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Utilization of Individual Cellodextrins by Three Predominant Ruminal Cellulolytic Bacteria

Y. Shi[1] and P. J. Weimer[2,3]*

Department of Dairy Science[1] and Department of Bacteriology,[2] University of Wisconsin—Madison, and U.S. Dairy Forage Research Center, Agricultural Research Service, U.S. Department of Agriculture,[3] Madison, Wisconsin 53706

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Growth of the ruminal bacteria *Fibrobacter succinogenes* S85, *Ruminococcus flavefaciens* FD-1, and *R. albus* 7 followed Monod kinetics with respect to concentrations of individual pure cellodextrins (cellobiose, cellotriose, cellotetraose, cellopentaose, and cellohexaose). Under the conditions tested, *R. flavefaciens* FD-1 possesses the greatest capacity to compete for low concentrations of these cellodextrins.

Cell wall polysaccharides are the major components of plant biomass and are thus a major energy source for forage-fed ruminant animals. Cellulose is the most abundant of these polysaccharides and is readily hydrolyzed in the rumen to soluble cellodextrins (β-1,4-linked oligomers of glucose) that can be utilized by both cellulolytic and noncellulolytic ruminal bacteria (17). Considerable differences exist among three predominant ruminal cellulolytic bacteria (*Fibrobacter succinogenes* S85, *Ruminococcus flavefaciens* FD-1, and *R. albus* 7) with respect to the utilization patterns of these substrates. *F. succinogenes* reportedly transports cellobiose (G2) simultaneously with, but more slowly than, glucose (G1) (12). By contrast, *R. albus* grows more rapidly on G2 than on G1 and shows a clear preference for G2 when both are available (21). Russell (17) has reported that, on a mixture of cellodextrins, *F. succinogenes* S85 and *R. flavefaciens* FD-1 display maximum growth rates (0.44 to 0.48 h^{-1}) similar to those on G2. *R. albus* 7 has been variously reported to have a maximum growth rate on G2 of 0.42 h^{-1} (17) or 1.19 h^{-1} (21) and to grow more rapidly on a mixture of cellodextrins than on G2 (17). *R. flavefaciens* FD-1 (which does not grow on G1 [7]) reportedly grows more rapidly under some conditions on the cellodextrin cellopentaose (G5) or cellotriose (G3) than on G2, and resting cell suspensions of *R. flavefaciens* hydrolyze these pure cellodextrins primarily to G2 and G3 (16). These data are in accord with those of Russell (17), who noted that pure cultures of many ruminal bacteria grown on mixed cellodextrins display complex changes in the concentrations of individual cellodextrins over time and who suggested that cellodextrins longer than four glucose units are hydrolyzed extracellularly to produce smaller oligomers that are then used as growth substrates.

In contrast to bacteria that hydrolyze cellodextrins, *F. succinogenes* has recently been shown to synthesize and excrete longer cellodextrins when supplied with moderate concentrations of shorter oligomers (25), suggesting that this ruminal cellulolytic bacterium may be capable of feeding cellodextrins to other rumen microbes (including, presumably, its own daughter cells during the planktonic phase of their life cycle).

Although the utilization of cellodextrins appears to be complicated, it is apparent that cellodextrins have the potential to be major growth substrates for ruminal cellulolytic bacteria and thus may be important determinants of competition among these organisms in the rumen. The purpose of this study was to obtain quantitative growth parameters for several cellodextrins for three predominant ruminal cellulolytic species.

Mixed cellodextrins were obtained by partial acid hydrolysis of cellulose by a modification of the method of Freer and Detroy (6). Sigmacell 20 microcrystalline cellulose (10 g) was first mixed with 100 ml of ice-cold concentrated HCl in a 500-ml flask and stirred to give a uniform suspension; ice-cold fuming HCl (100 ml) was then poured into the flask to completely dissolve the cellulose. A slightly yellow, viscous solution formed after 2 h of incubation at room temperature. The HCl was partially removed by application of a vacuum for 0.5 h. Cellodextrins were precipitated by the addition of 10 volumes of acetone. The precipitate was washed four times with 5 volumes of acetone and collected by centrifugation (10,000 × *g*, 0°C, 15 min). The pellet was resuspended in 600 ml of deionized distilled water to extract water-soluble cellodextrins, and the solution was stirred overnight in a chemical hood to remove residual acetone. The supernatant (water-soluble cellodextrins) was collected after centrifugation (10,000 × *g*, 0°C, 15 min), neutralized by passage through an anion-exchange column (1.7 by 7.5 cm; Dowex AG1-X8; Bio-Rad, Richmond, Calif.), and concentrated to 10 to 15 ml by lyophilization. The yield of total mixed water-soluble cellodextrins from the hydrolysis was 15% by weight. The composition of this mixture was determined by high-performance liquid chromatography (HPLC), essentially by the method of Russell (17) except that the solvent flow rate was 0.40 ml/min. The retention times of the cellodextrins (in minutes, determined from authentic standards from Sigma [St. Louis, Mo.]) were as follows: celloheptaose (G7), 11.8; cellohexaose (G6), 12.8; G5, 14.1; cellotetraose (G4), 15.8; G3, 18.0; G2, 20.8; and G1, 24.3. The proportions of the cellodextrins in the mixture (by weight) were 1.3% G1, 8.3% G2, 15.8% G3, 27.6% G4, 34.3% G5, 11.2% G6, and 1.4% G7.

The homologous series of cellodextrins was purified from the mixture described above by a modification of the method of Miller (13). Approximately 350 to 400 mg of hydrolysate was loaded onto a column (86-cm length by 5-cm diameter) of 50% Darco G60 activated charcoal (Aldrich, Milwaukee, Wis.)–50% acid-washed Celite 545 (Alltech, Deerfield, Ill.) saturated with stearic acid (Kodak, Rochester, N.Y.). Cellodextrins were separated by stepwise, reversed-phase elution with 2 liters of

* Corresponding author. Mailing address: U.S. Dairy Forage Research Center, 1925 Linden Dr. West, Madison, WI 53706. Phone: (608) 264-5408. Fax: (608) 264-5275. Electronic mail address: pjweimer@macc.wisc.edu.

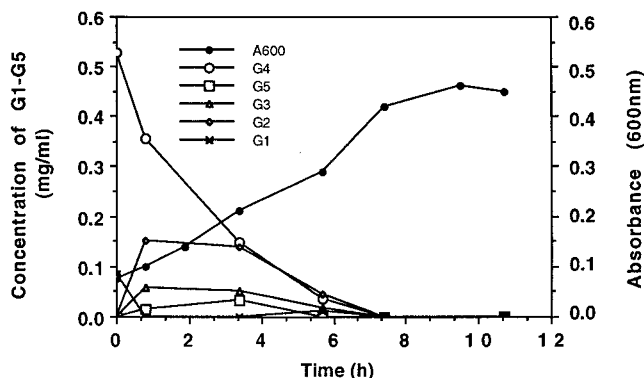

FIG. 1. Cellodextrin utilization by *F. succinogenes* S85 grown in batch culture on G4. Samples were collected at the indicated time points and analyzed by HPLC. While the results shown are from a single tube culture, similar trends were observed in two additional experiments.

deionized distilled water, 4 liters of 20% (vol/vol) ethanol, 4 liters of 30% (vol/vol) ethanol, and 4 liters of 40% (vol/vol) ethanol. The flow rate was controlled by a peristaltic pump connected to the bottom of the column. The eluate was collected in 800 to 900 fractions of 15 ml each. Peaks were identified by testing every fifth fraction with phenol-sulfuric acid reagent (5). Generally, each cellodextrin peak was collected in 10 to 40 fractions (~150 to 600 ml in total) and was pooled to three or four fractions. Each pooled fraction was concentrated to 10 to 20 ml by rotary evaporation under a vacuum at 45°C.

Analysis of these pooled fractions by HPLC revealed that G2, G3, and G4 eluted successively with 20% ethanol; the first 100 ml of G3 always contained a trace (1 to 5%) concentration of G2 due to diffusional band broadening of these small molecules. G5 and G6 were eluted by 30% ethanol. G4 and G5, which together represented ~60% of the total mixed cellodextrins in the hydrolysate sample, eluted at two different ethanol concentrations, and thus their resolutions were much better than those of G3 and G6, which were often contaminated by low concentrations (1 to 7%) of G2 and G5, respectively. In all cases, these contaminants were removed by rechromatographing by the procedure described above, and only purified cellodextrins (>99.9% purity, determined by HPLC) were used for growth experiments. The final concentrations of purified G3, G4, G5, and G6 were determined by the phenol-sulfuric acid method (5) with glucose as a standard.

To determine the cellodextrin utilization patterns of different cellulolytic species, *F. succinogenes* S85, *R. flavefaciens* FD-1, and *R. albus* 7 were grown in Balch-type anaerobic tubes sealed with butyl rubber stoppers (1). The tubes contained a CO_2 gas phase and 5 ml of modified Dehority medium (23) supplemented with 4 to 5.5 g of cellodextrin per liter. The tubes were inoculated with 0.1 or 0.2 ml of G1- or G2-grown culture (grown to stationary phase to reduce carryover of sugars). Batch culture experiments revealed that growth of *F. succinogenes* S85 (Fig. 1) or *R. flavefaciens* FD-1 (data not shown) on G4 was accompanied by the transient, extracellular accumulation of substantial amounts of shorter glucans (G1, G2, and G3), which were consumed later in the incubation. Similar results were also observed during growth on G5 with both species. The data suggest hydrolysis of G4 and G5 prior to growth on the shorter oligomers, as observed by Russell (17) for these strains grown on mixed cellodextrins and by Rasmussen et al. (16) for nongrowing cells of *R. flavefaciens* FD-1 incubated with pure cellodextrins. Such hydrolysis is apparently mediated by periplasmic (*F. succinogenes*) or extracellular (*F. succinogenes* and *R. flavefaciens*) cellodextrinases (2, 10). Hydrolysis of G3 prior to uptake was also observed for *R. albus* 7 (G4 and G5 were not tested with this strain).

We also observed the extracellular accumulation of small amounts of G5 in *F. succinogenes* cultures grown on G4 (Fig. 1), in accord with similar data of Wells et al. (25). Accumulation of G5 was not observed in *R. flavefaciens* cultures grown on low concentrations (<1 mg/ml) of G4, although we have observed some accumulation of G3 by both batch and continuous cultures of this strain fed a fairly high concentration of G2 (3.3 mg/ml), indicating that synthesis and efflux of longer cellodextrins may be general properties of cellulolytic bacteria containing a cellobiose phosphorylase or cellodextrin phosphorylase (25).

Although hydrolysis of longer-chain (G3 to G6) oligomers prior to uptake appears to be the dominant route of cellodextrin metabolism in all three cellulolytic species, we cannot at present exclude the possibility that at least a portion of these cellodextrins can be transported directly without hydrolysis. Demonstration of such direct transport is difficult because of the simultaneous occurrence of cellodextrin hydrolysis and synthesis of longer cellodextrins (Fig. 1). However, the extracellular accumulation, in cultures of *F. succinogenes* (25) or *R. flavefaciens* (see above), of longer cellodextrins produced by a reversal of an intracellular cellodextrin phosphorylase reaction (25) suggests a capacity to transport these longer oligomers across the cell membrane (and, in the case of *F. succinogenes*, across the outer membrane). Direct transport of the longer cellodextrins warrants further study to determine possible parallels with other oligosaccharide transport systems (e.g., the energy-driven unidirectional uptake of maltodextrins in *Escherichia coli* [8]).

TABLE 1. μ_{max} and $S_{0.5\mu_{max}}$ values for growth of *R. flavefaciens* FD-1, *R. albus* 7, and *F. succinogenes* S85 on different cellodextrins

Cellodextrin	n[a]			μ_{max} (h^{-1})[b]			$S_{0.5\mu_{max}}$ (mM)[b]		
	FD-1	7	S85	FD-1	7	S85	FD-1	7	S85
G1	—[c]	3	2	—	0.58 A	0.49 A	—	4.16 E	0.49 G
G2	2	4	4	0.49 A	0.48 A	0.41 A	0.29 C	1.21 F	0.53 G
G3	2	2	2	0.45 A	0.61 A	0.43 A	0.14 D	0.41 G	0.37 G
G4	4	2	2	0.42 A	0.53 A	0.52 A	0.11 D	0.54 G	0.47 G
G5	2	2	2	0.50 A	0.46 A	0.22 B	0.10 D	0.20 D	0.20 D
G6	2	ND[d]	2	0.40 A	ND	0.20 B	0.18 D	ND	0.11 D

[a] Number of replicate growth experiments for each combination of strain and cellodextrin.
[b] Values with different capital letters in a row or a column are significantly different ($P < 0.05$).
[c] —, FD-1 cannot grow on G1.
[d] ND, not determined.

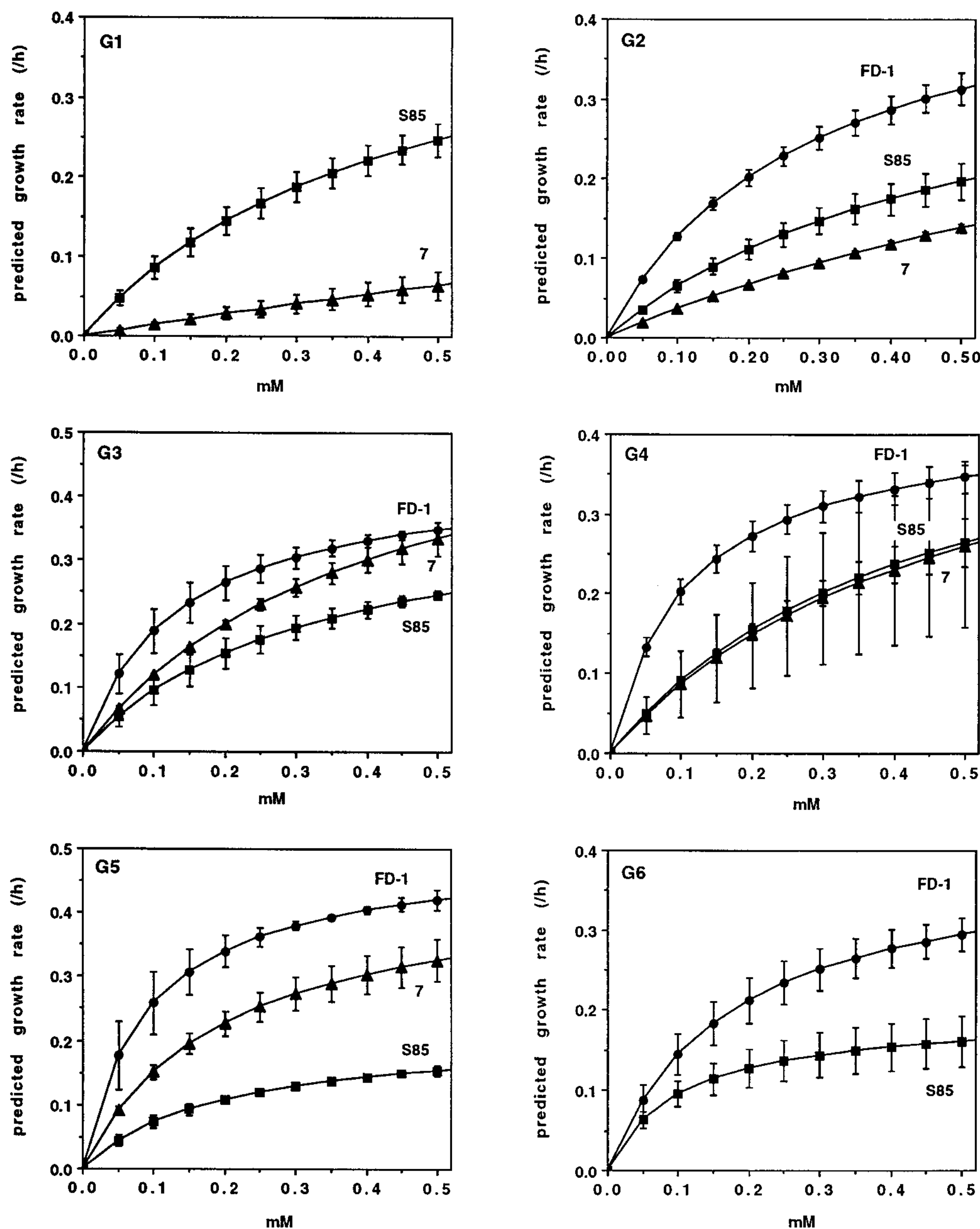

FIG. 2. Relationship between predicted growth rate and substrate concentration for *R. flavefaciens* FD-1, *R. albus* 7, and *F. succinogenes* S85 on different cellodextrins. Each growth curve contains 10 calculated datum points. Each of these datum points is a mean value calculated from the Monod equation with two to four paired values of μ_{max} and $S_{0.5\mu_{max}}$ that were experimentally determined from separate growth curves. The error bars indicate 90% confidence intervals.

The kinetics of growth of each species on each cellodextrin was determined from a series of trials, each conducted with a single bacterial strain and a series of individual cellodextrins. For each individual cellodextrin substrate, growth data were collected for two to four culture tubes, each having initial cellodextrin concentrations of 1, 0.5, 0.33, 0.25, 0.20, 0.17, and 0.14 mg/ml. To eliminate temperature fluctuations, incubations (with shaking at 150 rpm) and culture turbidity measurements at 600 nm were conducted in a 39°C room. Observed growth rates (μ) were calculated as the maximum slopes of the plots of ln A_{600} versus time for each culture tube, and the values from the replicate tubes were randomly assigned to separate groups. These growth rates were then transformed with Monod plots ($1/\mu$ versus 1/substrate concentration), resulting in replicate plots from which the Monod parameters μ_{max} ($1/y$ intercept; the maximum specific growth rate at the saturating substrate concentration) and $S_{0.5\mu_{max}}$ ($-1/x$ intercept; the concentration of substrate permitting growth at half the maximal rate) were determined. Statistical analysis on these parameters was then performed by the least significant difference test of the SAS statistical analysis software package (19).

Despite the complexity of utilization of different individual cellodextrins, the growth of each pure culture followed simple Monod kinetics with respect to substrate concentration (the r^2 of Monod plots ranged from 0.900 to 0.999 and averaged 0.955). From the Monod plots we obtained, for each bacterial species-cellodextrin combination, replicate values of two growth parameters: μ_{max} and $S_{0.5\mu_{max}}$. Most of the μ_{max} values

of the three species grown on G1 to G6 were similar (Table 1), except for those of *F. succinogenes* S85 grown on G5 or G6. The data suggest that the rate of extracellular hydrolysis of longer cellodextrins (G4 to G6) does not in most cases limit the rate of bacterial growth. The significant decrease in the μ_{max} of *F. succinogenes* S85 grown on G5 and G6 may be due to restricted movement of G5 (molecular weight = 828) or G6 (molecular weight = 990) across the outer membrane of this gram-negative bacterium or to slower hydrolysis of these longer dextrins in the periplasm.

Although a trend of decreasing $S_{0.5\mu_{max}}$ with increasing chain length of cellodextrins was observed with all three strains (Table 1), statistical analysis revealed that the $S_{0.5\mu_{max}}$ values of G3 to G6 were not significantly different for *R. flavefaciens* FD-1. The $S_{0.5\mu_{max}}$ values for *F. succinogenes* S85 were basically constant from G1 to G4 but significantly decreased for G5 or G6. The $S_{0.5\mu_{max}}$ values for *R. albus* 7 dramatically decreased from G1 to G5 (3.4-fold from G1 to G2 and 6.1-fold further from G2 to G5). The $S_{0.5\mu_{max}}$ values for *R. flavefaciens* were significantly lower than those for the other two species for most cellodextrins tested.

Our calculated $S_{0.5\mu_{max}}$ value for *F. succinogenes* S85 on G1 (0.49 mM) is very close to the transport coefficient (K_t) of 0.51 mM reported for this strain by Chow and Russell (4). On the other hand, our calculated $S_{0.5\mu_{max}}$ values of G1 and G2 (4.16 and 1.21 mM, respectively) for *R. albus* 7 appear to differ considerably from the K_s values (0.97 and 3.16 mM, respectively) reported for this species by Thurston et al. (21). This apparent disparity may be due in part to the use of different experimental methods (continuous versus batch culture). However, it should also be noted that our study differs from most growth kinetic studies in that we applied replication and tests of statistical significance in reporting values for μ_{max} and $S_{0.5\mu_{max}}$. Because previous studies do not report replicated data or any measure of variation, direct comparison with our data for statistically significant similarities or differences is not possible.

Unless a cellodextrin is taken up directly by a single transport mechanism, the $S_{0.5\mu_{max}}$ value lacks mechanistic significance (i.e., it is not an affinity constant [K_s]). However, μ_{max} and $S_{0.5\mu_{max}}$ together permit estimation of growth rates at a particular substrate concentration by use of a variation of the Monod equation: $\mu = (\mu_{max} \cdot S)/(S + S_{0.5\mu_{max}})$, where S is the substrate concentration. Calculations of predicted growth rates for each species at various concentrations of each cellodextrin were performed to assess which species would predominate if interactions were based solely on competition for a single substrate. The results of such calculations (Fig. 2) indicate that *F. succinogenes* S85 would be expected to outcompete *R. albus* 7 for G1 and that *R. flavefaciens* FD-1 would be expected to outcompete *R. albus* 7 and *F. succinogenes* S85 for low concentrations of G2, G3, G4, or G5. Significantly, at any G2 concentration lower than 1 mM, the predicted outcome of the competition for G2 was not altered by replacing our values of μ_{max} and $S_{0.5\mu_{max}}$ obtained for *R. albus* 7 with the above-mentioned values reported by Thurston et al. (21). Overall, the data are in accord with our observations that *R. flavefaciens* FD-1 outcompetes *F. succinogenes* S85 in both cellulose- and cellobiose-limited continuous cultures (20).

The ability to compete for soluble substrates, reflected in the values of μ_{max} and $S_{0.5\mu_{max}}$, provides only one of several strategies for competition among cellulolytic ruminal species. In the rumen, competition can be affected by several other factors, including the ability to hydrolyze different cell wall components of different feeds (11), kinetics of attachment to feed particles (15), production of inhibitory biochemicals (14), survival of nonadherent cells during temporary starvation (22, 24), pH sensitivity (18), and availability of other nutrients (3, 9). Experiments are in progress to assess the effect of these parameters on competition among these three cellulolytic species.

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