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Evaluation by Electron Microscopy and Anaerobic Culture of Types of Rumen Bacteria Associated with Digestion of Forage Cell Walls

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Different morphological types of rumen bacteria which degraded cell walls of forage grasses with various in vitro digestibilities were evaluated with electron microscopy. The majority of these bacteria (i.e., about 70% or more) consisted of two distinct types: (i) encapsulated cocci and (ii) irregularly shaped bacteria, resembling major fiber digesters found in the rumen. Each type was capable of degrading structurally intact cell walls. Differences ($P \leq 0.02$) in the percent ratio of encapsulated cocci to irregularly shaped bacteria were observed between Bermuda grass and fescue; the ratio of encapsulated cocci to irregularly shaped bacteria between Bermuda grass and orchard grass was similar and variations were high. The proportion of irregularly shaped bacteria usually increased with increased time of digestion. Differences ($P > 0.1$) were not found in the percentage ratio of encapsulated cocci to irregularly shaped bacteria attached to specific tissue types in either Bermuda grass or fescue. However, encapsulated cocci tended to be more prevalent on sclerenchyma than other tissues in Bermuda grass, but less prevalent on sclerenchyma than other tissues in fescue. Transmission electron microscopy of tissue digestion of rapidly degraded orchard grass blades revealed that mesophyll, parenchyma bundle sheath, and parts of the epidermal cell wall apparently were degraded without direct attachment of bacteria although bacteria were near the cell walls undergoing digestion. Anaerobic growth studies showed that the total culturable bacteria developing on medium 10 and media containing carbohydrates similar to those in forage cell walls (i.e., pectin, xylan, and cellobiose) were 80% higher from rumen bacterial populations adapted in vitro to cell walls of orchard grass compared to those from Bermuda grass; the number of colonies from the orchard grass-adapted population was significantly ($P \leq 0.05$) greater on the medium containing xylan. Filter paper tests showed that the cellulolytic activity of populations adapted to fescue was greater than that of orchard grass or Bermuda grass.

Attachment of cell wall-degrading rumen bacteria to forage tissues has been shown by light microscopy (12) and by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (4, 7), as well as by elution techniques (45). Differences in the mode of attachment to forage cell walls exist among various morphological types of the rumen bacteria (2).

Plant tissues that make up the forage diet differ in chemical composition (14), anatomy (15, 44), amounts present (6), and ease of in vitro digestibility by and association with rumen microorganisms (5-7). Although changes in the bacterial population with various types of diets have been reported (18, 25, 40, 49, 53), data on the morphological types of rumen bacteria associated with the dietary fiber of various forage grasses are few.

Recent electron microscopic studies have shown propensities for attachment to specific tissues in ryegrass by known cellulolytic rumen bacteria (42) and variations in the rumen bacterial populations associated with plant tissues and cellulosic substrates (27). However, comparisons of the predominant types of fiber-digesting bacteria from the mixed rumen microflora associated with specific tissues of various forage grasses are not available.

Electron microscopy permits in situ visualization of forage cell wall degradation and does so without physical or chemical pretreatment that might modify factors affecting the plant/microbe association during digestion. Microscopic studies have shown that cell walls vary in their relative rates of digestion (6, 34). Other research has shown variability in the digestibility of similar cell wall components in different forages (23). Hemicellulose, in particular, ap-

peared to be influential in affecting cell wall digestion (23, 50).

The objectives of this study were (i) to quantitate the morphological types (by TEM) of rumen bacteria involved in the initial degradation of forage cell walls which vary in digestibility, (ii) to quantitate the predominant morphological types attached to specific cell walls within forage species, (iii) to investigate the mode of attack of forage cell walls which vary in ease of digestion, and (iv) to identify relative differences in the number of culturable bacteria developing on specific substrates from populations adapted in vitro to forages of various digestibilities.

MATERIALS AND METHODS

Substrate. Samples consisted of Coastal Bermuda grass (*Cynodon dactylon* [L.] Pers.), Kentucky-31 tall fescue (*Festuca arundinacea* Schreb.), and Boone orchard grass (*Dactylis glomerata* L.). All forages were from well-managed, experimental fields and were harvested at 4 to 6 weeks' regrowth. Samples were quick-frozen after harvest and maintained at −30°C until used.

Preparation for TEM evaluation of in vitro-digested leaf blades. Samples of inoculum were taken from a cannulated steer maintained predominately on Bermuda grass hay and pasture. Each sample of rumen fluid was strained twice through cheesecloth and mixed at a ratio of 1:2 with McDougall carbonate buffer (43) and incubated in 125-ml flasks or 50-ml centrifuge tubes or prepared as washed-cell preparations in Cheng buffer as described earlier (5). Attachment by morphological types of rumen bacteria was similar with both types of preparations of inoculum, so data are not distinguished by inoculum. From 30 to 100 leaf sections 2 to 3 mm long from the center of the blade were used as substrate and incubated with the bacterial inoculum at 39°C with continuous CO_2 bubbling. Sections of these leaf blades undergoing digestion by rumen microorganisms were fixed and prepared for thin-sectioning as described (2, 7). Rumen bacteria that associated with forage cell walls were enumerated on these bases: (i) directly attached to eroded zones in the plant cell walls shown by modification in shape of the bacterial wall or by the presence of a capsular material, or (ii) near delimited, eroded zones in the plant wall similar in shape to the nearby bacteria that would result from detached bacteria or carbohydrases active in close proximity to bacteria. Bacteria were counted from electron micrographs in which sites with several bacteria (average of seven) were present, or from thin sections observed directly under the TEM, in which all bacteria present were enumerated. The *t* test was used to compare the percentages of attaching types of bacteria (28).

In vitro adaptation of rumen bacterial populations with forage cell walls. Rumen bacterial populations adapted by growth on various forage cell walls were evaluated for numbers of fermentative types by the following procedure. Rumen fluid was collected from the cannulated steer as described above and mixed 1:2 with McDougall buffer. A 30-ml amount of this inoculum was dispensed into 50-ml glass centrifuge tubes containing 0.4 g of either whole, freeze-dried and ground forage or the forage cell walls as represented by neutral detergent fiber (54). The tubes were gassed with CO_2 and then sealed with a Bunsen valve and incubated at 39°C for 6 to 24 h to select populations of rumen bacteria active in degrading the forage fiber.

Preparation of roll tube media for enumeration of fermentative types of rumen bacteria. To use a reproducible medium, the semisynthetic basal medium of Caldwell and Bryant (20) was chosen. Carbohydrates used to evaluate fermenting types of rumen bacteria included those of medium 10, i.e., 0.05% each glucose, cornstarch, and cellobiose (20), and basal medium (medium 10 minus carbohydrates) with either pectin, xylan, or cellobiose to simulate those components and linkages found in the forage cell walls. The pectin was purified to remove contaminating simple sugars (i.e., sucrose) by dialyzing a 2% solution against three changes of distilled water overnight at 3°C and freeze-drying the residue. The xylan was either a 1% solution purified as was the pectin or was a commercially pure source. Other carbohydrates used as substrates were those commercially available. The anaerobic media were prepared with 0.15% total carbohydrate. After the ingredients were boiled, a reducing agent consisting of sodium sulfide-cysteine hydrochloride (20) was added, and the media were bubbled with CO_2 for 15 min. Media were adjusted to a pH of 6.6 to 6.8 if necessary.

The general procedure for roll tube preparation (39) was used to enumerate culturable bacteria. The procedure was modified to use 1.8 ml of appropriate medium dispensed under CO_2 into Hungate tubes (16 by 125 mm; catalog no. 2047, Bellco Glass, Inc.) containing 40 mg of agar. Black butyl rubber septa and screw caps (Hungate type, Bellco) were fitted on the tubes, which were autoclaved (121°C, 15 lb/in^2, 15 min) under fast exhaust. Media were made fresh and with the same basal medium before the carbohydrates were added for each study. Media were maintained at 56°C until used, which facilitated the inoculation, mixing, and rolling procedures for this study. Dilution tubes were made, using an anaerobic dilution solution (17) dispensed under CO_2 into screw-cap vials (16 by 125 mm), which were fitted with septa and caps and autoclaved as described above. Usually the autoclaved media and dilutions were reduced as shown by resazurin, and any pink tubes were discarded.

Determination of relative cellulolytic activity. A 5-ml amount of the basal medium (without carbohydrate) was dispensed under CO_2 into screw-cap vials (16 by 125 mm) containing a strip of Whatman no. 541 (ashless) filter paper (12.5 by 114 mm) as substrate. Tubes were fitted with the Hungate septa and caps and autoclaved as described above.

Inoculation of media with adapted populations of rumen bacteria. After populations were adapted to forages, the rumen bacteria were removed from fiber by incubating fiber with 0.1% Tween 80 (except in one early test) for 30 min with continuous or intermittent shaking, and the bacteria were carried through a dilution series under CO_2, using disposable syringes with 25-gauge needles. (Preliminary tests on the effi-

cacy of Tween 80 in detaching bacteria from forage cell walls were carried out using the described TEM and anaerobic culture procedures). A 0.2-ml amount from the last dilution was then inoculated into each of three to six Hungate tubes of each of the media. Preliminary tests had shown that the 10^6 dilution gave well-isolated, countable colonies, so 0.2 ml of this dilution was inoculated (except for one test completed earlier). Tubes were then mixed by shaking, carefully turned to spread the agar onto the sides of the tubes, spun rapidly and briefly in an ice bath to harden the agar, and incubated at 39°C for 7 days, which was previously found to give sufficient time for growth. Colonies were counted with a Digimatic Colony Counter (Labline Instruments, Inc.).

To compare the relative cellulolytic activities of adapted populations, 1 ml of the Tween 80-treated bacterial suspensions (10^1 dilution) was inoculated into each of three to six filter paper-medium tubes. Preliminary tests using 0.2, 0.5, 1.0, and 2.0 ml of a 1:2 ratio of rumen fluid to McDougall buffer were found to indicate filter paper clearing or a break in filter paper usually within 10 to 15 days. Tubes were evaluated for times of initial digestion (i.e., holes, pitting, clearing), and for final break of filter paper at the liquid junction in tubes.

RESULTS

Morphological types of adhering rumen bacteria associated with forage cell walls. Several morphological types of bacteria were always involved in the attachment to (or close association with) and degradation of forage cell walls (Fig. 1). However, the majority of attaching, cell wall-degrading bacteria were of two distinct, easily recognized morphological types: (i) encapsulated cocci (EC [Fig. 1]) and (ii) irregular-shaped bacteria (IB [Fig. 1]). Other types included regular, evenly shaped bacilli (with and without extracellular material), bacteria with "knobby" (i.e., periodic electron-dense particles) coats, and nonencapsulated cocci (Fig. 1, arrows). The most tightly attached bacteria (as shown by proximity to forage walls or modification of the bacterial wall) were EC and IB. For enumeration of morphological types by TEM, four categories were chosen: (i) EC, distinctive type of evenly shaped cells usually less than 1 μm in diameter with a gram-positive-type cell wall anatomy (Fig. 1) (2); (ii) IB, distinctive type of irregular cells having an irregular, electron-dense outer layer of a gram-negative-type cell wall (Fig. 1) (2); (iii) regularly shaped bacteria, a heterogenous group consisting of bacilli (with and without capsules), bacteria with a knobby coat, and nonencapsulated cocci (Fig. 1, arrows); and (iv) other, i.e., bacteria indistinguishable as to type.

The percentages and standard deviations of these four groups of rumen bacteria in in vitro digestion studies at incubation times of 6 to 72 h for Bermuda grass and fescue leaf blades are shown in Table 1. Some of these data have been reviewed earlier (1) but are included here to clarify present findings. Furthermore, in a test using leaf blades of both grasses digested for 24 h in a single container, the percentages of EC and IB were 32 and 21%, respectively, for Bermuda grass and 10 and 66%, respectively, for fescue.

The data in Table 1 and other data (1) indicated that EC and IB made up the majority (i.e., 70% or more) of the fiber digesters intimately associated with forage cell walls, and therefore the association of these types was further evaluated for variations on forages varying in digestibility. To obtain a statistic for comparison, the relative proportion of these two types was expressed as a percentage ratio in the following manner: percent EC of total attached bacteria divided by percent IB of total attached bacteria; a higher percent ratio, therefore, indicated a greater proportion of EC.

During the course of these studies, more IB were apparent as digestion time increased. In a test of Bermuda grass sampled at different digestion times from a single container, the percentage ratios decreased from 4.61 to 0.83 to 0.32 at 6, 12, and 24 h, respectively. Furthermore, evaluation of several digestion studies with fescue showed percentage ratios of 1.56, 0.14, 0.16, and 0.27 at 6-, 12-, 24-, and 72-h incubation times, respectively. These tests, therefore, indicated a trend of a higher proportion of IB attached to fiber in the later stages of digestion.

The percentage ratio of major fiber digesters was also tested against specific cell wall types in Bermuda grass and fescue (Table 2). Variations were high, and tests of significance did not show differences ($P > 0.1$) in attachment to tissues within a species. However, EC tended to be more prevalent on sclerenchyma than other tissues in Bermuda grass, whereas in fescue fewer EC adhered to sclerenchyma than other tissues.

In studies that assessed the major fiber-digesting types attached to cell walls of forages with various digestibilities (Table 3), the average ratios were similar for Bermuda grass and orchard grass but lower for fescue.

TEM of forage cell wall degradation by rumen bacteria. Each of the major types of bacteria appeared capable of penetrating structurally intact forage cell walls. TEM showed digestion zones in the parenchyma bundle sheath of Bermuda grass (Fig. 2A, B) and a cell wall of fescue (Fig. 3) by both EC and IB. A large portion of the cell wall area remained intact and electron dense where defined zones of degradation associated with adhering bacteria did not occur. However, zones of clearing (i.e., diges-

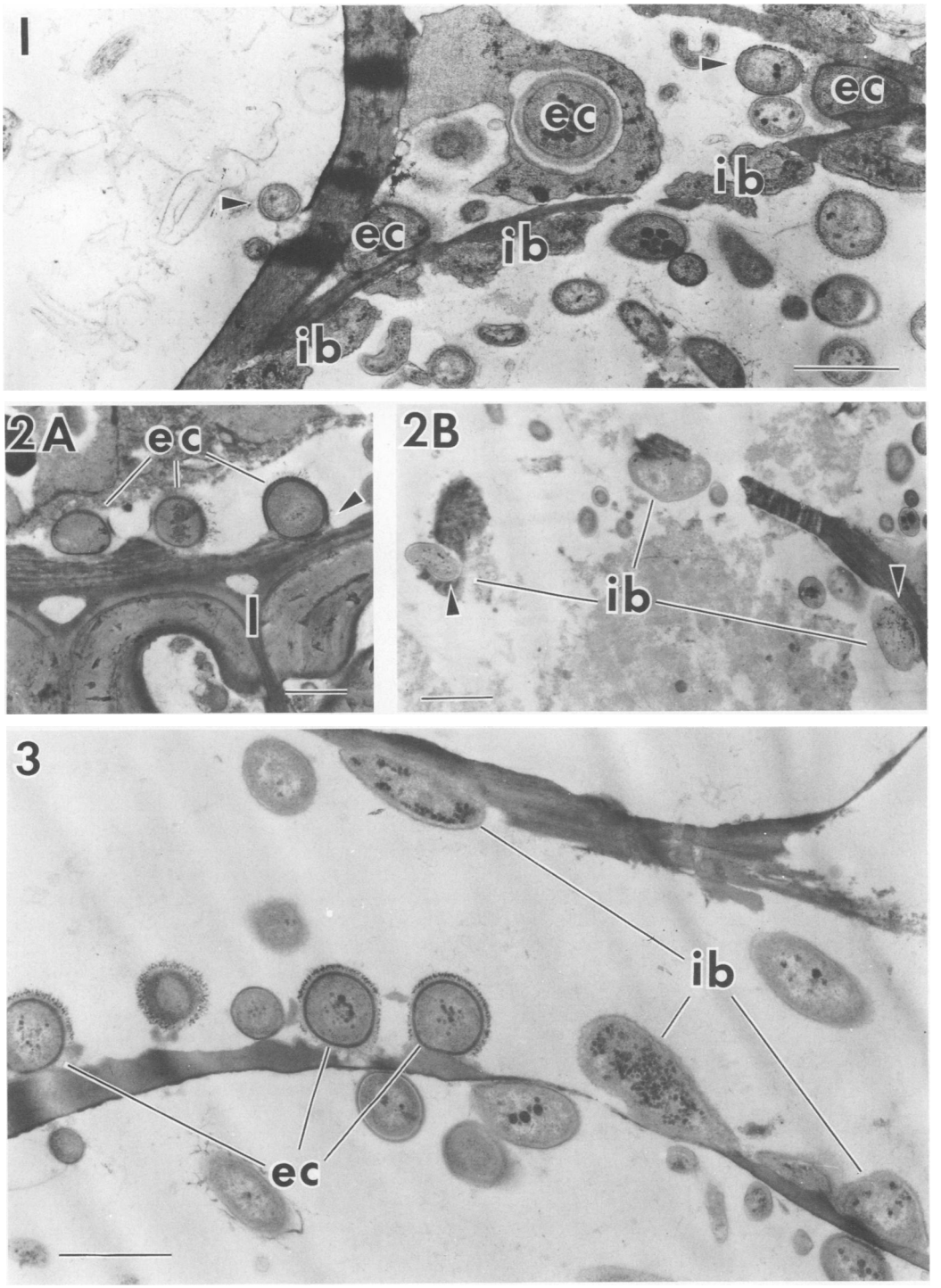

FIG. 1. *TEM of Kentucky-31 tall fescue showing the preponderance of encapsulated cocci (ec) and irregularly shaped bacilli (ib) attached to and degrading the cell wall. Other types of bacteria (arrows) are near degraded zones. Bar = 1 μm.*

FIG. 2. *TEM of the degradation of Bermuda grass parenchyma bundle sheath by the major attaching fiber digesters. (A) ec penetrated the cell wall (arrow) to the nondegradable inner bundle sheath (I). (B) ib degraded through the entire cell wall, producing clearly defined zones of erosion (arrows). Bar = 1 μm.*

FIG. 3. *TEM of penetration and degradation through the entire wall in fescue by ec and ib. Bar = 1 μm.*

TABLE 1. *Morphological types of rumen bacteria attached to cell walls of forage leaf blades during in vitro digestion*

Forage	No. of trials	No. of forage sampled	No. of leaves sampled	No. of bacteria counted	No. of micrographs (sampling sites)	% of the following bacterial types:			
						EC	IB	Regular-shaped bacteria	Other
Bermuda grass	6	3	33	1492	194	39 ± 19[a]	30 ± 19[a]	26 ± 9[a]	5 ± 5[a]
Fescue	5	3	23	1415	162	8 ± 3[b]	64 ± 11[b]	20 ± 6[a]	7 ± 3[a]

[a,b] Differing superscripts within columns mean values are significantly different ($P < 0.02$), using the t test.

TABLE 2. *Percentage ratio of EC to IB attached to specific tissues after 24 h of digestion*

Grass	Ratio[a]			
	Sclerenchyma	Epidermis	Parenchyma bundle sheath	Mesophyll
Bermuda grass	1.19 (0.61-2.17)	0.28 (0-0.69)	0.17 (0-0.46)	No attachment
Fescue	0.04 (0-0.08)	0.22 (0.18-0.26)	0.11 (0.07-0.15)	0.10 (0.03-0.16)

[a] Values represent averages of three tests for Bermuda grass and two tests for fescue; ranges are in parentheses.

TABLE 3. *Percentage ratio of EC to IB attached to forage cell walls at 12 to 28 h of in vitro digestion*[a]

Grass	Total no. of bacteria counted	% EC and IB of total bacteria[a]	% Ratio, EC/IB[a]
Bermuda grass	788	77 (70-92)	0.74 (0.21-1.06)
Fescue	1,005	88 (80-94)	0.16 (0.12-0.21)
Orchard grass	1,208	82 (80-87)	0.77 (0.14-1.86)

[a] Values represent averages of three trials; ranges are in parentheses.

tion) occasionally were observed at a slight distance (<0.5 μm) from the cocci, possibly indicating that fiber-digesting enzymes had diffused from these bacterial walls (Fig. 1 and 3). TEM of orchard grass revealed that mesophyll, parenchyma bundle sheaths, and the inner part (i.e., contiguous with the mesophyll) of the epidermal cell wall were degraded often without direct adherence of bacteria, although bacteria were near (Fig. 4). EC and IB occasionally adhered to these tissues, but often morphological types other than EC and IB made up most of the bacterial population near to the generalized digestion zones (Fig. 4A, C); these other types would not be counted as associated bacteria based on the criteria used for enumerating bacterial types. In particular, note the unattached bacterial types within the degraded intercellular area of Fig. 4C (arrow).

TEM observations suggested that fewer bacteria were associated with Bermuda grass than with either of the cool-season grasses. For example, the average numbers of associated bacteria per leaf blade evaluated in the three incubations (Table 3) were 80 and 60% higher for fescue and orchard grass, respectively, compared with Bermuda grass. Therefore, bacterial populations adapted to cell walls of these forages during in vitro digestion were compared by using anaerobic culture techniques.

Enumeration of fermentative types by anaerobic culturing techniques. Average viable counts in anaerobic cultures (adjusted for all, including the initial 1:2 dilutions) of about 0.8×10^9 to 1.0×10^9 bacteria per ml on medium 10 for three tests each of whole forage samples of the three forage grasses are similar to those reported for ruminants on grass (38). Furthermore, assessment of viable counts on the basal medium without added carbohydrate showed that bacterial colonies were never higher than 3.6% of the number on medium 10 for two tests each of Bermuda grass and fescue. These data indicated, then, that high numbers of rumen bacteria were cultivated and that the majority of colonies utilized the specific added carbohydrates for growth.

Tween 80 (0.1%) added to the incubation media to release fiber-digesting bacteria from forage cell walls before diluting increased viable counts on medium 10 compared to growth on this medium without pretreatment with Tween 80. Results from three to four tests showed a 70% increase in culturable bacteria after incubation with Tween 80 for 25 to 30 min, an increase of 59% after 45 to 55 min, but a reduction of 19% after incubation for longer than 60 min. In particular, the number of bacteria culturable on cellulose or cellulolytic activities (as determined by filter paper degradation) was increased by prior treatment with Tween 80. Comparisons, using TEM, of Tween 80-treated (7 min with intermittent vigorous shaking by hand) leaf blades with untreated controls showed proportionately more EC removed than IB; the

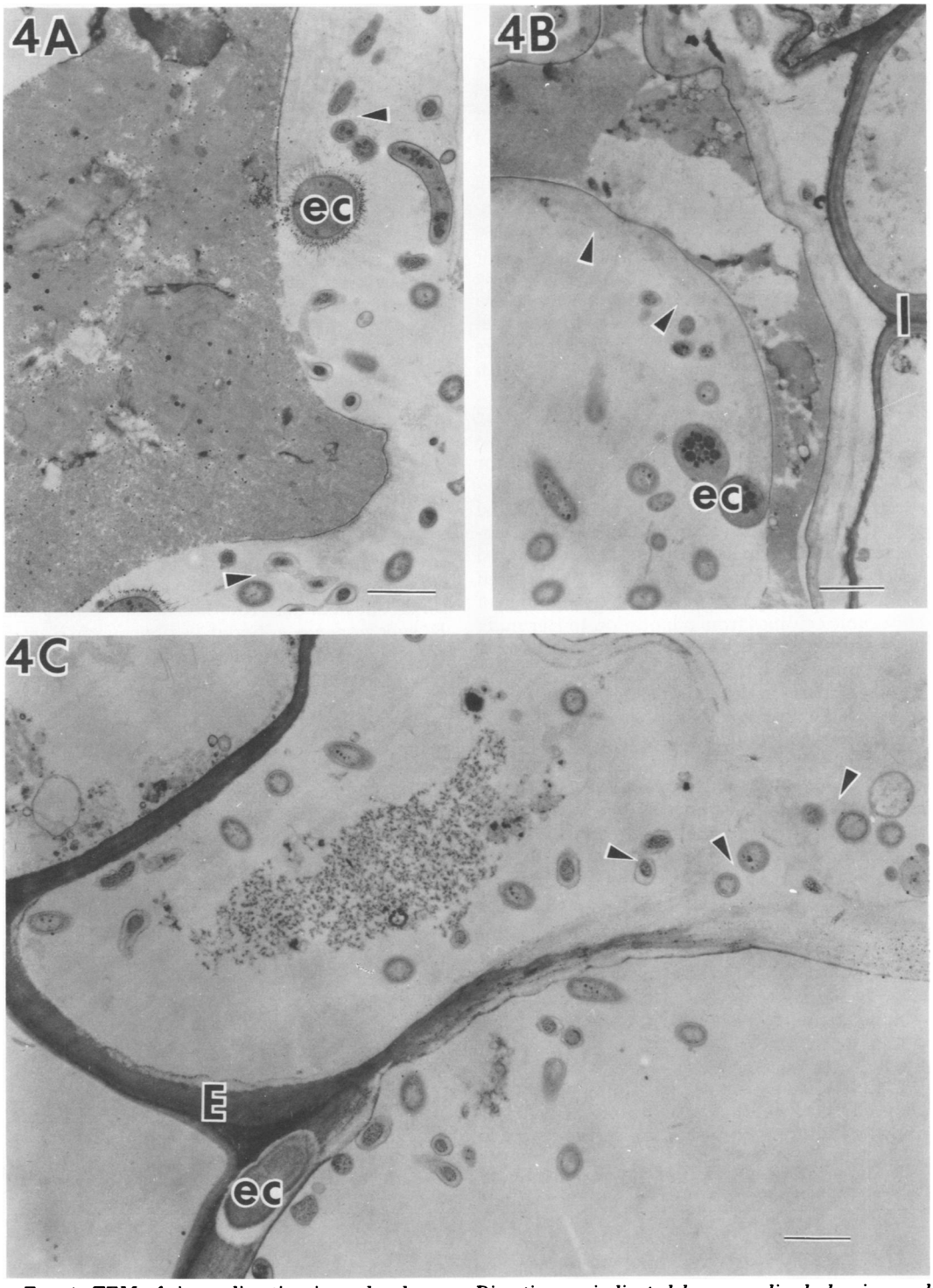

FIG. 4. *TEM of tissue digestion in orchard grass. Digestion as indicated by generalized clearing and diminished electron density of cell walls apparently occurred in many areas without direct bacterial adherence. (A) Mesophyll cell wall digested by nearby bacteria (arrows) morphologically unlike the attaching encapsulated cocci (ec). (B) Parenchyma bundle sheath with attached ec and generalized erosion (arrows). Compare with the lignified, nondegradable inner sheath (I). (C) Epidermal cell wall. The inner wall, contiguous with the mesophyll wall, shows generalized erosion and loss of electron density. Bacteria, unlike ec and irregularly shaped bacteria (ib), are near to and within these generalized areas of digestion (arrows). The thicker portion of the epidermis (E) underneath the cuticle retained electron density, except in clearly defined zones produced by ec. Bar = 1 μm.*

percentage ratio of EC to IB decreased from 0.23 to 0.16 for Bermuda grass and 0.12 to 0.01 for fescue. In another study to evaluate release of rumen bacteria from Bermuda grass cell walls, treatment with Tween 80 (45-min incubation in shaker bath at 150 rpm) compared with untreated control forage resulted in a 27% increase in culturable bacteria and a decrease in percentage ratio of EC to IB of 0.78 to 0.23. These studies indicated Tween 80 did release bacteria from fiber, but TEM observations indicated that many rumen bacteria, and proportionately more IB, still adhered to forage cell walls even with these procedures.

Rumen bacterial populations elicited during in vitro digestion with various forage cell walls (as neutral detergent fiber) were evaluated for viable counts developing on specific carbohydrate media (Table 4). The data were calculated as comparisons of the ratio of colonies developing from either fescue- or orchard grass-adapted bacterial populations divided by those adapted with Bermuda grass. Therefore, the number of culturable colonies from adaptation to Bermuda grass served as an internal control to standardize rumen fluid samples collected from the steer. Ratios greater than 1.00 indicated higher viable counts from fescue- or orchard grass-adapted populations compared with those from Bermuda grass-adapted populations. In general, higher numbers of culturable bacteria developed on medium 10 and specific carbohydrate media from populations adapted with orchard grass than with Bermuda grass, and the number of xylanolytics was significantly ($P \leq 0.05$) greater. Bacterial populations adapted to fescue were markedly higher only for pectinolytics. The average total culturable bacteria developing on pectin, xylan, or cellobiose media were 20 and 80% greater for fescue- and orchard grass-adapted populations, respectively; the 80% increase for orchard grass was significant ($P \leq 0.05$).

Cellulolytic activities of the populations adapted to cell walls of various forages as determined by filter paper assays are shown in Table 5. No consistent differences were apparent in the degradation of filter paper with populations from Bermuda grass and orchard grass. Filter paper digestion was fastest with fescue-adapted populations.

DISCUSSION

The EC and IB types of bacteria have always been observed in the rumen inocula used in our laboratory (4, 7) and their morphologies have previously been described (2). The characteristics of EC agree with those for *Ruminococcus* species (19, 38) and TEM of EC showed morphological similarities to pure cultures of *Ruminococcus flavefaciens* (41) and *R. albus* (48). The IB type had similar characteristics to (19) and closely resembled TEM observations of (21, 42) *Bacteroides succinogenes*. *B. succinogenes*, *R. albus*, and *R. flavefaciens* are the three most prevalent fiber digesters in the rumen (16). Furthermore, studies involving differential elution with Tween 80 of rumen bacteria from digesta solids showed that *Ruminococcus* species and *B. succinogenes* adhered to fiber, with *B. succinogenes* adhering more tightly (45). Similar results were found in this study with Tween 80 for EC and IB. Therefore, possibly EC and IB are *Ruminococcus* species and *B. succinogenes*, respectively.

Zones of digestion were occasionally observed at a slight distance from EC, which might indicate that cell wall-degrading enzymes had diffused from the bacteria (Fig. 1, 3). *R. albus* has

TABLE 5. *Time of filter paper degradation by rumen bacterial populations adapted in vitro to neutral detergent fiber from different forage grasses*[a]

Forage	Initial digestion observed (day)	Break in filter paper strip (day)
Bermuda grass	7.3	11.0–13.7
Fescue	4.7	9.7–13.0
Orchard grass	6.3	12.3–13.7

[a] Values represent averages from three trials.

TABLE 4. *Ratio of number of culturable colonies utilizing specific carbohydrates from rumen bacterial populations adapted in vitro to neutral detergent fiber from different forage grasses*

Forages compared	Ratio of colonies that developed on:[a]			
	Medium 10 (n = 6)	Pectin (n = 5)	Xylan (n = 5)	Cellobiose (n = 5)
Fescue/Bermuda grass	1.05 (0.60–1.43)	1.87 (0.43–4.80)	0.74 (0.37–0.87)[b]	0.84 (0.22–1.45)
Orchard grass/Bermuda grass	1.75 (0.45–3.77)	2.05 (0.81–5.00)	1.82 (1.04–2.50)[c]	1.54 (0.66–3.29)

[a] Ratio calculated as: (colonies from population adapted to fescue or orchard grass)/(colonies from population adapted to Bermuda grass), expressed as the average with range in parentheses.

[b] Significantly different at $P = 0.05$, using the one-sided t test to test mean value less than 1.00.

[c] Significantly different at $P = 0.05$, using the one-sided t test to test mean value greater than 1.00.

been shown to digest 65% of a small amount of ground cellulose by extracellular enzymes (52), and cell-free filtrates of cellulolytic species have been shown to degrade xylan, cellulose, and cellodextrins (24), The IB type adhered more closely to its substrate than EC or other morphological types (Fig. 2B and 3). This tight adherence of irregularly shaped bacterial cells to plant walls has been shown for *B. succinogenes* (42), some strains of which do not produce extracellular cellulases (33). Perhaps close adherence is necessary for cell-bound enzymes of IB to degrade plant cell walls. Synergism has been shown to be involved in the digestion of cell wall constituents (23, 26) and could contribute to overall fiber digestion, particularly in the later stages after initial digestion has occurred.

Several reports have shown that dietary variations influenced the population of cellulolytic rumen bacteria (18, 49, 53). In this (Table 1) and another study (1), TEM consistently revealed that the proportions of EC and IB attached to Bermuda grass and fescue varied. However, significant differences were not found in attachment by bacterial types to different cell wall types within either forage, although consistent trends for more EC on Bermuda grass sclerenchyma and less EC on fescue sclerenchyma were observed. Conversely, Latham et al. (42) reported that *R. flavefaciens* predominated on epidermis, phloem, and sclerenchyma cell walls and that *B. succinogenes* predominated on mesophyll of ryegrass in studies with an inoculum containing only these two species. Dinsdale et al. (27) concluded that cell walls of leaves seemed to be digested by cocci, whereas plant residues and cotton fibers elicited a rodlike population. In the present study and another study of several other grasses (1), no consistent relationship between the predominant adhering fiber digesters and the ease of forage digestion was found, although the proportion of IB tended to be higher at later digestion times for many forages. Additionally, EC tended to attach in greater proportions to Bermuda grass sclerenchyma, which is a lignified tissue digested usually only at the periphery by the predominant fiber digesters (Table 2) (7). It cannot be ruled out that conditions in the in vitro tubes changed with increased digestion times and affected the proportions of fiber digesters rather than the remaining tissue types per se.

Forage grasses vary in amount of fiber and, in general, warm-season forages are higher in cell wall content than are cool-season forages (14). Similar tissue types are more slowly degraded in warm- than in cool-season forages (6, 34). Samples from the same and other harvests similar to those used in the present study have shown this relative order of forage cell wall digestion: Bermuda grass < fescue < orchard grass (6). Although differences in the percentage ratio of EC to IB were consistently found between Bermuda grass and fescue, cell walls of orchard grass, the most rapidly digested forage used in these studies, showed average ratios similar to the more slowly degraded Bermuda grass samples (Table 3). Thus, proportions of attaching rumen fiber digesters do not always correspond to differences in cell wall digestibility.

TEM, however, showed that the manner of digestion by rumen microbial populations of the cell walls differed in Bermuda grass and orchard grass. It appeared that the constituents in orchard grass cell walls were more easily available to the bacteria so that attachment was not a prerequisite for digestion of several of the cell walls, although bacteria were always near digested areas. Previous research from this laboratory showed that bacteria often degraded mesophyll and phloem without adhering to the forage cell wall (1, 4, 7). Furthermore, rumen bacteria are capable of degrading constituents of forage cell walls by extracellular enzymes (24, 31, 52). However, in the inocula used in the studies conducted in this laboratory, rumen fluid supernatant per se did not have marked cell wall-degrading activity without viable bacteria present as tested by TEM of tissues digested by centrifuged rumen fluid supernatant (unpublished data) and streptomycin-treated washed rumen bacteria (8). Perhaps inhibitors to the cell wall-degrading enzymes were present in the rumen fluid (29), which prevented the activity of cell-free enzymes except for those from bacteria extremely near to the forage cell walls. Although direct adherence of bacteria to certain cell walls (e.g., parenchyma bundle sheath of Bermuda grass) is required for degradation, other apparently less rigidly structured cell walls (e.g., mesophyll, parenchyma bundle sheath, and parts of the epidermis in orchard grass) appear to be available to the extracellular enzymes produced by nearby but unattached bacteria. However, the bacteria closest to the cell wall undergoing degradation may not necessarily be those involved in cell wall digestion, since bacteria could be displaced during TEM preparation. Additionally, many noncellulolytic rumen bacteria digest or utilize constituents such as pectin and xylan (23, 38) present in forage cell walls, and their contribution could be significant with particular forages or in conjunction with cellulolytic bacteria (23).

Constituents in forage cell walls are organized in a complex manner. Cell wall compositions

differ among tissues within a forage (32) and among forages (9), and data indicate that differences in digestibility of cell wall fractions exist (26, 47). Results have suggested that cell wall organization, and in particular the hemicellulose component, does influence forage degradation (11, 23). Furthermore, research has shown that the various carbohydrates in intact forage cell walls are not equally available to the degradative enzymes (22, 29).

In general, higher numbers of culturable bacteria developed on medium 10 in populations adapted to orchard grass than in those adapted to Bermuda grass (Table 4). Increased total viable counts from orchard grass-adapted populations were reflected in higher counts for those carbohydrates (i.e., pectin, xylan, and cellobiose) found in hemicelluloses (9). The significantly ($P \leq 0.05$) higher proportion of xylanolytics in orchard grass-adapted than in Bermuda grass-adapted populations could result from either a higher amount or a greater availability of xylan in the cell walls in orchard grass. Since compositional data indicated that the hemicellulose of grass is mostly xylan and that the percentage of hemicellulose in the cell walls is less in cool-season grasses, including orchard grass, than in Bermuda grass (9, 13, 22, 23; F. E. Barton II, personal communication), the significantly higher number of culturable bacteria on xylan from the orchard grass-adapted population apparently results from greater availability of xylan or similar components. Similarly, higher numbers of culturable bacteria developing on pectin and cellobiose of orchard grass also suggest that, in general, the cell wall constituents are less rigidly complexed in the cell wall and, therefore, more available to the bacteria.

Culturable bacteria from fescue-adapted populations developing on specific substrates did not confirm the greater number of fiber-digesting bacteria observed by TEM for fescue compared with the other grasses. This discrepancy might be explained by differences in the proportion of the major types of fiber digesters associated with fescue cell walls. The significantly ($P \leq 0.05$) low number of xylanolytics might result from the high proportion of IB associated with fescue. If IB is indeed *B. succinogenes*, then these bacteria would not grow on xylan medium since they degrade but do not utilize xylan (23). Similarly, greater cellulolytic activity (as measured by the filter paper test) for fescue-adapted populations compared with the other grasses might also be explained by higher ratios of *B. succinogenes*, which has a greater cellulolytic activity than *Ruminococcus* species (26, 33).

The study presented herein and other work (6) indicate that the greater in vitro digestion of fescue compared to Bermuda grass could result from a higher amount of easily digested tissues and a different proportion of fiber-digesting bacteria. This different population of attaching, fiber-digesting bacteria might result in invalid comparisons of grasses with anaerobic techniques, and, therefore, the ratio of comparative culturable bacteria between fescue and Bermuda grass might not be accurate. Conversely, the rapid digestion of orchard grass compared with Bermuda grass (6) did not appear to result from variations in types of adhering fiber digesters, but apparently more responsible was a markedly greater availability of hemicellulose-type cell wall components to the rumen bacteria.

The higher percentages of culturable bacteria on these carbohydrates found in hemicellulose for orchard grass-adapted populations (80%) than in Bermuda grass-adapted populations, especially the significant increase in xylanolytics from orchard grass populations, whereas lower amounts of these components appear in the cell walls of cool-season grasses, suggest that the hemicellulose is less rigidly complexed into the cell wall of orchard grass than Bermuda grass. Furthermore, degradation of cell walls of cool-season (including fescue and orchard grass) but not warm-season grasses using aliquots of the same inoculum by the rumen entodiniomorph *Epidinium ecaudatum* form *caudatum* (3, 8), a strongly xylanolytic and hemicellulolytic rumen protozoan (10), also indicated that components of these cool-season grass cell walls were more available to enzymatic degradation than those in Bermuda grass.

"Lignin-carbohydrate complexes" have been found in various lignified as well as unlignified (as determined histochemically with acid phloroglucinol) forage cell walls (30, 35, 36). These complexes show a significant negative correlation to cell wall degradation (37). In ryegrass, lignin-carbohydrate complexes include *p*-coumaric and ferulic acids linked to carbohydrate moieties of β-(1-4)-linked D-glucose residues and similarly linked chains of xylose residues (46). Lignin is thought to arise from enzymatic polymerization of the primary precursors of sinapyl, coniferyl, and *p*-coumaryl alcohols; hemicellulose has been implicated in cell wall lignification (51). Recently, lignification of cell walls as indicated by the chlorine-sulfite test has been found in the slowly or partially degraded parenchyma bundle sheath cell walls of Bermuda grass and portions of the parenchyma sheath in fescue but not in the rapidly degraded sheath walls of orchard grass (unpublished data). Therefore, it is possible that progressive

polymerization of lignin-carbohydrate complexes might occur and reduce the availability of cell wall carbohydrates for bacterial utilization in grasses like Bermuda grass. Indeed, delignification of forage cell walls increases the digestibility of cell wall components (13, 50).

Complex interrelationships exist between fiber-digesting rumen bacteria and forage cell walls during digestion. The data presented herein indicate that differences in the proportions of the major adherent fiber digesters do exist among forages and could influence cell wall degradation. However, similarities in the proportion of major attaching bacteria between other forages of different digestibilities indicate that such proportions are not always related to the ease of cell wall digestion, but rather that inherent variations in availability of cell wall constituents may be responsible for variations in digestibility. These inherent variations result in variations in the manner in which the rumen bacterial population associate with forage cell walls during digestion.

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