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Synergism in Degradation and Utilization of Intact Forage Cellulose, Hemicellulose, and Pectin by Three Pure Cultures of Ruminal Bacteria†

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Pure cultures of ruminal bacteria characterized as using only a single forage polysaccharide (*Fibrobacter succinogenes* A3c, cellulolytic; *Bacteroides ruminicola* H2b, hemicellulolytic; *Lachnospira multiparus* D15d, pectinolytic) were inoculated separately and in all possible combinations into fermentation tubes containing orchard grass as the sole substrate. Fermentations were run to completion, and then cultures were analyzed for digestion of cellulose plus degradation and utilization of hemicellulose and pectin. Addition of the noncellulolytic organisms, in any combination, to the cellulolytic organism *F. succinogenes* had little effect on overall cellulose utilization. *F. succinogenes* degraded but could not utilize hemicellulose; however, when it was combined with *B. ruminicola*, total utilization of hemicellulose increased markedly over that by *B. ruminicola* alone. *L. multiparus* was inactive in hemicellulose digestion, alone or in any combination. Although unable to degrade and utilize purified pectin, *B. ruminicola* degraded and utilized considerable quantities of the forage pectin. In contrast, *L. multiparus* was very active against purified pectin, but had extremely limited ability to degrade and utilize pectin from the intact forage. Both degradation and utilization of forage pectin increased when *F. succinogenes* was combined with *B. ruminicola*. Sequential addition of two cultures, allowing one to complete its fermentation before adding the second, was used to study synergism between cultures on forage pectin digestion. In general, synergistic effects did not appear to be related to a particular sequence of utilization. The ability of *F. succinogenes* to degrade and *B. ruminicola* to degrade and utilize forage pectin contradicts both previous and present data obtained with purified pectin. Thus, isolation and characterization of ruminal bacteria on purified substrates may be misleading with regard to their role in the overall ruminal fermentation.

Dehority and Scott (6) were able to demonstrate a synergistic increase in forage cellulose digestion by combining a hemicellulolytic, noncellulolytic organism with several cellulolytic strains. Synergism between cellulolytic and noncellulolytic species was postulated as a reason for the increased extent of cellulose digestion observed in vivo as compared with that observed in pure cultures of cellulolytic ruminal bacteria in vitro (9). Coen and Dehority (1) observed a slight increase in degradation (solubilization in acidified 80% ethanol) and a marked increase (from 6 to 80%) in utilization of intact forage hemicellulose by combining a cellulolytic, hemicellulose-nonutilizing strain with a hemicellulose-utilizing strain. Results indicated that the nonutilizing strains could degrade or solubilize the forage hemicellulose, which then was available to the utilizing strain. Similar or larger amounts of the hemicellulose were utilized when the hemicellulose was isolated from the forage by chemical means. Similar results were obtained for pectin digestion from intact forages; i.e., a slight increase in degradation and a large increase in utilization were obtained by combining a cellulolytic, limited-pectin-utilizing strain with a pectinolytic strain (7). From these results it was concluded that the hemicellulolytic and pectinolytic species can almost completely utilize either of these substrates when the substrates are freed from the intact forage, either chemically or biologically. Thus, in addition to utilizing cellulose, the cellulolytic species appear to contribute to the overall ruminal fermentation by solubilizing hemicellulose and pectin, making them available to other bacteria. The present study was undertaken to determine the extent of synergism among three ruminal organisms which were specific in their ability to digest one of the major carbohydrate constituents of intact forage (cellulose, hemicellulose, or pectin). An additional objective was to determine whether this synergism was dependent upon a particular sequence of substrate utilization.

MATERIALS AND METHODS

Fibrobacter succinogenes A3c, formerly *Bacteroides succinogenes* (10), *Bacteroides ruminicola* H2b, and *Lachnospira multiparus* D15d have been previously characterized (3–5) and were selected on the basis of their ability to utilize a single purified substrate, namely, cellulose, xylan, and pectin, respectively.

Intact orchard grass (OG) (*Dactylis glomerata* common), at two different stages of maturity, was used as the substrate. The forages were harvested from a pure stand, baled into large round bales, chopped in a tub grinder, and finally ground through a no. 40 screen (openings of 400 by 450 μm) in a small laboratory Wiley mill. In a separate study, degradation and utilization of purified pectin (pectin NF; Calbiochem-Behring) were determined.

The anaerobic technique of Hungate (8), with the modifications of Dehority (5), was used to culture the bacteria. The 0.5% forage fermentation test medium, containing 40% ruminal fluid, was prepared as described by Coen and Dehority

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TABLE 1. Percent utilization of cellulose from two maturity stages of OG in vitro

Organism(s)	% Utilization (± SD) of cellulose from[a]:	
	Immature OG	Mature OG
A3c	64.5 ± 13.4a	68.0 ± 13.0a
H2b	1.5 ± 3.1b	6.7 ± 6.4b
D15d	0.3 ± 0.5b	5.1 ± 6.1b
A3c + H2b	61.9 ± 12.9a	76.2 ± 8.0a
A3c + D15d	63.0 ± 11.6a	71.2 ± 3.9a
H2b + D15d	5.5 ± 7.1b	3.4 ± 4.4b
A3c + H2b + D15d	70.6 ± 13.5a	75.8 ± 5.6a

[a] Means within the same column followed by different letters differ significantly ($P < 0.05$).

(1). Test medium containing 0.3% purified pectin was prepared as described by Dehority (5).

All media were inoculated by the procedures detailed by Coen and Dehority (1), and an uninoculated tube was included as a control. All fermentations were incubated for 168 h at 38°C (6), after which time fermentation was complete. Forage cellulose analysis was performed by the procedure of Crampton and Maynard (2). The hemicellulose content was determined by the method described by Coen and Dehority (1). Degradation is defined as the solubilization of hemicellulose in 80% acidified ethanol, and utilization is defined as the loss of hemicellulose from the forage. The concentration of galacturonic acid was used to estimate both purified and forage pectin contents as described by Gradel and Dehority (7). Similar to the definitions for hemicellulose, degradation is defined as the solubilization of pectin in 88% acidified ethanol and utilization is defined as the loss of forage pectin.

Utilization and, when applicable, degradation were determined in the same fermentation series. Single tubes were used for each analysis, inoculum, and maturity stage. Cellulose digestion was based on four separate fermentations. All other hemicellulose and pectin digestibility values were estimated in three replicate experiments.

For the sequential inoculation with two different bacterial species, see Table 5. Following stage 1 inoculation, the fermentation tubes were incubated at 38°C for 30 days. The assumption was made that after 30 days, all stage 1 organisms would be nonviable. At that time, the fermentation tubes were inoculated with a second species (stage 2). Following stage 2 inoculation, the cultures were incubated at 38°C for 168 h and then analyzed for forage pectin as described above.

All data were analyzed by one-way analysis of variance followed by Tukey's procedure at the 0.05 probability level, using the *SPSS-X Release 3.0* statistical computer package (11).

RESULTS

In this study there was no apparent synergism between organisms in the utilization of cellulose from either maturity stage of OG hay (Table 1). No significant increase in cellulose utilization by *F. succinogenes* A3c was observed when this species was combined with *B. ruminicola* H2b, *L. multiparus* D15d, or both. Unexpectedly, utilization of cellulose did not decrease with increasing maturity of the forage as observed previously (1).

When the cellulolytic strain A3c was combined with the hemicellulolytic strain H2b, hemicellulose utilization was increased significantly ($P < 0.05$) (Table 2). The increase ranged from approximately 100% with immature OG to 250% with mature OG. Essentially, degradation was the same as or slightly increased above that observed with A3c alone. The increase in utilization by the combination of A3c and H2b was considerably greater than the additive responses of A3c and H2b alone. Addition of D15d to tubes with H2b or A3c did not affect the extent of degradation or utilization of hemicellulose. The percent degradation and utilization of hemicellulose decreased with increased maturity of the OG, but this decrease was not significant in all cases. The cellulolytic organism (strain A3c) solubilizes a large percentage of the total hemicellulose, presumably while degrading and utilizing cellulose for its own proliferation. In turn, the solubilized hemicellulose is available for utilization by H2b. Degradation appears to be the limiting step for utilization of hemicellulose by H2b alone.

Degradation and utilization of pectin from the two maturity stages of OG (Table 3) were both quite different than expected. The hemicellulolytic organism H2b degraded and utilized more of the forage pectin than the pectinolytic organism D15d did, although not significantly so with the mature OG. Additionally, D15d had the lowest percent degradation of forage pectin observed in this study. Pectin degradation and utilization decreased with increasing maturity of the forage, although not significantly ($P > 0.05$).

The cellulolytic organism *F. succinogenes* A3c degraded a large percentage of the total forage pectin but could not utilize this material as an energy source. However, when *B. ruminicola* H2b was added to the inoculum with A3c, the percent degradation significantly ($P < 0.05$) increased above the level observed with either organism alone and utilization by the combination was greater than the sum of utilization by the two individual organisms. The combination of all three organisms showed no additional increase in percent degradation or utilization above that observed with the combina-

TABLE 2. Percent degradation and percent utilization of hemicellulose from two maturity stages of OG hay in vitro

Organism(s)[a]	Immature OG[b]		Mature OG[b]	
	% Degradation ± SD	% Utilization ± SD	% Degradation ± SD	% Utilization ± SD
A3c*	76.6 ± 3.4a	3.1 ± 2.9a	59.7 ± 7.1a	6.8 ± 3.8a
H2b*,**	39.1 ± 4.1b	36.2 ± 3.9b	20.3 ± 2.8b	18.8 ± 2.4a
D15d	1.9 ± 1.7c	1.8 ± 1.6a	4.1 ± 3.9b	4.1 ± 3.9a
A3c + H2b	77.4 ± 2.6a	74.5 ± 1.9c	70.2 ± 10.9a	68.6 ± 10.9b
A3c + D15d	75.1 ± 0.2a	3.7 ± 3.2a	61.3 ± 9.5a	4.2 ± 4.8a
H2b + D15d*,**	41.2 ± 6.4b	37.8 ± 6.3b	21.6 ± 8.2b	19.5 ± 9.4a
A3c + H2b + D15d*	79.2 ± 2.7a	76.8 ± 2.8c	69.1 ± 5.4a	67.3 ± 6.6b

[a] Symbols: *, percent degradation of immature and mature OG differs significantly ($P < 0.05$); **, percent utilization of immature and mature OG differs significantly ($P < 0.05$).

[b] Means within the same column followed by different letters differ significantly ($P < 0.05$).

TABLE 3. Percent degradation and percent utilization of pectin from two maturity stages of OG in vitro

Organism(s)	Immature OG[a]		Mature OG[a]	
	% Degradation ± SD	% Utilization ± SD	% Degradation ± SD	% Utilization ± SD
A3c	68.5 ± 4.4a,c	0.0 ± 0.0a	61.2 ± 13.3a,c,d	4.3 ± 8.6a
H2b	54.9 ± 5.4a	46.1 ± 6.5b	40.9 ± 10.9a,b	29.5 ± 14.4a,b
D15d	18.9 ± 4.6b	6.8 ± 6.4a	28.3 ± 10.2b	13.1 ± 14.8a
A3c + H2b	83.9 ± 3.6d	75.3 ± 4.5c	76.2 ± 7.2d	61.9 ± 12.4c
A3c + D15d	78.3 ± 5.3c,d	0.0 ± 0.0a	66.7 ± 7.1c,d	4.8 ± 9.6a
H2b + D15d	56.6 ± 7.2a	49.4 ± 8.4b	47.2 ± 13.8a,b,c	33.6 ± 19.2a,b,c
A3c + H2b + D15d	85.4 ± 3.9d	76.8 ± 2.9c	73.1 ± 11.8d	58.6 ± 13.0b,c

[a] Means within the same column followed by different letters differ significantly ($P < 0.05$).

tion of A3c and H2b. The combination of H2b and *L. multiparus* D15d was not different from an inoculum with H2b alone in either degradation or utilization. The combination of A3c and D15d showed a slight increase in degradation above that by A3c alone; however, a nonsignificant decrease occurred in percent utilization as compared with that by D15d alone. Presumably either the soluble pectin degradation products produced by A3c are nonutilizable by the pectinolytic organism D15d or A3c produces specific substances which inhibit the pectinolytic enzymes of D15d.

Because degradation of forage pectin by A3c and both degradation and utilization by H2b contradict the published information on these two organisms (5, 7), an experiment was run to determine whether the abilities of these two organisms to degrade and utilize purified pectin had changed since they were first isolated and characterized (3–5). Strain D15d was the only organism that showed appreciable degradation or utilization of purified pectin (Table 4) or any growth response (data not shown) over a 7-day incubation period. There was no apparent synergism in either degradation or utilization of purified pectin when any two or more cultures were combined. These results are similar to those obtained in the previously mentioned studies (3–5, 7).

An experiment was designed to determine whether the sequence of substrate utilization by the different organisms might affect the percent degradation and utilization of pectin. A sequential inoculation scheme was used (Table 5). In general, all combinations of A3c and H2b, regardless of whether they were inoculated at the same time or one before the other, gave similar results. The results obtained with the sequential addition of A3c and D15d are more difficult to explain. When A3c was added first, there was a slightly higher percent degradation and lower percent utilization than when D15d was added first; however, these differences were not significant. There was almost a twofold decrease in percent utilization when A3c was combined with D15d in any order as compared with the use of D15d alone; however, there was no difference between adding the two organisms sequentially or together. The decreased utilization observed when A3c was added first agrees with earlier results (Table 3) and might be explained on the basis that A3c alters the degradation products in the process of solubilizing pectin. However, an explanation for the results observed when D15d was added first is not immediately obvious. It would be expected that D15d should be able to utilize pectin to the same extent whether or not A3c was added later. The decrease in utilization by any combination of A3c and D15d below that by D15d alone was significant at $P < 0.1$ but not at $P < 0.05$.

The viability of the stage 1 organisms was determined after the initial 30-day incubation. The results indicated that after 30 days, A3c had an increased lag time but did respond to fresh substrate, whereas H2b grew at the same rate as a fresh culture. The 30-day-old culture of D15d did not respond to fresh medium and was presumed nonviable.

TABLE 4. Percent degradation and percent utilization of purified pectin in vitro

Organism(s)	% Degradation ± SD[a]	% Utilization ± SD[a]
A3c	17.9 ± 1.6a	9.5 ± 3.2a
H2b	12.1 ± 3.2b	5.1 ± 3.9a
D15d	87.1 ± 1.0c	73.2 ± 2.6b
A3c + H2b	17.9 ± 1.5a	8.1 ± 2.3a
A3c + D15d	87.8 ± 1.2c	73.2 ± 2.8b
H2b + D15d	87.9 ± 0.9c	73.4 ± 2.2b
A3c + H2b + D15d	88.7 ± 0.9c	73.5 ± 2.4b

[a] Means within the same column followed by different letters differ significantly ($P < 0.05$).

DISCUSSION

Utilization of cellulose did not decrease with increasing maturity of the forage in this study, as had been observed previously (6). Variations in the year and stand of OG hay may account for these differences. The absence of synergism in cellulose digestion between the cellulolytic and hemicellulolytic organisms (*F. succinogenes* A3c and *B. ruminicola* H2b) disagrees with previously observed responses. Dehor-

TABLE 5. Percent degradation and percent utilization of pectin from OG hay in vitro with sequential addition of selected organisms

Organism(s)[a]		% Degradation ± SD[b]	% Utilization ± SD[b]
Stage 1	Stage 2		
A3c	H2b	81.5 ± 9.5a	74.7 ± 9.7a
H2b	A3c	82.5 ± 1.3a	76.8 ± 1.0a
A3c	D15d	82.4 ± 1.0a	18.5 ± 9.1b,d
D15d	A3c	79.9 ± 1.8a	21.6 ± 16.3b,d
A3c + H2b	–	81.2 ± 8.0a	77.9 ± 6.8a
A3c + D15d	–	79.9 ± 5.0a	16.3 ± 13.3b,d
A3c	–	70.5 ± 5.3a,b	4.7 ± 8.1d
H2b	–	60.8 ± 7.1a,b,c	57.6 ± 8.0a,c
D15d	–	47.1 ± 10.4b,c	38.3 ± 11.0b,c
–	A3c	61.8 ± 17.0a,b,c	1.8 ± 3.1d
–	H2b	61.3 ± 2.8a,b,c	57.7 ± 5.9a,c
–	D15d	41.6 ± 11.8c	33.7 ± 14.3b,c
–	A3c + H2b	82.4 ± 3.8a	77.2 ± 5.2a
–	A3c + D15d	80.3 ± 9.2a	17.7 ± 9.2b,d

[a] See Materials and Methods for details of inoculation procedures. –, None used in that stage.

[b] Means within the same column followed by different letters are significantly different ($P < 0.05$).

ity and Scott (6) reported that combining the hemicellulolytic *B. ruminicola* H8a with several different cellulolytic organisms, including A3c, resulted in a significant increase in total cellulose utilization over that by H8a alone. This difference was based on the mean percent cellulose digestion of 12 different forages. The combination of A3c and H2b with the mature OG and the combination of all three strains with both maturity stages gave a slightly higher mean cellulose digestibility than A3c alone; however, because of variation and limited numbers, this mean difference was not significant. Dehority and Scott (6) proposed that H8a may, in the process of digesting hemicellulose, expose cellulose for utilization by the cellulolytic organism. H2b, on the other hand, may degrade and utilize hemicellulose in such a way that no additional cellulose is made available.

The percent degradation and utilization of hemicellulose and of pectin decreased with increased maturity of OG. These results are similar to previous observations with brome grass (1, 7).

In general, *F. succinogenes* A3c and *B. ruminicola* H2b degrade relatively large amounts of forage pectin, and H2b utilizes about 75 to 80% of the pectin it degrades. Neither strain degrades or utilizes much of the purified pectin. When A3c and H2b are combined, the extent of both degradation and utilization of forage pectin is increased. This suggests that each organism degrades or solubilizes part of the pectin unavailable to the other or removes a barrier which blocks the activity of the second organism. The extent of degradation and utilization was similar whether the two cultures were added simultaneously or sequentially, which would support the concept of barrier removal. If the stage 1 organism was not viable after 30 days, a decreased utilization would be expected when H2b was added first; however, utilization increased approximately 20% when the nonutilizing strain A3c was added in stage 2. Because definite synergism occurs in utilization, with marked increases over the combined total of each individually, it would appear that H2b utilizes most of the solubilized pectin. The relative inability of these two organisms to degrade and utilize purified pectin suggests a chemical or structural difference between purified and intact forage pectins.

L. multiparus D15d degraded and utilized appreciable amounts of purified pectin, but showed very limited activity in the digestion of forage pectin. If the inability to degrade and utilize forage pectin were based on a physical barrier which could not be removed by D15d (i.e., cellulose or hemicellulose), combinations of D15d with A3c or H2b should have shown an increase in degradation and utilization over that by D15d alone. These findings might further support the concept of chemical or structural differences between purified and forage pectins. Consequently, the use of a purified substrate for isolation of bacteria may or may not be selective for the predominant or functionally active species which occur in the rumen itself.

Consistent throughout this study is the finding that the combination of all three organisms shows no increase in percent degradation or utilization above that of the best pair. In addition to utilizing cellulose, the cellulolytic organism appears to degrade large quantities of the other two major structural carbohydrates of forage, making these substrates available for further utilization. In this system, which might be considered a simple model of ruminal fermentation, the cellulolytic organism appears to play a very important role in overall forage digestibility.

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