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Inhibitory Effects of Methylcellulose on Cellulose Degradation by *Ruminococcus flavefaciens*

M. A. RASMUSSEN,[1†] R. B. HESPELL,[1,2*] B. A. WHITE,[1] AND R. J. BOTHAST[2]

Department of Animal Sciences, University of Illinois, Urbana, Illinois 61801,[1] and Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois 61604[2]

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Highly methylated, long-chain celluloses strongly inhibited cellulose degradation by several species of cellulolytic bacteria of ruminal origin. Specifically, the inhibitory effects of methylcellulose on the growth of *Ruminococcus flavefaciens* FD1 were concentration dependent, with complete inhibition at 0.1% (wt/vol). However, methylcellulose did not inhibit growth on cellobiose or cellulooligosaccharides. Mixtures of methylated cellulooligosaccharides having an average degree of polymerization of 6.7 to 9.5 inhibited cellulose degradation, but those with an average degree of polymerization of 1.0 to 4.5 did not. Similar inhibitory effects by methylcellulose and, to a lesser extent, by methyl cellulooligosaccharides were observed on cellulase activity, as measured by hydrolysis of *p*-nitrophenyl-β-D-cellobioside. *R. flavefaciens* cultures hydrolyzed cellulooligosaccharides to cellobiose and cellotriose as final end products. Cellopentaose and cellohexaose were cleaved to these end products, but cellotetraose was also formed from cellohexaose. Methylcellulose did not inhibit hydrolysis of cellulooligosaccharides. These data are consistent with the presence of separate cellulase (β-1,4-glucanase) and cellulodextrinase activities in *R. flavefaciens*.

The degradation of cellulose in the rumen is quite variable, in defiance of its apparent structural simplicity, relative to other plant cell wall polymers such as hemicelluloses and lignins. Much of this variability may be attributed to physical factors, such as crystallinity or association with other polymers such as lignin (43). In addition, carbohydrate derivatization with substituents that restrict degradation may also be important in altering degradation. For example, xylans have been shown to be esterified to ferulic and *p*-coumaric acids, the primary monomers of lignin (4, 40), and it is thought that this esterification inhibits xylan degradation. The incorporation of O-methylated monosaccharides into carbohydrate polymers results in increased resistance to biological degradation (8). Several workers, using a variety of aerobic fungi and bacteria, have demonstrated that an increasing degree of substitution (DS) of cellulose results in decreased degradation (28, 36, 44). However, little information is available on the effect of ether derivatives on anaerobic cellulose degradation. Minato and Suto (32) and Kudo et al. (27) have demonstrated that methylcellulose can interfere with the adherence of ruminal bacteria to cellulose. Groleau and Forsberg (22) have reported that methylcellulose inhibits the cellulolytic activity of *Bacteroides succinogenes*.

The cellulolytic system of *Ruminococcus flavefaciens* has been shown to be complex and involve several enzymes. The enzyme system exists in multiple forms, distinguished by molecular mass (35). The products of cellulolytic activity from *R. flavefaciens* are cellobiose, cellotriose, and a small amount of glucose resulting from the combined action of endo-β-1,4-glucanase, exo-β-1,4-glucanase, and cellulodextrinase (35; this study). Thiol-reducing agents and metal ions (Ca^{2+}) activate the cellulase complex. An aryl-β-glucosidase (4) and a cellobiose phosphorylase (3) have also been reported to be produced by this bacterium, and an exo-β-1,4-glucanase from *R. flavefaciens* FD1 has been purified (19). Recently, an endo-β-D-glucanase from *R. flavefaciens* FD1 has been cloned and expressed in *Escherichia coli* (6).

The effect of cellulose methyl ether derivatives on anaerobic cellulose hydrolysis by ruminal microorganisms was investigated in this research. Specifically, we examined the effect of methylcellulose and methyl cellulooligosaccharides on cell growth, cellulase activity, and hydrolysis of cellulose and of cellulooligosaccharides by *R. flavefaciens*.

(Preliminary reports of these findings have been presented [M. A. Rasmussen and R. B. Hespell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, K-146, p. 217; M. A. Rasmussen and R. B. Hespell, Abstr. Annu. Meet. Am. Soc. Anim. Sci. 1986, abstr. no. 608, p. 410].)

MATERIALS AND METHODS

Organisms. *R. flavefaciens* FD1, *Ruminococcus albus* 7, and *Bacteroides succinogenes* S85 were obtained from the culture collection of M. P. Bryant, Department of Animal Sciences, University of Illinois, Urbana. *Butyrivibrio fibrisolvens* 12 and *Clostridium polysaccharolyticum* B were obtained from N. O. van Gylswyk, Council for Scientific and Industrial Research, National Chemical Research Laboratory, Pretoria, Republic of South Africa. *R. flavefaciens* RF1 was freshly isolated from ruminal contents on cellulose-containing basal medium. All microorganisms were stored at −20°C in liquid medium containing 20% glycerol (41).

Media and growth measurements. Throughout the following experiments, techniques for anaerobiosis were maintained when necessary in the preparation of media, buffers, and inocula (11, 13). When it was necessary to perform open manipulations anaerobically, plastic gloveboxes (Coy Laboratories, Ann Arbor, Mich.) were used and contained atmospheres of 95% N_2 and 5% H_2 or 95% CO_2 and 5% H_2.

The microbiological medium used for all experiments

* Corresponding author.
† Present address: Animal Nutritional Supplements, Eastman Chemicals, P.O. Box 1955, Kingsport, TN 37664.

TABLE 1. Basal medium[a]

Ingredient	Amt (ml/liter)
Mineral 1 solution[b]	50.0
Mineral 2 solution[b]	50.0
Resazurin solution (0.1% [wt/vol])	1.0
Fatty acid solution[c]	30.0
Vitamin solution[c]	1.0
3-Phenylpropionic acid solution (2 mM)[d]	42.5
Phenylacetate solution (2 mM)[d]	37.5
Hemin and 1,4-naphthoquinone solution[e]	10.0
Trace mineral solution[f]	5.0
Sodium carbonate solution (8.0% [wt/vol])[g]	50.0
Cysteine hydrochloride and sodium sulfide solution[h]	20.0

[a] Prepared under 100% CO_2; final pH, 6.8.

[b] Solutions from Bryant and Burkey (12).

[c] Solutions from Lowe et al. (29), except that 1,4-naphthoquinone was omitted from the vitamin solution, which was filter sterilized (0.22-μm pore size) under N_2 and aseptically added to sterile medium.

[d] Dissolved in a minimal volume of ethanol before addition of water.

[e] Hemin (10 mg) and 1,4-naphthoquinone (25 mg) dissolved in 50 ml of ethanol–50 ml of 0.05 M NaOH.

[f] Trace mineral solution made in 20 mM HCl contained (in milligrams per liter): $MnCl_2 \cdot 4H_2O$, 250; $FeSO_4 \cdot 7H_2O$, 200; $ZnCl_2$, 25; $CuCl_2 \cdot 2H_2O$, 25; $CoCl_2 \cdot 6H_2O$, 50; SeO_2, 50; $NiCl_2 \cdot 6H_2O$, 250; $NaMoO_4 \cdot 2H_2O$, 250; $NaVO_3 \cdot 4H_2O$, 50; H_3BO_3, 250.

[g] Prepared as a separate, CO_2-equilibrated solution before addition to cooled medium.

[h] Cysteine hydrochloride (1.25 g) and $Na_2S \cdot 9H_2O$ (1.25 g) are prepared in 100 ml of boiled water under N_2. Cysteine hydrochloride is first dissolved, the pH is adjusted to 10.0 with NaOH, and sodium sulfide is added. The solution is then sterilized under N_2 and added aseptically to sterile medium.

unless otherwise indicated was basal medium (Table 1). Cellobiose was prepared separately as a 100 mM aqueous solution and added aseptically to sterile medium when appropriate. Cellulose (Whatman no. 1 filter paper) was included in the basal medium when appropriate before sterilization in one of two forms. For quantitative cellulose degradation experiments, a cellulose slurry (2% [wt/vol] filter paper in water, pebble-milled for 48 h) was used. This slurry was added to obtain a final concentration of 0.2% (wt/vol) cellulose. For all other experiments where cellulose was required as the substrate, acid-swollen disks (Whatman no. 1 filter paper, 6.5 mm diameter, 85% phosphoric acid for 1 h) were used. After neutralization with a sodium bicarbonate solution and washing with distilled water, these disks were stored at 4°C in water. When needed, the disks were added to culture media prior to autoclaving at a concentration of 0.15% (wt/vol).

Methylcellulose (15 and 400 cP; DS, 1.6 to 1.9; Sigma Chemical Co., St. Louis, Mo.) was washed before use to remove derivatizing agents and soluble sugars. Methylcellulose powder was suspended in hot water (100°C), filtered, and dried (40°C). This material was then suspended in absolute ethanol, filtered, and dried again. Carboxymethyl cellulose (CMC; 15 cP; DS, 0.7; Sigma Chemical Co.) was washed by the same procedure. Both cellulose derivatives were then prepared as aqueous solutions (1% [wt/vol]), sterilized under an N_2 atmosphere, and added to sterile medium when appropriate.

Cultures were grown anaerobically under 100% CO_2 at 39°C. Growth was monitored turbidimetrically, when appropriate, at 600 nm with a Bausch & Lomb Spectronic 70 or a Gilford model Stasar II. Residual cellulose in cultures was determined after acid hydrolysis by the phenol-sulfuric acid method of Ashwell (1).

HPLC. High-performance liquid chromatography (HPLC) analysis of various samples for sugars and oligosaccharides was performed with a Beckman model 324 chromatograph fitted with a model 100-A pump, a Hewlett-Packard model 79877A refractive index detector, and a Bio-Rad Aminex HPX-42A oligosaccharide column (300 by 7.8 mm) set at 84°C. HPLC-grade water was used as the eluant at a flow rate of 1 ml/min. When required, samples for HPLC analysis were desalted by treatment with ion-exchange resins to avoid damage to the HPLC column (37).

Acid hydrolysis of methylcellulose. A procedure was developed for the partial hydrolysis of methylcellulose (400 cP) to obtain methylated cellulooligosaccharides. This procedure followed the principles of Gibbons (21). A methylcellulose solution (200 ml; 2% [wt/vol]) was mixed with 200 ml of 4 N HCl. This acidic solution was then incubated at 50°C for up to 21 days, depending on the batch. This procedure allowed gentle, slow hydrolysis of methylcellulose. After incubation, the solution was neutralized with 4 N NaOH and then frozen at −70°C and lyophilized to dryness. The residue was extracted with two 500-ml portions of absolute ethanol (to separate the methylated cellulooligosaccharides from the remaining methylcellulose and salts of neutralization), and the insoluble fraction was removed by centrifugation. The supernatant fluid was concentrated to dryness by vacuum evaporation at 45°C. The dried material was then taken up in 10 ml of water and assayed for total carbohydrates (1) and reducing sugars (33). Glucose was used as the standard for both assays. The average degree of polymerization (DP) of the acid hydrolysates was calculated as the ratio of total to reducing carbohydrate concentrations.

A complete hydrolysis of methylcellulose to methylglucose (DP of 1) was also performed by an alternative procedure with 5.5% (vol/vol) sulfuric acid at 60°C (15). All acid hydrolysates were filter sterilized (0.22-μm pore size) and stored at 4°C until needed.

Preparation of unsubstituted cellulooligosaccharides. Mixtures of unsubstituted cellulooligosaccharides were obtained from α-cellulose powder (Sigma) by the method of Hamacher et al. (23). The percent composition by weight of the resultant mixture as determined by HPLC was: cellobiose, 1.9%; cellotriose, 19.1%; cellotetraose, 28.2%; cellopentaose, 25.2%; cellohexaose, 18.0%; and celloheptaose, 7.6%.

Individual unsubstituted cellulooligosaccharides were obtained by chromatography of the mixture on a carbon-celite-stearic acid column (106 by 4.5 cm) by the method of Miller (31). The initial mixture was prepared by acid hydrolysis of Whatman cellulose powder. Isolated oligosaccharides were tentatively identified by thin-layer chromatography (TLC) in a Keiselguhr model F254 with isopropanol-ethyl acetate-water (42:35:23) (24). TLC plates were developed with 10% (vol/vol) sulfuric acid and heat (120°C, 5 min). The individual oligosaccharides were subsequently analyzed for purity by HPLC.

Methylation of cellulooligosaccharides. Methyl cellulooligosaccharides were synthesized from their unsubstituted counterparts by the Haworth procedure for methylation and extraction as described by Hirst and Percival (24). Dimethyl sulfate was used as the methylation agent. After methylation, the products were extracted with chloroform, clarified by filtration (0.65-μm pore size), and dried by vacuum evaporation at 45°C. The methyl cellulooligosaccharides obtained by this procedure were fully substituted (i.e., >3 methyl substituents per hexose) as verified by TLC (silica gel GF; doubly developed in chloroform-methanol [17:3]).

Depolymerization experiments. An assay procedure was

developed to determine whether *R. flavefaciens* FD1 was capable of depolymerizing methylcellulose without bacterial growth. Reducing sugar assays proved inadequate, and an alternate procedure was developed. This procedure exploited the fact that methylcellulose is insoluble in ethanol, whereas shorter cellulooligosaccharides are ethanol soluble. Cells harvested from cellulose-grown cultures were suspended in the basal medium (Table 1) which contained methylcellulose (15 cP, 0.1% [wt/vol]). Tubes of suspended cells were incubated at 39°C, and samples were collected at 0 and 24 h. Absolute ethanol (2 ml) was then added to 0.5 ml of cell suspension to precipitate the remaining methylcellulose. The mixture was clarified by centrifugation, and the carbohydrate content of the supernatant was determined (1). CMC was used as a positive control for these experiments. An increase in the ethanol-soluble carbohydrate concentration was interpreted as evidence of biological hydrolysis of the cellulose derivative.

Cellulodextrin hydrolysis experiments. A cellulooligosaccharide mixture or individual cellulooligosaccharides were incubated with an active culture of *R. flavefaciens* FD1 to determine hydrolysis patterns. The incubation mixture (1.25 ml total volume) contained (i) 0.5 ml of PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer solution and, when appropriate, 0.4% (wt/vol) methylcellulose (400 cP), (ii) 0.5 ml of PIPES buffer solution with the desired cellulooligosaccharide (5 mg/ml) or cellulooligosaccharide mixture (10 mg/ml, composition given above), and (iii) 0.25 ml of an active, cellulose-grown culture of *R. flavefaciens* FD1. The mixture was allowed to incubate anaerobically at 39°C in a glovebox (95% N_2, 5% H_2). Sample tubes were collected for analysis at 0, 1, and 2 h and temporarily placed on ice. The tubes were then rapidly heated to 100°C for 5 min to stop activity. The samples were then desalted as previously described for analysis by HPLC.

Cellulase assay. The typical assays for measuring cellulase activity (e.g., reducing sugar release from CMC, glucose release from cellulose, and viscosity reduction of CMC solutions) were unsuited for use when methylated carbohydrates were introduced as inhibitors.

The substrate *p*-nitrophenyl-β-D-cellobioside (PNPC) was chosen for use based on the work of Deshpande et al. (16). However, PNPC has one major disadvantage common to the study of cellulolytic enzymes. This chromophoric substrate has been shown to be nonspecific for enzyme type, as demonstrated by Deshpande et al. (16) and Ohmiya et al. (34). All three classes of fungal cellulolytic enzymes (endocellulases, exocellulases, and β-glucosidases) have been shown to be active towards PNPC. Therefore, the results obtained with the use of this substrate have to be interpreted with some caution. We have assumed that PNPC hydrolysis reflects overall cellulolysis, and only cellobiose and *p*-nitrophenol were found as products in the assay procedure described below.

Assay mixtures (250 μl total volume) contained (i) 100 μl of a substrate solution which contained 20 mM PNPC in 50 mM anaerobic PIPES buffer (pH 6.8), 15% (vol/vol) each mineral nos. 1 and 2, and 1% cysteine hydrochloride solution (Table 1), (ii) 100 μl of water or an aqueous solution of the appropriate inhibitor or additive, and (iii) 50 μl of enzyme source. Assay mixtures were incubated for various times up to 1 h at 39°C in an anaerobic glovebox (95% N_2, 5% H_2). After cooling the mixture on ice to stop the reaction, 100 μl of water was added to bring the total volume to 350 μl. The concentration of *p*-nitrophenol was determined at 410 nm with a Gilford Response spectrophotometer fitted with 500-μl thermal cuvettes (26°C). One unit of enzyme activity was defined as 1 μmol of nitrophenol produced per min, based on a standard curve obtained by using actual nitrophenol concentrations. The assay was determined to be linear with respect to time and protein concentration. Addition of alkali, which can be used to stop the reaction and increase color development, was not used because of the turbidity caused when methylcellulose was present.

TABLE 2. Effect of methylcellulose on degradation of cellulose by cultures of ruminal bacteria

Organism	% Degradation of pebble-milled cellulose[a]	
	No methylcellulose added	Methylcellulose added[b]
R. flavefaciens FD1	100	7
R. flavefaciens RF1	100	2
Bacteroides succinogenes S85	80	0
Clostridium polysaccharolyticum B	100	34
Butyrivibrio fibrisolvens 12	30	0
Ruminal inoculum	100	2

[a] Initial concentration of cellulose, 0.1% (wt/vol).
[b] Methylcellulose (400 cP); 0.1% (wt/vol) final concentration.

The enzyme source used in these assays was whole-cell cultures of *R. flavefaciens* FD1 grown in basal medium containing cellulose disks until the disks had disintegrated. In certain cases, activity was measured on cell and supernatant fractions separately. Cultures were harvested anaerobically (10,000 × *g*, 10 min, 10°C), and the supernatant fluid and resuspended cells were used for analysis.

Protein determination. Protein content was determined by the method of Bradford (9) after hydrolysis of cells in base (0.1 N NaOH, 70°C, 30 min). Cytochrome *c* (horse heart) was used as the standard for protein determination.

RESULTS

Growth experiments. Methylcellulose was a strong inhibitor of cellulose degradation. Several species of cellulolytic bacteria as well as mixed species from ruminal contents showed pronounced sensitivity to methylcellulose (Table 2). Methylcellulose as the sole substrate did not support growth of a variety of bacterial species (including *Bacteroides succinogenes* S85, *Butyrivibrio fibrisolvens* 12, *R. albus* 7, and *R. flavefaciens* FD1 and RF1). The recalcitrant nature of methylcellulose was also observed in enrichment cultures (basal medium plus 0.2% [wt/vol] methylcellulose) inoculated with ruminal contents, horse manure, or sewage sludge. These enrichment cultures failed to grow within 30 days of incubation. Parallel cultures having either ruminal contents or horse manure as inoculum and containing both methylcellulose and cellulose disks showed no growth, and the cellulose remained undegraded. In contrast, methylcellulose failed to inhibit the growth of a ruminal inoculum when soluble starch or oats spelt xylan was present as the sole substrate.

When CMC was tested for inhibition of cellulose degradation with a ruminal content inoculum, no inhibition was observed. In contrast to the effects of methylcellulose, cellulose disks were rapidly degraded in the presence of CMC. However, CMC, like methylcellulose, did not support growth when used as the sole substrate.

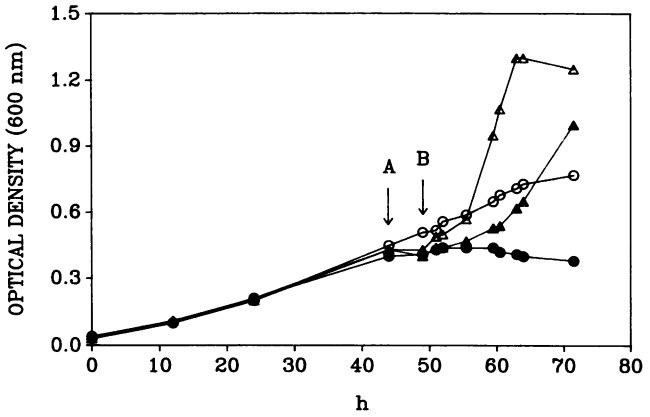

FIG. 1. Growth of cellulose-grown *R. flavefaciens* FD1 on cellobiose or cellulooligosaccharides after the addition of methylcellulose. Cells were grown with cellulose to 44 h (point A), at which time methylcellulose (0.2% [wt/vol]) was added. After a short incubation period (5 h), cellobiose (4.75 mM) or cellulooligosaccharides (DP 3 to 7, 8.3 mM glucose equivalent) were added (point B). Symbols: ○, cellulose-grown cells with no methylcellulose or soluble substrates added: ●, addition of methylcellulose only; △, addition of methylcellulose and cellobiose; ▲, addition of methylcellulose and a cellulooligosaccharide mixture.

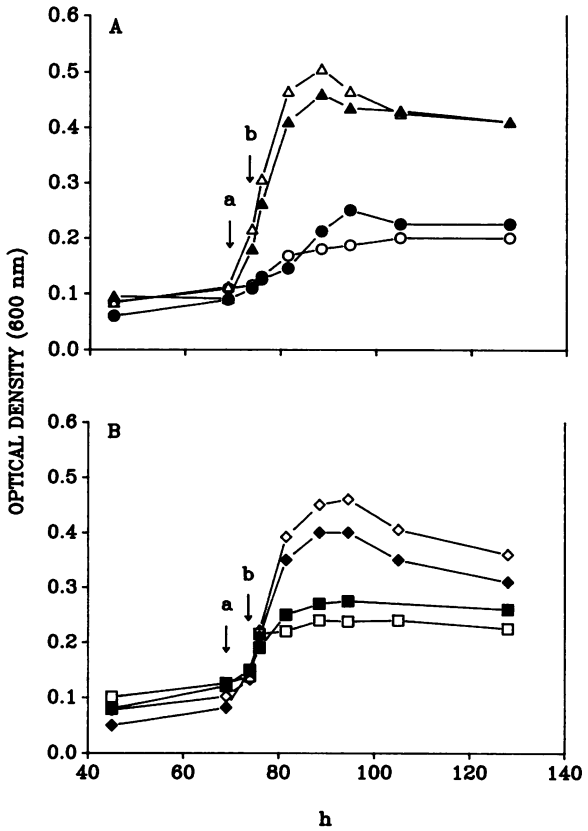

FIG. 2. Effect of methyl cellulodextrins on the growth of cellulose-grown *R. flavefaciens* FD1 on individual cellulooligosaccharides. Cells were grown with cellulose to 69 h (point a), at which time (A) cellobiose (○) or cellotriose (△) was added or (B) cellotetraose (□) or cellopentaose (◇) (10 mM final concentration) was added. After a 5-h incubation (point b), a mixture of methyl cellulooligosaccharides (10 mM final concentration, DP 3 to 7) was added to some cultures (solid symbols).

To study the effect of methylcellulose more thoroughly, *R. flavefaciens* FD1 was chosen for further experimentation. The inhibition of growth of *R. flavefaciens* was concentration dependent, and 0.1% (wt/vol) methylcellulose was required for complete inhibition on pebble-milled cellulose. The growth response of *R. flavefaciens* FD1 on cellulose with various concentrations of methylcellulose (maximal optical density and concentration of methylcellulose, respectively) was: 0.86 at 0% (wt/vol); 0.65 at 0.01%; 0.58 at 0.02%; 0.40 at 0.03%; 0.24 at 0.04%; 0.16 at 0.05%; 0.1 at 0.6%; and 0.05 at 0.1%. In contrast, the growth of *R. flavefaciens* FD1 on soluble substrates was unaffected by the presence of methylcellulose. Active, cellulose-degrading cultures of this bacterium suppressed by the addition of methylcellulose could reinitiate growth when cellobiose or cellulodextrins were added (Fig. 1). Furthermore, the growth rates of batch cultures of *R. flavefaciens* FD1 on cellobiose and cellulooligosaccharides were 0.23 h^{-1} and 0.24 h^{-1}, respectively. When methylcellulose was added (0.1% [wt/vol] methylcellulose 400 cP), the growth rates were essentially unchanged (0.25 and 0.22 h^{-1}, respectively). When *R. flavefaciens* was grown on cellulose disks for about 69 h and then either cellobiose, cellotriose, cellotetraose, or cellopentaose was added (10 mM, final concentration), the growth rate increased dramatically (Fig. 2). The rates were about the same for cellotriose and cellopentaose, and these were about threefold greater than those observed with cellobiose. The concomitant addition of chemically synthesized methyl cellulooligosaccharides (DP, 3 to 7) with these substrates resulted in slight stimulation of growth on cellobiose and cellotetraose and only slight inhibition of growth on cellotriose and cellopentaose (Fig. 2).

Methylcellulose was resistant to biological depolymerization by *R. flavefaciens* FD1. The concentration of ethanol-soluble carbohydrates (i.e., release of sugars from methylcellulose) after incubation with cultures was unchanged (111 mM at 0 h versus 115 mM at 24 h). Similar experiments with CMC as a positive control indicated rapid hydrolysis. To investigate further the effects of methylcellulose, short fragments of methylcellulose (i.e., methylated cellulooligosaccharides) were made to determine whether the inhibitory activity of methylcellulose could be relieved by its hydrolysis to small fragments. The inhibitory activity of the hydrolysates on cellulose degradation was related to the size of the cellulooligosaccharides present in the hydrolyzed preparations (Table 3). Hydrolysate preparations of larger size

TABLE 3. Inhibition of cellulose degradation by methylcellulose or its acid hydrolysates[a]

Inhibitor[b]	% Degradation of pebble-milled cellulose[c]
None	98
Methylcellulose, 15 cP	9
Methylcellulose hydrolysate	
DP 9.5	35
DP 8.5	46
DP 6.7	71
DP 4.5	96
DP 1.0	99

[a] Incubated for 14 days with *R. flavefaciens* FD1.
[b] Inhibitor concentrations, 9 to 10 mM glucose equivalent.
[c] Initial concentration of cellulose, 0.1% (wt/vol).

demonstrated greater degrees of inhibition than the smaller preparations. Little or no inhibition of cellulose degradation was observed with methylcellulose completely hydrolyzed to methylglucose. Additionally, 3-*O*-methyl-D-glucose did not inhibit growth of *R. flavefaciens* FD1 on cellulose.

Although it appeared that inhibitory activity was related to DP, it was recognized that the acid hydrolysates were mixtures with a wide range of different-sized cellulooligosaccharides present. To address this problem, unsubstituted cellulooligosaccharides (see Materials and Methods) were prepared from cellulose and subsequently methylated with dimethyl sulfate. The methylated materials were then assayed for growth inhibition. These experiments demonstrated that methyl cellulooligosaccharides with a DP range of 3 to 7 were partially inhibitory to cellulose degradation, and longer times (4 versus 8 days) were required for degradation of the cellulose substrate in their presence. However, methylcellobiose (13 mM) had no effect on growth or cellulose degradation.

Cellulase studies. In addition to the growth studies just described, a series of enzymatic assays were conducted to determine the effect of the methyl derivatives on cellulase activity as measured with PNPC as the substrate (16). For *R. flavefaciens* FD1, the end products of PNPC hydrolysis were determined to be cellobiose and *p*-nitrophenol. Both methylcellulose and CMC were shown to inhibit cellulase activity in a concentration-dependent manner (Table 4). With methylcellulose hydrolysates, only those with a DP of 6.7 or greater caused significant inhibition of cellulase activity. The inhibitory activity of CMC was transitory, as this compound was very inhibitory in immediate assays but a portion of this inhibition was relieved by preincubating CMC with the enzyme source (Table 5). In contrast, preincubation did not alter the inhibitory activity of methylcellulose.

Cellulase inhibition data were obtained for individual methylated cellulooligosaccharides and indicated that both methylglucose and methylcellobiose were not inhibitory (Table 6). The other individual methyl oligosaccharides (DP, 3 to 7) displayed degrees of inhibition (33 to 53%) which were less than that of the methyl cellulooligosaccharide mixture (71%). When the concentration of the mixture was very high (124 mM), the degree of inhibition (85%) was comparable to that observed with unhydrolyzed methylcellulose (Table 5).

In other experiments, it was found that maltose, glucose, and 3-*O*-methylglucose did not inhibit enzymatic activity. Pebble-milled cellulose (0.2%) was a weak inhibitor (25%) of activity when the enzyme source was preincubated (2 h) with the cellulose prior to addition of PNPC. In contrast, high concentrations of unsubstituted cellobiose (20 mM) or cellulooligosaccharide mixtures (10 mM glucose equivalent) were found to cause inhibitions of 43.1 and 25%, respectively.

No cellobiase activity was detected in cultures of *R. flavefaciens* FD1 as indicated by the lack of glucose as an end product of cellulooligosaccharide hydrolysis. Only cellobiose and cellotriose accumulated as extracellular products (Table 7). Cellotetraose was degraded to cellobiose, and cellopentaose was degraded to cellobiose plus cellotriose. Interestingly, cellohexaose was degraded by random cleavage to cellobiose, cellotriose, and cellotetraose. The enzyme(s) which hydrolyzed the cellulooligosaccharides to cellobiose and cellotriose was insensitive to inhibitory concentrations of methylcellulose (Table 8). Both the rate of formation and the types of oligosaccharides formed in the presence of methylcellulose were very similar to those found with the hydrolysis of cellulooligosaccharide mixture alone. These data were consistent with the other data which indicated that the growth of *R. flavefaciens* FD1 on cellobiose and cellulooligosaccharides was unaffected by the presence of methylcellulose (Fig. 1).

TABLE 4. Inhibition of cellulase activity by CMC and by methylcellulose and its acid hydrolysates

Inhibitor and concn (%, wt/vol) or DP	Mean cellulase sp act (U/mg of protein) ± SD	Relative activity[a] (%)
Methylcellulose (400 cP)		
0.04	0.019 ± 0.001	14.6
0.004	0.059 ± 0.006	51.5
0.0004	0.112 ± 0.004	96.5
CMC (15 cP)		
0.4	0.012 ± 0.001	10.3
0.04	0.062 ± 0.005	53.4
0.004	0.099 ± 0.019	84.9
Methylcellulose hydrolysate[b]		
DP 9.5	0.036 ± 0.002	31.4
DP 8.5	0.044 ± 0.002	37.7
DP 6.7	0.046 ± 0.003	39.6
DP 4.5	0.095 ± 0.004	81.9
DP 1.0	0.096 ± 0.003	82.7

[a] 100% activity was 0.116 U/mg of protein.
[b] 8 mM glucose equivalent.

TABLE 5. Effect of preincubation on cellulase inhibition by CMC and methylcellulose

Inhibitor[a] (%, wt vol)	Cellulase activity (% of control)[b]	
	Immediate assay	Preincubation[c]
Methylcellulose		
0.16	17.0	18.2
0.08	18.3	14.0
CMC[d]		
0.16	20.3	27.4
0.08	22.3	50.2

[a] CMC, 15 cP; methylcellulose, 400 cP.
[b] Specific activity of control, 0.142 U/mg of protein.
[c] Inhibitor incubated for 2 h with the enzyme preparation before addition of PNPC.
[d] Final concentration in assay mixture.

DISCUSSION

The resistance of methylcellulose to biological degradation appears to be common in anaerobic environments, since

TABLE 6. Inhibition of cellulase activity by synthetically methylated cellulooligosaccharides[a]

Methylated cellulooligosaccharide	Concn[b] (mM)	Mean cellulase sp act (U/mg of protein) ± SD	Relative activity[c] (%)
Glucose	12.4	0.116 ± 0.034	103.8
Cellobiose	12.4	0.111 ± 0.005	99.1
Cellotriose	12.4	0.070 ± 0.002	62.2
Cellotetraose	12.4	0.077 ± 0.004	69.0
Cellopentaose	12.4	0.066 ± 0.003	59.1
Cellohexaose	12.4	0.058 ± 0.002	51.1
Celloheptaose	12.4	0.056 ± 0.015	50.2
Cellulooligosaccharide mixture	12.4	0.032 ± 0.003	28.6
	124.0	0.017 ± 0.001	15.2

[a] Enzymatic assay with PNPC as the chromophoric substrate.
[b] Based on glucose equivalents.
[c] 100% activity was 0.112 U/mg of protein.

TABLE 7. Hydrolytic products of individual cellulooligosaccharides when incubated with *R. flavefaciens* FD1

Initial substrate	Incubation time (h)	Cellulooligosaccharide(s) formed (mM)				
		Cellobiose	Cellotriose	Cellotetraose	Cellopentaose	Cellohexaose
Cellobiose	0	8.17				
	1	8.22				
	2	8.34				
Cellotriose	0		4.60			
	1		4.74			
	2		4.65			
Cellotetraose	0	0		3.28		
	1	1.57		2.97		
	2	3.21		2.14		
Cellopentaose	0	0	0		2.44	
	1	1.87	1.68		0.98	
	2	1.90	1.90		0.14	
Cellohexaose	0	0	0	0		1.68
	1	1.40	1.11	0.73		0
	2	1.60	1.22	0.54		0

methylcellulose enrichment cultures from several sources failed to grow (Table 1). Limited information from the literature suggests that these derivatives are resistant in aerobic environments as well (28, 36, 39). Several characteristics of cellulose derivatives may determine their resistance to biological hydrolysis, including DS, type of substitution, site of substitution, and uniformity of substitution. The high DS (>1.3) of water-soluble methylcellulose results in few glucose monomers that are completely unsubstituted (17, 38). This high DS confers resistance, since it appears that hydrolysis is dependent on unsubstituted hydroxyls at one or more adjacent monomers (7, 20). With respect to CMC, an indirect relationship exists between DS and extent of hydrolysis (26). Lowly substituted CMC (DS, 0.4) can support the growth of cellulolytic microbes, since a major portion of the glucose is unsubstituted and can be metabolized for growth (30).

Cellulose derivatives vary greatly in uniformity of substitution because derivatization is essentially a random process governed by the reactivity of the hydroxyl group and by the accessibility of the cellulose to derivatizing reagents. Theoretically, a uniformly substituted cellulose with a DS of 1.0 should be resistant to hydrolysis, according to current proposals. However, CMC and hydroxyethyl cellulose with a DS of >1.0 have been used in enzymatic assays (14, 42). Such observations suggest nonuniform substitution in these polymers, as is the case with hydroxyethyl cellulose (in which the substituents can self-polymerize, resulting in only 50 to 60% of the glucose residues being substituted) or the importance of other factors, such as type of substituent or site of substitution.

The biological resistance that substituted celluloses acquire when the secondary hydroxyls are derivatized is analogous to the current concept of biological resistance as a result of plant lignification. Lignin-carbohydrate complexes, which consist of phenolic acids ester linked to hemicellulose, appear to be equally undegradable (18). Similar observations have been made for acetylated xylans (5).

The recalcitrance of methylcellulose and methyl cellulooligosaccharides to degradation and their widespread inhibitory activities make these compounds useful tools in the study of bacterial adherence to cellulose and of the enzymology of cellulose degradation. In the current study, methylcellulose and methyl cellulooligosaccharides were used to explore the mechanisms of cellulose degradation and growth in *R. flavefaciens* FD1. Methylcellulose inhibited growth on cellulose in a dose-dependent manner. Similar inhibitions were observed for cellulase activity, as measured by PNPC hydrolysis (Tables 4 and 5). In contrast, methylcellulose did not inhibit growth on or hydrolysis of cellulooligosaccharides (Table 8). These data suggest that methylcellulose acts as a nonhydrolyzed, competitive inhibitor of those enzymes displaying cellulase (β-1,4-glucanase) activity. Another ruminal cellulolytic bacterium, *Bacteroides succinogenes*, has been reported to possess a CMC-hydrolyzing activity that is inhibited by methylcellulose (22). These glucanase enzymes might also be involved in attachment, as methylcellulose inhibits attachment of ruminal bacteria to cellulose and plant materials (27, 32; M. A. Rasmussen and S. D. Farlin, Univ. Neb. Inst. Agric. Q. **26**:15–16, 1979).

With *R. flavefaciens* FD1, we think that PNPC hydrolysis

TABLE 8. Effect of methylcellulose on hydrolysis of a cellulooligosaccharide mixture by *R. flavefaciens* FD1

Treatment	Incubation time (h)	Cellulooligosaccharide(s) detected (mM)						
		Glucose	Cellobiose	Cellotriose	Cellotetraose	Cellopentaose	Cellohexaose	Celloheptaose
None	0	Tr	0.4	1.1	1.3	0.9	0.6	P[a]
	1	Tr	1.2	1.8	1.5	0.7	0.1	O
	2	Tr	2.1	2.5	1.5	0.4	0	O
Methylcellulose (400 cP, 0.16% [wt/vol])	0	Tr	0.4	1.0	1.2	0.8	0.5	P
	1	Tr	1.2	1.8	1.6	0.8	0.2	O
	2	Tr	1.7	2.3	1.7	0.7	0	O

[a] P, Present in small amounts, but quantity could not be accurately determined.

is a representative substrate for the cellulase complex (i.e., both exoglucanase and endoglucanase) from this bacterium. The inhibition of PNPC hydrolysis by both CMC and pebble-milled cellulose is consistent with this appraisal, as CMC is more specific for endoglucanases and pebble-milled cellulose is more specific for exoglucanases. Analysis of the PNPC hydrolysate indicated that cellobiose was the only product, demonstrating that the agluconic bond and not the holosidic bond was cleaved by the preparation. Furthermore, recent studies indicate that *R. flavefaciens* FD1 has at least one exo-β-1,4-glucanase capable of hydrolyzing PNPC (19). Moreover, at least three CMCase components have been identified, and these hydrolyze PNPC with various efficiencies (K. C. Doerner and B. A. White, personal communication). It is clear that this substrate can be used to measure overall cellulase activity with this organism.

The effects of methyl cellulooligosaccharides on cellulose hydrolysis differ from those of polymerized methylcellulose. Methylglucose and methylcellulose hydrolysate mixtures up to a DP of about 6 to 7 had little or no effect on degradation of cellulose (Table 3) or on cellulase activity (Table 4). When fully methylated, individual cellulooligosaccharides were added to a cellulase assay mixture, methyl cellobiose was found to have no effect on activity, but methyl cellotriose through celloheptaose caused inhibition of 40 to 50% (Table 6). The maximal inhibition (85%) that was observed occurred only with high concentrations (124 mM) of the methyl oligosaccharides. These data are consistent with the hypothesis that there are at least two levels of enzyme systems in *R. flavefaciens*. One system is at the level of β-1,4-glucanase activity and acts primarily on cellulose polymers as a substrate. The second system is at the level of cellulodextrinase activity and acts mainly on cellulooligosaccharides as substrates.

The cellulodextrinase enzyme(s) was capable of hydrolyzing cellohexaose to cellotetraose, cellotriose, and cellobiose, whereas cellopentaose was converted to cellotriose and cellobiose (Table 7). Neither cellobiose nor cellotriose was hydrolyzed further. The cellulodextrinase activity was not inhibited by methyl cellulose (Table 8). These data are consistent with the properties of the periplasmic cellobiosidase (cellulodextrinase) that has been purified from another ruminal cellulolytic species, *Bacteroides succinogenes* (25). This enzyme has substrate specificities and product formation similar to the *R. flavefaciens* activity.

The final end products of cellulose hydrolysis by the β-1,4-glucanase and cellulodextrinase activities of *R. flavefaciens* were cellobiose and cellotriose, although trace quantities of glucose was detected from cellulooligosaccharide degradation (Table 8) (37). As shown by our studies (Fig. 1 and 2) and Russell (37), *R. flavefaciens* can grow rapidly on cellulooligosaccharides. Thus, it is reasonable to assume that transport mechanisms exist in *R. flavefaciens* for the uptake of cellobiose and cellotriose. Whether a single or two or more separate transport mechanisms exist is unknown. The reported presence of a cellobiose phosphorylase in *R. flavefaciens* (2) and the observation that extracellular glucose is not formed after growth on cellulose or cellobiose suggest that transport and phosphorylytic cleavage may be linked. If true, this would represent a considerable increase in ATP formed per mole of hexose fermented and an overall increase in growth efficiency for this anaerobe. The rapid growth rates and yields observed with cellotriose and cellopentaose (Fig. 2) are consistent with this hypothesis, but further experiments on transport of cellulooligosaccharides are needed to establish whether phosphorylytic cleavage does occur.

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