159	178	211	208	231
β-D-Cellobiosidase	−	1.4	2.1	2.0	3.7	7.9	8.2	8.4
	+	11.5	24.1	32.7	36.9	46.6	45.2	48.1
α-L-Arabinofuranosidase	−	6.1	8.8	14.2	27.7	33.5	37.3	39.1
	+	7.5	13.4	16.2	34.7	49.3	42.3	49.7
β-D-Xylosidase	−	79.2	104	140	244	315	287	348
	+	86.5	283	297	374	507	483	549
Carboxymethyl cellulase	−	30.1	85.2	96.9	154	139	114	197
	+	127	351	324	321	416	292	386
Cellulase	+	41.8	102	85.2	85.2	127	75.2	76.8
Hemicellulase	−	184	409	357	515	496	640	618
	+	752	1321	1077	1037	2054	1787	1602
Xylanase	−	209	202	279	605	658	479	852
	+	554	1237	1151	1566	2121	1408	1581

[a] −, Fungus alone; +, coculture of fungus and *M. smithii*.
[b] Specific activities are milliunits per milligram of protein per minute. The reducing sugars released in the polysaccharidase determinations were quantified with glucose standards (carboxymethyl cellulase and cellulase) or xylose standards (hemicellulase and xylanase).
[c] ND, Not detected.

considerably slower rate than degradation occurred, and as a consequence, xylose and arabinose, the monomer constituents of the xylan, and xylobiose accumulated in the cultures. Accumulation of xylose and arabinose has previously been observed during hemicellulolysis by *Neocallimastix* spp. (18, 23), but there have been no reports of xylobiose as an intermediate. Xylobiose was present only at the early stages of xylan fermentation when fungal biomass was low, suggesting that as an intermediate of fungal xylanolysis, xylobiose either was rapidly taken up by cells or was rapidly hydrolyzed to xylose by β-xylosidase action. The xylose accumulation period in *N. frontalis* PNK2 cultures was of a considerably shorter duration than that in *S. communis* B7 cultures, despite the similar rates and extent of degradation in the two cultures.

S. communis B7 and *N. frontalis* PNK2 did not utilize the released arabinose, which is in accord with observations that ruminal fungi cannot utilize arabinose (30). The release of essentially all the arabinose component of the xylan into the medium early in the incubation (Fig. 1) and at a stage when only approximately 60% of the xylan was degraded suggests that xylanases of ruminal fungi are impeded by arabinose side chains. Removal of these side chains appears to be essential for xylanolysis to proceed efficiently. A similar effect has been observed during xylanolysis by other ruminal microorganisms (36).

Release of arabinose by ruminal fungi during degradation of hemicellulose of plant cells walls allows cross-feeding of arabinose and supports nonfibrolytic microorganisms in the rumen. Some cellulolytic bacteria and certain ciliate protozoa also are able to degrade hemicellulose but not fully utilize degradation products (8, 38).

The present study has shown, for the first time, that xylanolysis by ruminal fungi is markedly stimulated when fungi are grown in association with a methanogenic bacterium. Both the rate and extent of xylan utilization increased. This provides a further example of the improvement in fungal abilities to degrade polysaccharides when methanogens are cocultured with ruminal anaerobic fungi. Previously, it has been found that the rate and extent of cellulolysis by an *N. frontalis* isolate was stimulated by growth with *Methanobrevibacter ruminantium* (3), and Joblin et al. (15) have shown that straw solubilization by *Neocallimastix* spp. and *Piromonas* spp. is enhanced in methanogenic cocultures.

The specific activities of extracellular xylanase, α-L-arabinofuranosidase, and β-D-xylosidase increased upon coculturing; the effect was evident when fungi were grown on xylan, glucose, or plant tissue, indicating that the stimulation of xylanolytic enzymes is independent of substrate. Measurements from *N. frontalis* PNK2 cultured on xylan revealed that the specific activities of nonxylanolytic enzymes such as cellulase, carboxymethyl cellulase, β-cellobiosidase, β-D-glucosidase, and β-D-galactosidase were also increased

TABLE 3. Specific activities of cell-associated fibrolytic activities in cultures of *N. frontalis* grown in the absence or presence of *M. smithii*

Enzyme	Methanogen[a]	Sp act after the following periods of growth (days)[b]:						
		3	4	5	8	12	15	18
α-D-Galactosidase	−	2.2	1.3	1.4	0.6	0.9	0.9	0.5
	+	2.4	4.5	4.7	3.5	4.0	5.7	4.0
β-D-Galactosidase	−	2.6	1.2	1.5	1.5	1.5	1.3	0.9
	+	2.6	6.0	7.9	6.9	7.9	10.5	9.0
α-D-Glucosidase	−	3.6	2.6	2.1	2.8	3.0	3.3	2.8
	+	1.6	2.9	2.8	2.9	2.5	3.2	3.7
β-D-Glucosidase	−	40.1	45.1	46.4	55.6	32.1	55.0	50.7
	+	77.4	111	118	132	157	172	184
β-D-Cellobiosidase	−	4.4	6.2	7.7	8.1	9.5	7.6	6.5
	+	17.7	25.6	27.7	30.2	32.3	33.4	32.2
α-L-Arabinofuranosidase	−	45.5	73.9	34.5	15.3	9.0	10.5	7.4
	+	61.8	77.4	31.0	18.9	17.5	20.8	20.2
β-D-Xylosidase	−	517	754	400	152	54.1	77.5	81.7
	+	696	716	294	146	129	148	155
Carboxymethyl cellulase	−	122	117	100	132	114	114	102
	+	92	127	165	247	287	356	329
Cellulase	−	3.5	11.7	11.5	14.7	10.4	10.7	5.0
	+	41.8	48.4	33.4	28.4	35.1	38.4	35.1
Hemicellulase	−	511	606	433	347	301	347	412
	+	374	441	553	731	773	696	615
Xylanase	−	514	501	497	391	352	284	254
	+	548	611	695	583	805	773	840
Endopeptidase	−	0.8	1.6	1.5	1.3	0.9	0.7	0.7
	+	1.7	1.8	1.2	1.0	0.9	0.9	1.0

[a] −, Fungus alone; +, coculture of fungus and *M. smithii*.

[b] Specific activities are milliunits per milligram of protein per minute. The reducing sugars released in the polysaccharidase determinations were quantified with glucose standards (carboxymethyl cellulase and cellulase) or xylose standards (hemicellulase and xylanase).

in cocultures (Tables 2 and 3). The magnitude of the stimulation was enzyme dependent. Coculturing of *N. frontalis* PNK2 with *M. smithii* did not increase the specific activity of cell-bound endopeptidase (aminoacylamidase). The specific activities of extracellular proteinases did not increase when *N. frontalis* PNK2 was cocultured with *M. smithii* (R. J. Wallace and K. N. Joblin, unpublished data). It appears, then, that the specific activities of only polysaccharidases and glycohydrolases are increased on coculturing of fungi with methanogens. This conclusion is supported by observations that crystalline cellulose-solubilizing activity (47) and the activities of carboxymethyl cellulase and β-glucosidase (21) of *N. frontalis* strains are increased by coculturing with methanogens.

The reason for the stimulation of fungal activities by methanogens is likely to be complex and involve several factors. Syntrophic removal of hydrogen by methanogens cocultured with anaerobic bacteria (35, 44) or ruminal fungi (3, 11) growing on cellulose shifts metabolic production away from lactate, ethanol, and formate toward acetate and methane. A similar shift in fermentation end products was found in this study for *N. frontalis* and *S. communis* growing on xylan in coculture with *M. smithii*. The adverse effects of fermentation products on enzyme synthesis or activity may therefore be less pronounced in the cocultures, and as a consequence, the activities would appear to be stimulated. Lactate, for example, has an adverse effect on ruminal cellulolysis (10) and on the metabolic activity of ruminal ciliate protozoa (36). Also, it has been reported that formate can inhibit fungal degradation of plant tissue (33).

Increased production of acetate by the syntrophic association between fungi and methanogenic bacteria improves the potential yield of fungal ATP from 3 to 4 mol/mol of hexose fermented (45); both the rate and extent of fungal growth are thus increased in fungal cocultures. We noted that the fungal biomass in cocultures used as inocula was higher (but only by 16 to 33%) than that in the equivalent monocultures. Increased fungal biomass in cocultures would not explain the increases in enzyme specific activities. Fungal growth rate, however, may make a contribution. In the case of fibrolytic ruminal bacteria, the activities of polysaccharidases are growth rate dependent and vary with the stage of growth (32, 37, 42, 43).

The major determinant affecting the polysaccharolytic activities of ruminal fungi, however, is likely to be the level of certain sugars released into growing cultures. In monocultures and cocultures, the increases in specific activities of xylanolytic enzymes with increasing incubation times for *N.*

frontalis and *S. communis* correlated with decreasing xylose levels in cultures. Changes in specific activity did not correlate with changes in arabinose levels. The greatest increases in the specific activities of enzymes in cocultures relative to those in equivalent monocultures occurred about 3 days after inoculation. At those times, the amount of free xylose in monoculture was greater than that in coculture (Fig. 1 and 3). Hemicellulosic monosaccharides do not inhibit fungal hemicellulase activity (39), but these results can be explained by repression of enzyme synthesis by xylose.

Catabolite repression of sugar utilization and polysaccharidase and glycohydrolase production may be a general feature of ruminal fungi (20, 39, 40). Repression by xylose of xylanase production by *N. frontalis* has been reported previously (23). Similarly, glucose represses the formation of amylase, carboxymethyl cellulase, and β-glucosidase in strains of *N. frontalis* (21, 22). Borneman et al. (5) were unable to detect activity by polycentric or monocentric ruminal fungi against plant tissue until reducing sugars were utilized. We conclude that the stimulation of many enzyme activities in cultures of ruminal fungi grown in coculture with methanogenic bacteria occurred mainly because fungi in such cocultures are better able to utilize sugars (Fig. 3), and thus, enzyme formation is not as constrained by catabolite repression. The increased fungal biomass in cocultures would aid sugar uptake, but this is unlikely to be the complete explanation for enhanced sugar utilization. Further studies are needed to determine the factors that control the rate of fungal sugar uptake and utilization.

In this study, we demonstrated that the activities of ruminal fungal polysaccharolytic enzymes and xylanolysis by the fungi are significantly increased by cocultivation with methanogenic bacteria. As methanogens are often coisolated with fungi from ruminal contents (2; K. N. Joblin, unpublished data), it is probable that such interactions also occur in the ruminal ecosystem. As the effects of the interspecies interactions are so marked, it appears that the production of fungal fibrolytic enzymes for biotechnological applications could best be achieved by the cocultivation of the fungus with methanogenic bacteria.

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LITERATURE CITED

1. **Appel, W.** 1974. Amino acid arylamidases (leucine-nitroanilidase), p. 958–963. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, vol. 2. Academic Press, Inc. (London), Ltd., London.
2. **Bauchop, T.** 1981. The anaerobic fungi in rumen fibre digestion. Agric. Environ. **6**:239–348.
3. **Bauchop, T., and D. O. Mountfort.** 1981. Cellulose fermentation by a rumen anaerobic fungus in both the absence and presence of rumen methanogens. Appl. Environ. Microbiol. **42**:1103–1110.
4. **Biely, P.** 1985. Microbial xylanolytic systems. Trends Biotechnol. **3**:286–290.
5. **Borneman, W. S., D. E. Akin, and L. G. Ljungdahl.** 1989. Fermentation products and plant cell wall-degrading enzymes produced by monocentric and polycentric anaerobic ruminal fungi. Appl. Environ. Microbiol. **55**:1066–1073.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
7. **Dehority, B. A.** 1965. Degradation and utilization of isolated hemicellulose by pure cultures of cellulolytic rumen bacteria. J. Bacteriol. **89**:1515–1520.
8. **Dehority, B. A.** 1973. Hemicellulose degradation by rumen bacteria. Fed. Proc. **32**:1819–1825.
9. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric methods for determination of sugars and related substances. Anal. Chem. **28**:350–356.
10. **Fay, J. P., and F. M. A. Ovejero.** 1986. Effect of lactate on the *in vitro* digestion of *Agropyron elongatum* by rumen microorganisms. Anim. Feed Sci. Technol. **16**:161–167.
11. **Fonty, G., P. Gouet, and V. Sante.** 1988. Influence d'une bactérie méthanogene sur l'activité cellulolytique et le métabolisme de deux espèces de champignons cellulolytiques du rumen in vitro. Résultats préliminarires. Reprod. Nutr. Dev. **28**:133–134.
12. **Hébraud, M., and M. Fèvre.** 1988. Characterization of glycoside and polysaccharide hydrolases secreted by the rumen anaerobic fungi *Neocallimastix frontalis*. *Sphaeromonas communis* and *Piromonas communis*. J. Gen. Microbiol. **134**:1123–1129.
13. **Hungate, R. E.** 1969. A roll-tube method for cultivation of strict anaerobes. Methods Microbiol. **3B**:117–132.
14. **Joblin, K. N.** 1981. Isolation, enumeration and maintenance of rumen anaerobic fungi in roll tubes. Appl. Environ. Microbiol. **42**:1119–1122.
15. **Joblin, K. N., G. P. Campbell, A. J. Richardson, and C. S. Stewart.** 1989. Fermentation of barley straw by anaerobic rumen bacteria and fungi in axenic culture and in co-culture with methanogens. Lett. Appl. Microbiol. **9**:195–197.
16. **Joblin, K. N., and G. E. Naylor.** 1989. Fermentation of woods by rumen anaerobic fungi. FEMS Microbiol. Lett. **65**:119–122.
17. **Lever, M.** 1977. Carbohydrate determination with 4-hydroxybenzoic acid hydrazide (PAHBAH): effect of bismuth on the reaction. Anal. Biochem. **81**:21–27.
18. **Lowe, S. E., M. K. Theodorou, and A. P. J. Trinci.** 1987. Cellulases and xylanase of an anaerobic fungus grown on wheat straw, wheat straw holocellulose, cellulose and xylan. Appl. Environ. Microbiol. **53**:1216–1223.
19. **Morrison, I. M.** 1974. Changes in the hemicellulosic polysaccharides of ryegrass with increasing maturity. Carbohydr. Res. **36**:45–51.
20. **Mountfort, D. O., and R. A. Asher.** 1983. Role of catabolite regulatory mechanisms in control of carbohydrate utilization by the rumen anaerobic fungus *Neocallimastix frontalis*. Appl. Environ. Microbiol. **46**:1331–1338.
21. **Mountfort, D. O., and R. A. Asher.** 1985. Production and regulation of cellulase by two strains of the rumen anaerobic fungus *Neocallimastix frontalis*. Appl. Environ. Microbiol. **49**:1314–1322.
22. **Mountfort, D. O., and R. A. Asher.** 1988. Production of α-amylase by the ruminal anaerobic fungus *Neocallimastix frontalis*. Appl. Environ Microbiol. **54**:2293–2299.
23. **Mountfort, D. O., and R. A. Asher.** 1989. Production of xylanase by the ruminal anaerobic fungus *Neocallimastix frontalis*. Appl. Environ. Microbiol. **55**:1016–1022.
24. **Mountfort, D. O., R. A. Asher, and T. Bauchop.** 1982. Fermentation of cellulose to methane and carbon dioxide by a rumen anaerobic fungus in a triculture with *Methanobrevibacter* sp. strain RA1 and *Methoansarcina barkeri*. Appl. Environ. Microbiol. **44**:128–134.
25. **Orpin, C. G.** 1976. Studies on the rumen flagellate *Sphaeromonas communis*. J. Gen. Microbiol. **94**:270–280.
26. **Orpin, C. G.** 1977. The rumen flagellate *Piromonas communis*: its life-history and invasion of plant material in the rumen. J. Gen. Microbiol. **99**:107–117.
27. **Orpin, C. G., and K. N. Joblin.** 1988. The rumen anaerobic fungi, p. 129–150. *In* P. N. Hobson (ed.), The rumen microbial ecosystem. Elsevier Applied Science, London.
28. **Orpin, C. G., and A. J. Letcher.** 1979. Utilization of cellulose, starch, xylan and other hemicelluloses for growth by the rumen phycomycete *Neocallimastix frontalis*. Curr. Microbiol. **3**:121–124.
29. **Pearce, P. D., and T. Bauchop.** 1985. Glycosidases of the rumen anaerobic fungus *Neocallimastix frontalis* grown on cellulosic

substrates. Appl. Environ. Microbiol. **49**:1265–1269.

30. **Phillips, M. W., and G. L. R. Gordon.** 1988. Sugar and polysaccharide fermentation by rumen anaerobic fungi from Australia, Britain and New Zealand. BioSystems **21**:377–383.
31. **Stewart, C. S., J. Gilmour, and M. L. McConville.** 1986. Microbial interactions, manipulation and genetic engineering, p. 243–257. *In* New developments and future prospects for research on rumen function. Commission of European Communities report EUR 10054. Commission of European Communities, Brussels.
32. **Strobel, H. J., and J. B. Russell.** 1987. Regulation of β-glucosidase in *Bacteroides ruminicola* by a different mechanism: growth rate dependent derepression. Appl. Environ. Microbiol. **53**:2505–2510.
33. **Theodorou, M. K., S. E. Lowe, and A. P. J. Trinci.** 1988. The fermentative characteristics of anaerobic rumen fungi. BioSystems **21**:371–376.
34. **Wallace, R. J., and K. N. Joblin.** 1985. Proteolytic activity of a rumen anaerobic fungus. FEMS Microbiol. Lett. **29**:19–25.
35. **Weimer, P. J., and J. G. Zeikus.** 1977. Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence and presence of *Methanobacterium thermoautotrophicum*. Appl. Environ. Microbiol. **33**:289–297.
36. **Williams, A. G.** 1986. Rumen holotrich ciliate protozoa. Microbiol. Rev. **50**:25–49.
37. **Williams, A. G.** 1989. Hemicellulose utilization by micro-organisms in the alimentary tract of ruminant and non-ruminant animals, p. 183–219. *In* M. P. Coughlan (ed.), Enzyme systems for lignocellulose degradation. Elsevier Applied Science, London.
38. **Williams, A. G., and G. S. Coleman.** 1985. Hemicellulose-degrading enzymes in rumen ciliate protozoa. Curr. Microbiol. **12**:85–90.
39. **Williams, A. G., and C. G. Orpin.** 1987. Polysaccharide-degrading enzymes formed by three species of anaerobic fungi grown on a range of carbohydrate substrates. Can. J. Microbiol. **33**:418–426.
40. **Williams, A. G., and C. G. Orpin.** 1987. Glycoside hydrolase enzymes present in the zoospore and vegetative growth stages of the rumen fungi *Neocallimastix patriciarum*, *Piromonas communis* and an unidentified isolate, grown on a range of carbohydrates. Can. J. Microbiol. **33**:427–434.
41. **Williams, A. G., and N. H. Strachan.** 1984. The distribution of polysaccharide-degrading enzymes in the bovine rumen digesta ecosystem. Curr. Microbiol. **10**:215–220.
42. **Williams, A. G., and S. E. Withers.** 1985. Formation of polysaccharide depolymerase and glycoside hydrolase enzymes of *Bacteroides ruminicola* subsp. *ruminicola* in batch and continuous culture. Curr. Microbiol. **12**:79–84.
43. **Williams, A. G., and S. E. Withers.** 1989. Factors affecting the formation of polysaccharide depolymerase and glycoside hydrolase enzymes by *Butyrivibrio fibrisolvens* NCDO 2249. J. Appl. Bacteriol. **67**:299–308.
44. **Wolin, M. J., and T. L. Miller.** 1983. Interactions of microbial populations in cellulose fermentation. Fed. Proc. **42**:109–113.
45. **Wolin, M. J., and T. L. Miller.** 1988. Microbe-microbe interactions, p. 343–359. *In* P. N. Hobson (ed.), The rumen microbial ecosystem. Elsevier Applied Science, London.
46. **Wood, T. M.** 1988. Preparation of crystalline, amorphous, and dyed cellulase substrates. Methods Enzymol. **160**:19–25.
47. **Wood, T. M., C. A. Wilson, S. I. McCrae, and K. N. Joblin.** 1986. A highly active extracellular cellulase from the anaerobic rumen fungus *Neocallimastix frontalis*. FEMS Microbiol. Lett. **34**:37–40.