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p-Coumaroyl and Feruloyl Arabinoxylans from Plant Cell Walls as Substrates for Ruminal Bacteria

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Growth of the ruminal bacteria *Ruminococcus flavefaciens* FD1, *Selenomonas ruminantium* HD4, and *Butyrivibrio fibrisolvens* 49 was limited by ester-linked feruloyl and *p*-coumaroyl groups. The limitation of growth on phenolic acid-carbohydrate complexes varied with individual bacteria and appeared to be influenced by ability to hydrolyze carbohydrate linkages.

Ferulic and *p*-coumaric acids limit the biodegradation of plant fiber. In vitro studies (2) indicated that concentrations of these phenolic acids above 1 mM inhibited growth of many species of ruminal bacteria. Further, research (6) has shown that these phenolic acids are esterified to arabinoxylans within the plant cell wall, and digestibility of plant cell walls has been related to amounts of phenolic acids released by alkali treatment (5). Formation of ester bonds between phenolic acids and plant wall polysaccharides through in vitro syntheses, while not entirely representative of naturally occurring esters, reduced biodegradation of carbohydrates (1, 11), further supporting the contention that phenolic esters limit carbohydrate degradation by ruminal microorganisms. Studies to date have focused on the inhibition of carbohydrate degradation by the presence of phenolic acids, but little work has been done on the microbial utilization of sugars that are bound to phenolic acids through ester bonds. Information on this phenomenon is hindered by the lack of appropriate naturally occurring esters to test as substrates. Recently, [5-*O*-((*trans*)-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAXX) and [5-*O*-((*trans*)-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (PAXX) were isolated from cell walls of the warm season grass coastal bermudagrass (*Cynodon dactylon* L. Pers) by enzymatic treatment with driselase (Sigma Chemical Co., St. Louis, Mo.) (3). This enzyme mixture is derived from a basidiomycete and contains cellulases and hemicellulases but lacks phenolic acid esterases. These complexes afford an opportunity to evaluate limitations to microbial utilization of plant carbohydrates by specific esters that naturally exist within plant cell walls. The objective of our work was to investigate the growth of pure cultures of ruminal bacteria that have different substrate propensities on specific phenolic acid-carbohydrate complexes.

Pure cultures of anaerobic bacteria, which were originally isolated from the rumen and maintained in the culture collection of S. A. Martin, included *Ruminococcus flavefaciens* FD1, *Selenomonas ruminantium* HD4, and *Butyrivibrio fibrisolvens* 49. Cultures were maintained on basal medium (see below) with 0.2% cellobiose (*R. flavefaciens* and *B. fibrisolvens*) or 0.2% glucose (*S. ruminantium*). The phenolic acid-arabinoxylan esters tested were FAXX and PAXX. Methyl-5-*O*-feruloyl-α-L-arabinofuranoside (8) was supplied by R. F. Helm and J. Ralph (Agricultural Research Service, U.S. Department of Agriculture, Madison, Wis.). L-Arabinose, D-(+)-xylose, oat spelt xylan, *p*-coumaric acid, and ferulic acid were commercial products (Aldrich Chemical Co., Milwaukee, Wis.) of at least 98% purity.

Hungate tubes (Bellco Biotechnology, Vineland, N.J.) were matched for optical density at 520 nm, using a Spectronic 21D spectrophotometer (Milton Roy, Rochester, N.Y.). Basal medium, consisting of minerals, tryptone, yeast extract, hemin, and volatile fatty acids without carbohydrates, as published by Caldwell and Bryant (4), was prepared with modification of the concentration of Na_2CO_3 to provide a pH of about 6.5 to 6.7 when the medium was equilibrated in a glove box with an atmosphere of 97% CO_2–3% H_2. An aliquot of 4.5 ml of medium was dispensed into each matched, Hungate tube, which was capped and autoclaved (121°C, 103.5 × 10^3 Pa, 15 min). The following substrates were added to separately autoclaved, dry vessels: (i) 17.7 mg of FAXX; (ii) 16.8 mg of PAXX; and (iii) 15 mg of arabinose plus 30 mg of xylose (A/X). Anaerobic stock solutions of each substrate were prepared by aseptically dispensing in the glove box 3 ml of autoclaved basal medium to each tube containing FAXX or PAXX or 10 ml to tubes with A/X. Then, a 0.5-ml aliquot from each stock solution was aseptically dispensed to the matched tubes containing 4.5 ml of basal medium. Tubes were fitted with black butyl rubber septa and a retaining cap and removed from the glove box. This procedure provided tubes with unautoclaved substrates at 1 mM concentrations of FAXX or PAXX or equivalent concentrations of arabinose (1 mM) and xylose (2 mM). Identical procedures were used in testing equimolar concentrations of arabinose, xylose, xylan, A/X plus free ferulic and *p*-coumaric acids, methyl 5-*O*-feruloyl-α-L-arabinofuranoside (FA), and arabinose plus ferulic acid (F/A). To test the effect of saponification of ferulic acid from FAXX on bacterial growth, 1 mM concentrations of FAXX and A/X (as a control) were incubated with 0.5 ml of 0.1 M NaOH at room temperature for 30 or 60 min before the addition of basal medium for the anaerobic stock solutions of substrates. In these comparisons, 0.5 ml of distilled water was added to 1 mM concentrations of substrates before the addition of basal medium for stock solutions.

Starter cultures 18 to 24 h old from maintenance media

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TABLE 1. Phenolic acid esterase activity of ruminal bacteria

Microorganism[a]	Protein ($\mu g\ ml^{-1}$)	FAXX esterase		PAXX esterase	
		Ferulic acid from FAXX ($ng\ min^{-1}$)	Sp act (mU mg of protein^{-1})[b]	*p*-Coumaric acid from PAXX ($ng\ min^{-1}$)	Sp act (mU mg of protein^{-1})
R. flavefaciens	26.3	0	0	0	0
S. ruminantium	37.2	0	0	0	0
B. fibrisolvens	17.9	16.39	47.15	1.34	4.56

[a] *R. flavefaciens* and *B. fibriosolvens* were grown to stationary phase on oat spelt xylan, and *S. ruminantium* was grown to stationary phase on A/X.
[b] U = micromoles of product released per minute.

were inoculated (0.2 to 0.3 ml) into each test substrate in duplicate (triplicate in Fig. 3). Growth at 39°C was monitored spectrophotometrically at 520 nm, and the absorbance value at zero time for each tube was subtracted from those reported for the growth period. For statistical comparisons, values were analyzed by *t* test or one-way analysis of variance, with means tested by least significant difference.

To test for esterase activities, *R. flavefaciens* and *B. fibrisolvens* were grown to stationary phase on xylan and *S. ruminantium* HD4 was grown to stationary phase on A/X. Supernatants were clarified by filtration (0.45- or 0.2-μm pore size), dialyzed, and assayed for esterase activity, using FAXX or PAXX as the substrate. The methods were those published previously (3). Briefly, *p*-coumaric and ferulic acids released from PAXX and FAXX, respectively, were separated from the assay mixture by reverse-phase high-performance liquid chromatography and quantitated by UV A_{313}. Control treatments for assays were undertaken with heat-denatured enzyme preparations. Each assay was performed with duplicate replications of triplicate tubes.

R. flavefaciens did not grow well on A/X, FAXX, or PAXX, with maximum absorbances of 0.019, 0.014, and 0.013, respectively, during incubation for 24 h. This bacterium, which is a major fiber degrader in the rumen, produces cellulases and xylanases, but many strains use relatively few free sugars as substrates (13). In our study, although *R. flavefaciens* did not grow on A/X, some growth occurred on oat spelt xylan as indicated by protein content (Table 1). However, Hespell et al. (10) reported that strain FD1 did not utilize xylan well, having low growth efficiencies on this substrate. Lack of growth on FAXX and PAXX appeared to be primarily due to the inherent inability to utilize arabinose or the xylobiose moiety of the complex, rather than an inhibition due to esterification. Phenolic acid esterases were not detectable in supernatants from growth on xylan (Table 1).

S. ruminantium grew rapidly on A/X, completing logarithmic growth by 3 to 5 h (Fig. 1 and 2). The addition of 1 mM concentrations of ferulic or *p*-coumaric acid to A/X supported growth similar to that on the carbohydrates alone, indicating that this level of phenolic acid was not inhibitory or stimulatory to growth (Fig. 1). However, FAXX supported only scarce growth, and absorbance was only slightly higher than that on basal medium without carbohydrates (Fig. 1). Another study confirmed the lack of growth on FAXX and further established that *S. ruminantium* also did not grow on PAXX; in this study, maximum absorbances were 0.338, 0.072, and 0.060 for growth on A/X, FAXX, and PAXX, respectively, after incubation over a 20-h period. *S. ruminantium* had no detectable feruloyl or *p*-coumaroyl esterase activity (Table 1), which was consistent with the lack of growth on FAXX and PAXX. Oat spelt xylan did not support growth of *S. ruminantium*.

The effect of ester-linked phenolic acid-carbohydrate complexes on growth of *S. ruminantium* was further investigated (Fig. 2). Growth was not supported by FAXX, FAXX treated with 0.1 M NaOH, or FA. Growth was supported, however, by 1 mM concentrations of arabinose plus free phenolic acid. Absorbance on F/A was about one-third that of growth on A/X, which corresponded to the fact that F/A contained one-third the substrate concentration as A/X. The pH at stationary growth in this study was 6.6 to 6.7 for all substrates, suggesting that growth ceased because of exhaustion of substrates rather than production of inhibitory concentrations of fermentation acids and subsequent drop in pH.

S. ruminantium uses a wide range of sugars, including arabinose and xylose, but not xylan (10, 13). Our work indicates that this strain grew well on A/X (Fig. 1 and 2) but could not grow on oat spelt xylan. The present study indicated that linkages within the FAXX and PAXX complexes prevented the utilization of arabinose and xylose by *S. ruminantium*. Esterification of the phenolic group to arabinose appeared to prevent utilization of the carbohydrate for growth on FA. The apparent inability of *S. ruminantium* to hydrolyze the arabinofuranoside or xylobiose moiety contributed to the total lack of bioavailability of FAXX or PAXX.

B. fibrisolvens grew on phenolic-carbohydrate substrates and on pentoses alone (Fig. 3), but growth was slower than that with *S. ruminantium*, completing logarithmic growth by ca. 20 h. *B. fibrisolvens* grew faster on FAXX or PAXX compared with A/X (Fig. 3). The slopes during logarithmic

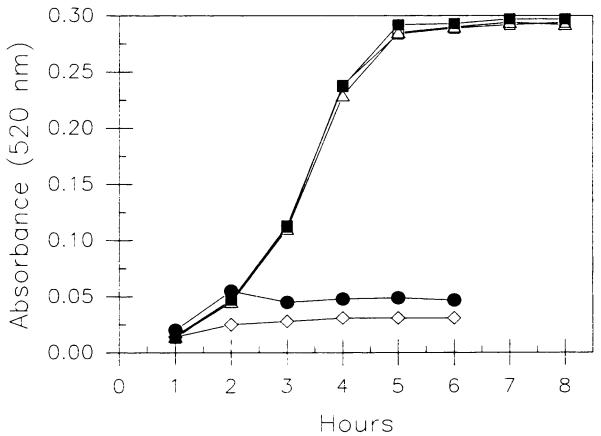

FIG. 1. A_{520} of *S. ruminantium* HD4 grown with the following substrates: 1 mM arabinose plus 2 mM xylose A/X, △; A/X plus 1 mM ferulic acid, ■; A/X plus 1 mM *p*-coumaric acid, □; 1 mM FAXX, ●; basal medium (no added carbohydrate), ◇.

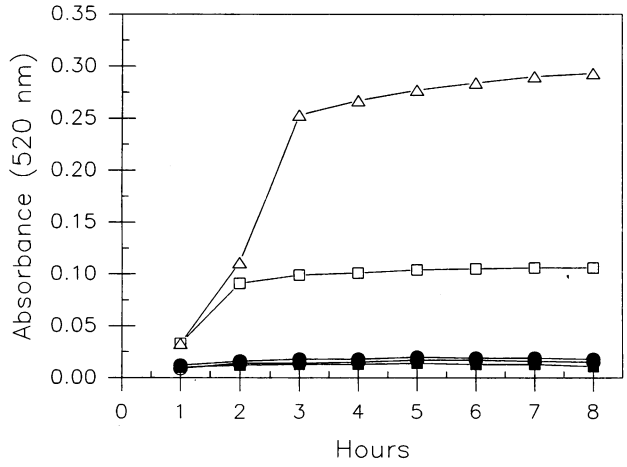

FIG. 2. A_{520} of *S. ruminantium* HD4 grown with the following substrates: 1 mM arabinose plus 2 mM xylose A/X, △; 1 mM FAXX, ●; 1 mM FAXX saponified with 0.1 M NaOH, ○; 1 mM FA, ■; 1 mM arabinose plus 1 mM ferulic acid F/A, □.

growth (i.e., 4 to 10 h for FAXX or PAXX and 8 to 12 h for A/X) in Fig. 3 were 0.017, 0.017, and 0.015 h^{-1} for FAXX, PAXX, and A/X, respectively. Growth on FAXX or PAXX ceased before that on A/X, on which absorbance was eventually greater than on FAXX or PAXX. Since absorbance was declining after 24 h (Fig. 3), the maximum absorbance on A/X possibly occurred between 18 and 24 h.

Growth patterns of *B. fibrisolvens* on FAXX, PAXX, and A/X were compared in additional experiments (Table 2). The ratio of absorbance on FAXX or PAXX to that on A/X was calculated during active growth and for the maximum absorbance for two studies in addition to data of Fig. 3. Results confirmed a faster growth on FAXX or PAXX and a greater maximum absorbance on A/X.

The ability of *B. fibrisolvens* to grow on phenolic acid-carbohydrate complexes was studied further in a series of experiments. Treatment with 0.1 M NaOH for 30 min to saponify the ester linkage in FAXX (7) tended to result in slightly greater absorbance over that with untreated FAXX at 16, 18, and 20 h, but results were not significant ($P > 0.05$)

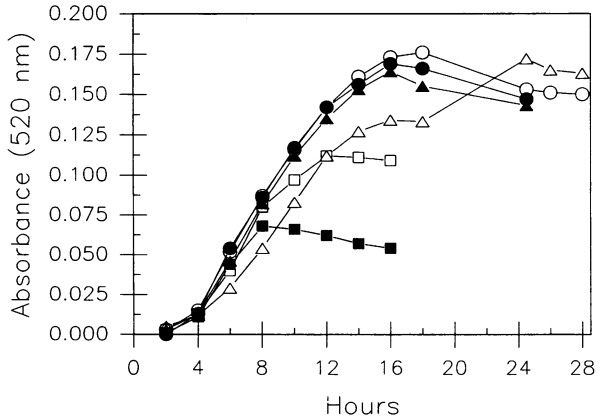

FIG. 3. A_{520} of *B. fibrisolvens* 49 grown with the following substrates: 1 mM arabinose plus 2 mM xylose A/X, △; 1 mM FAXX, ●; 1 mM FAXX saponified with 0.1 M NaOH, ○; 1 mM PAXX, ▲; 1 mM FA, ■; 1 mM arabinose plus 1 mM ferulic acid F/A, □.

TABLE 2. Comparative growth of *B. fibrisolvens* on feruloyl and *p*-coumaroyl arabinoxylans

Substrate comparison	Expt	Ratio of A_{520} for:	
		Active growth[a]	Maximum absorbance
FAXX:A/X	1	1.24	0.83
	2	1.23	0.85
PAXX:A/X	1	1.17	0.77
	2	1.16	0.77

[a] Active growth is mid- to late logarithmic stage, with absorbance read at 12 (experiment 1) and 16 (experiment 2) h for all substrates.

at any hour. Growth on A/X incubated with 0.1 M NaOH as a control was not different from that on untreated A/X and, therefore, this curve is not presented in Fig. 3. A second run, in which FAXX was incubated with 0.1 M NaOH for 60 min, resulted in a slight but nonsignificant ($P > 0.05$) increase in absorbance at 20 h (maximum absorbance) over that on untreated FAXX (data not shown). Growth on F/A was substantially higher than that on FA, suggesting a limitation by *B. fibrisolvens* to break the ester bond and utilize all of the arabinose for growth. The FA supported growth to 8 h of incubation; the slope for growth from 4 to 6 h was 0.017 h^{-1} for arabinose with or without ester-linked ferulic acid. This growth and the abrupt cessation of growth at 8 h on FA suggested that some available carbon was present in the esterified arabinose substrate. Possibly an accumulation of nonmetabolizable intermediates prevented further growth on FA.

As with *S. ruminantium*, the pH at the end of the incubation period in Fig. 3 (i.e., 28 h) was 6.6 to 6.7 for all incubation tubes for all substrates. Apparently, 1 mM arabinose (F/A) supported less growth compared with A/X because of a lower total concentration of substrate. Growth on basal medium was scarce (maximum absorbance of 0.056).

In contrast to *R. flavefaciens* and *S. ruminantium*, *B. fibrisolvens* produced esterase activities against both FAXX and PAXX, with substantially higher amounts of feruloyl esterase (Table 1). This finding was consistent with the fact that *B. fibrisolvens* grew well on FAXX and PAXX and that absorbance on FAXX was slightly but consistently greater than on PAXX at comparable hours (Fig. 3 and Table 2). Growth over a 26-h period indicated that rates of growth were similar between A/X and A/X supplemented with either ferulic or *p*-coumaric acid. These results indicate that phenolic acids at 1 mM concentrations were not inhibitory or stimulatory to growth of *B. fibrisolvens*.

B. fibrisolvens is a major contributor to hemicellulolytic activity in the rumen as well as in the human colon, utilizing xylans and pentoses (12, 13). In addition to degrading xylans (10), this bacterium produces an arabinofuranosidase (9), which cleaves arabinose from arabinoxylans. However, Hespell and O'Bryan (9) reported that the arabinofuranosidase from *B. fibrisolvens* GS113 did not cleave arabinose that was esterified to ferulic or *p*-coumaric acid. Results of the present study indicated that *B. fibrisolvens* 49 utilizes these phenolic acid-carbohydrate esters for growth. The production of FAXX and PAXX esterases likely was important in this context. Results further suggested that the organism may derive energy from the xylobiose moiety of FAXX and PAXX, thus growing faster initially on the esters compared with growth on A/X. However, growth was limited by the esters (i.e., lower maximum absorbance), per-

haps due to incomplete availability of arabinose as indicated by growth on FAXX, PAXX, and FA compared with growth on equimolar concentrations of sugars.

These results are the first report directly establishing the limitation of pentose bioavailability in phenolic acid-arabinoxylan esters of plant cell walls. The extent of limitation appeared to be dependent upon individual bacterial species and the diversity of enzymatic potential to hydrolyze ester and carbohydrate linkages.

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