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Evidence that Cellulolysis by an Anaerobic Ruminal Fungus Is Catabolite Regulated by Glucose, Cellobiose, and Soluble Starch

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A *Piromyces*-like ruminal fungus was used to study preferential carbohydrate utilization of [U-^{14}C]cellulose, both alone and in combination with several soluble sugars. For cells grown on cellulose alone, cellulolytic activity was immediate and, initially, greater than that observed in the presence of added carbohydrate. Cellulolytic activity remained minimal in cultures containing cellulose plus glucose or cellobiose until the soluble sugar was depleted. Soluble starch also regulated cellulose activity but to a lesser extent. The results presented suggest that some fungal cellulases are susceptible to catabolite regulatory mechanisms.

Preferential utilization of soluble carbohydrates by ruminal microorganisms has been previously demonstrated (6, 9), and these preferences allow ruminal microorganisms to occupy separate niches even though they are capable of utilizing the same substrates. When cellulose or some other insoluble carbohydrate has been included in similar studies (3, 5), time-consuming analytical techniques and interference from microbial biomass have limited the sensitivity of the assays. Recently, duPreez and Kistner (2) described the application of [U-^{14}C]cellulose for the study of total cellulase activity for a growing culture of *Ruminococcus flavefaciens* FD-1. We present here an extension of the technique, which should simplify study of the regulation of cellulose hydrolysis in the presence of an alternative carbohydrate source. This report describes the first such experiments with a pure culture of an anaerobic ruminal fungus previously maintained upon cellulose.

Ruminal fluid was obtained from a goat fed a diet of kikuyu grass (*Pennisetum clandestinum*) and strained through two layers of cheesecloth. Samples (0.1 ml) were taken to inoculate 10-ml volumes of 10X broth (8) containing 1.5×10^4 IU of penicillin per ml and 2 mg of streptomycin sulfate (Sigma Chemical Co., St. Louis, Mo.) per ml. Following several days of incubation at 39°C, 0.1-ml samples of the cultures were spotted onto anaerobic plates of the same medium and incubated at 39°C in an anaerobic cabinet (1024; Forma, Marietta, Ohio) with a 30% CO_2–65% N_2–5% H_2 atmosphere. Isolated fungal colonies were picked and transferred to 10X broth. Purity was ascertained by microscopic examination, and cultures were maintained by transferring subsamples to freshly prepared 10X medium every 3 or 4 days. The isolate chosen for study produced zoospores with a single flagellum (Fig. 1), while the vegetative morphology appeared branched, with singular sporangia developing at multiple sites (Fig. 2).

Carbohydrate utilization studies were performed with medium 10 (1) with carbohydrates deleted. [U-^{14}C]cellulose was prepared by culturing the gram-negative aerobe *Acetobacter xylinum* on rich medium containing [U-^{14}C]glucose as previously described (2). The cellulose pellicle was boiled in 1 M KOH for 1 h to remove bacterial biomass and then washed repeatedly with deionized water until the pH of the washings was less than 7. Radiolabelled cellulose (specific activity, 8.5 μCi/mg) was included in the growth medium at a final concentration of 1.5 g/liter before sterilization. When cellulose was the sole carbohydrate source, 0.5 ml of sterile, anaerobic diluent was added to the culture. Carbohydrate combinations were similarly established by aseptic addition of glucose, cellobiose, or soluble starch to a final concentration of 1.5 g/liter. For each treatment, duplicate tubes 9.5 ml in volume were inoculated with 0.5 ml of a 4-day-old culture grown upon pebble-milled Whatman no. 1 cellulose filter paper for three successive transfers before experimentation. One-milliliter volumes were immediately collected from all cultures, and over a period of 120 h, a further eight 0.7-ml volumes were taken from each culture. The samples were made alkaline by addition of concentrated NaOH and then stored at −20°C before analysis. Solubilization of ^{14}C was determined from 0.2-ml volumes of supernatant collected after two centrifugations at 10,000 × *g* for 2 min in an Eppendorf microcentrifuge. The second centrifugation step was performed to ensure removal of small particles. The sample was then diluted to 2.0 ml with Scintillator 299 (Packard Instrument Co., Inc., Rockville, Md.) scintillation fluid for counting with a Packard 2000 CA liquid scintillation counter. Samples were automatically corrected for background and plotted against incubation time. The remaining supernatant was used to estimate the concentrations of alternative, soluble carbohydrates. Samples containing either cellobiose or starch were assayed for glucose concentration and then acid hydrolyzed to convert the sugars to their glucose equivalents (0.5 M H_2SO_4 at 121°C for 1 h). Glucose concentrations in all samples were determined with a glucose oxidase kit (catalog no. 139 106) as recommended by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The limit of detection was 0.01 g/liter.

The morphology of the fungal isolate chosen for study was consistent with that of *Piromonas* spp. (7) (now reclassified as *Piromyces* spp.), which are thought to produce cellulolytic and xylanolytic enzymes constitutively (4). However, the pattern of cellulose hydrolysis by our isolate was markedly different. When cellulose was provided as the sole energy source (Fig. 3), cellulose hydrolysis was initiated rapidly. For at least 24 h, it was significantly greater than that observed for all other treatments. The initial rates of

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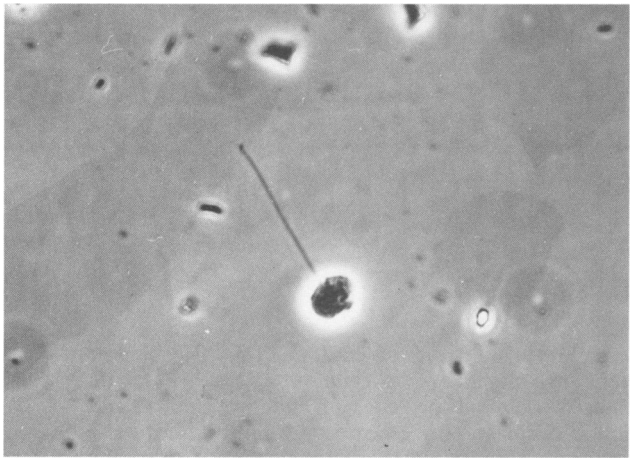

FIG. 1. Phase-contrast micrograph of the zoospore produced by the anaerobic ruminal fungus isolated from a goat and used in the studies reported here. The singular flagellum is clearly evident. Magnification, ×500.

cellulose hydrolysis, considered to be reflected by the rate constant for release of ^{14}C disintegrations per minute within the first 24 h, were 0.056 with cellulose alone and 0.005, 0.019, and 0.025 with cellulose in combination with cellobiose, glucose, and starch, respectively. However, between 24 and 48 h, the fungal isolate, when grown on dual carbohydrate combinations, rapidly approached maximal levels of cellulose hydrolysis, and visually, cellulose had completely disappeared by 48 h. A similar level of cellulose solubilization with cellulose alone required 96 h (Fig. 3). Analyses showed that cellulose solubilization did not produce detectable concentrations of free glucose (Fig. 4a). The patterns of utilization of alternate carbohydrate source are illustrated in Fig. 4b to d. Glucose and cellobiose were rapidly depleted from these batch cultures, concomitant with minimal solubilization of cellulose (Fig. 4b and 4c). The concentration of glucose, measured before acid hydrolysis, for the cellobiose-cellulose combination was increased slightly, from 0.07 g/liter at time zero to a maximum concentration of 0.34 g/liter

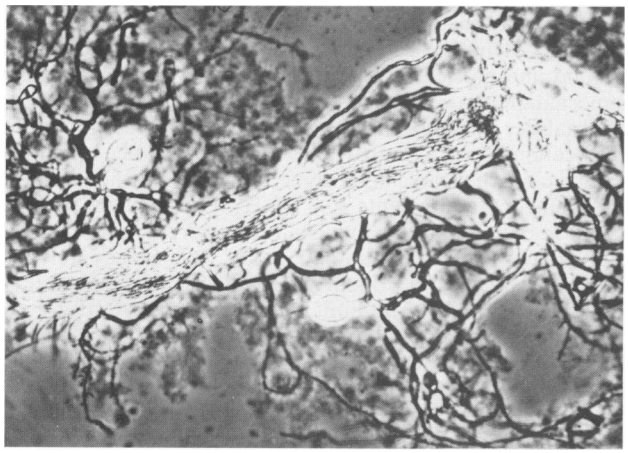

FIG. 2. Phase-contrast micrograph of the vegetative growth stage of the anaerobic ruminal fungus used. A branching mycelium, with multiple sites of singular sporangium development, is illustrated. Magnification, ×125.

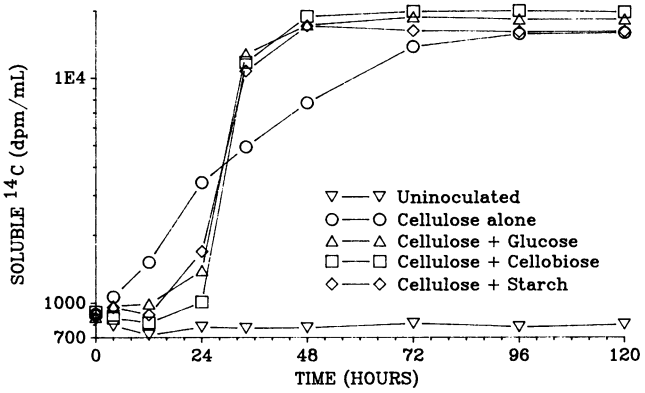

FIG. 3. The pattern of cellulose solubilization, measured by the solubilization of ^{14}C from radiolabeled cellulose, by an anaerobic ruminal fungus grown on cellulose alone or in combination with glucose, cellobiose, or starch.

after 24 h. During the same period, 92% of the added cellobiose was fermented; thus, cellulase activity was catabolite regulated by cellobiose per se and not by substantial accumulation of glucose. Starch appeared to be more slowly utilized than the simple sugars. A substantial amount was still unutilized at the time cellulolytic activity increased, and subsequently, cellulose and starch disappeared at similar rates (Fig. 4d).

It has been documented that ruminal fungi utilize a variety of simple sugars and exhibit preferential utilization of such sugars (6). Radiolabeled cellulose provided a rapid, sensitive method to determine the breakdown of cellulose in the presence of other glucose-containing sugars. The rapid yet sequential utilization of substrates by the fungal isolate when grown on cellulose in combination with either glucose or cellobiose is consistent with effective regulation of cellulase activity by simple sugars. The pattern of substrate utilization by the isolate when grown on cellulose plus starch was different, perhaps because of the complexity of both of the available substrates. These data indicate that any inhibitory effect from starch was weaker than that found with either glucose or cellobiose. Whereas additional carbohydrates ultimately resulted in reduction of the time required to solubilize cellulose completely, cellulolysis appeared to be inhibited for as long as 24 h in these batch cultures for all of the soluble carbohydrates tested. Substantial differences in biomass production were likely to have existed between cultures provided two carbohydrate sources and cultures provided cellulose alone; furthermore, the patterns of cellulolysis after 24 h are probably influenced to some extent by such differences.

There is general agreement that a contribution from the rumen fungi to animal production lies with their active colonization and degradative action on plant fiber. Defaunation of the rumen environment has been promoted as a means to increase fungal biomass and, thus, fiber digestion. In light of the present results and the range of simple sugars found to support the growth of many different strains of fungi, the cellulase enzymes of many strains could be readily inhibited when the niche occupied by the ruminal protozoa is exposed.

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The technical assistance provided by C. Hudson and the Depart-

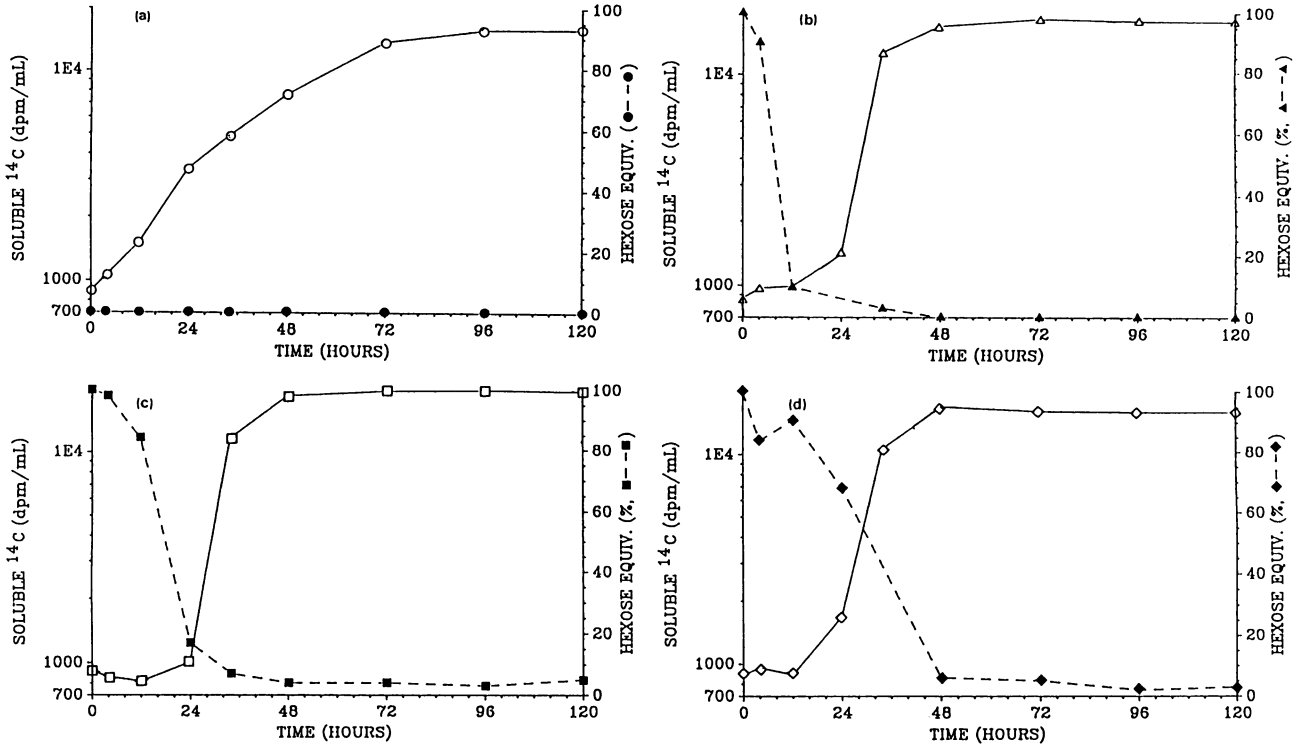

FIG. 4. The patterns of carbohydrate utilization by an anaerobic ruminal fungus grown on [U-^{14}C]cellulose alone (a) or in combination with glucose (b), cellobiose (c), or starch (d). Disappearance of glucose, cellobiose, and starch was determined by the decline of the glucose concentration in culture fluids with time. The initial concentrations of glucose, cellobiose, and starch were determined by analysis to be 1.2, 1.9, and 3.0 g/liter, respectively.

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ERRATA

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Volume 56, no. 10, p. 3227, column 2, line 4: "8.5 μCi/mg" should read "8.5 μCi/g."

Chlorophenol Degradation Coupled to Sulfate Reduction

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Volume 56, no. 11, p. 3257, column 1, Results, line 56: "86 to 124%" should read "89 to 129%."
Page 3258: Table 1 should read as follows:

TABLE 1. Sulfate depletion during chlorophenol degradation[a]

CP fed	CP metabolized (mM)	Sulfate loss (mM)		% of expected
		Predicted[b]	Measured[c]	
Freshwater				
2-CP	0.86	2.8	2.5	89
3-CP	0.82	2.7	2.4	89
4-CP	0.85	2.8	3.6	129
Saline				
2-CP	0.83	2.7	3.2	118
3-CP	1.03	3.3	4.2	127
4-CP	0.87	2.8	2.9	103

[a] All values are means from duplicate cultures, except for 2-CP (freshwater), for which activity in one of the duplicates was lost.
[b] Calculated from the following equation: $C_6H_5ClO + 3.25\ SO_4^{2-} + 4\ H_2O \rightarrow 6\ HCO_3^- + 3.25\ H_2S + Cl^- + 0.5\ H^+$.
[c] Sulfate loss (1.5 mM) in background cultures subtracted.