Ruminococcus flavefaciens Cell Coat and Adhesion to Cotton Cellulose and to Cell Walls in Leaves of Perennial Ryegrass (*Lolium perenne*)

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Ruminococcus flavefaciens was shown to possess a prominent glycoprotein coat, which contained rhamnose, glucose, and galactose as its principal carbohydrates. Periodate-reactive carbohydrate occurred as a surface layer of the coat. The ruminococci adhered strongly by means of this coat to cotton cellulose and to cell walls in leaf sections of *Lolium perenne* L. (perennial ryegrass). The coat was diffuse at the point of contact so that the bacterial cell wall was in close contact with the substrate. Adhesion was influenced by the availability of damaged plant cell walls and by the cell wall type and occurred most rapidly to cell walls of the epidermis and sclerenchyma, followed by the phloem and mesophyll. Plaques of bacteria with filamentous coat extensions developed on all these tissues. The bacteria did not readily adhere to the walls of the bundle sheath cells or metaxylem or protoxylem vessels and did not adhere to the cuticle or chloroplasts. The epidermal and phloem cell walls were more rapidly digested than the walls of other cell types.

Carbohydrate components of the plant cell wall, chiefly cellulose, hemicellulose, and pectin, provide a major source of energy for ruminants. The carbohydrates are degraded and fermented by the combined activities of many species of bacteria and protozoa in the rumen. However, the primary attack on cell wall cellulose depends largely upon the activity of only a few species of cellulolytic bacteria (18).

Since the early light microscope studies of Baker and Hariss (5), it has been known that cavities containing many bacteria develop in plant particles undergoing digestion by rumen organisms. Recently, the excellent studies of Akin and co-workers (1, 2, 3), using transmission electron microscopy (TEM) and scanning electron microscopy, have revealed the presence of many different morphological types of rumen bacteria attached to plant particles. Predominant among the surface flora were coccoid bacteria, which were surrounded by extracellular coat material (1). Unfortunately, in mixed culture systems the identity of the bacteria associated with the plant fragments is uncertain, and nothing is known of the propensity of individual species of rumen bacteria to attach to and digest the various types of plant cell wall.

Two species of cellulolytic cocci, *Ruminococcus flavefaciens* and *R. albus*, occur in the normal rumen microflora, and ruminococci are fre-

quently the most numerous of the cellulolytic bacteria isolated from the bovine rumen (18, 20). Both species actively degrade a wide range of forages (28). Patterson et al. (27), in describing the ultrastructure of *R. albus*, noted the presence of fibrous material surrounding bacterial cells attached to cotton fibers. We are currently investigating the mechanisms of cellulose digestion by *R. flavefaciens*, and we have observed that much extracellular material is associated with the organism when it is grown on cotton cellulose in the form of Whatman no. 1 filter paper. In this paper, we describe the mode of adhesion of this organism to cotton cellulose and to different types of plant cell wall in leaf laminae of *Lolium perenne* L. (perennial ryegrass) and describe some chemical and histochemical properties of an extracellular coat material that mediates in adhesion. A preliminary report of this work has appeared elsewhere (M. J. Latham, B. E. Brooker, G. L. Pettipher, and P. J. Harris, Proc. Soc. Gen. Microbiol. 4:42, 1976), and a description of the enzymes produced by *R. flavefaciens* that are involved in plant cell wall digestion is to be reported separately.

MATERIALS AND METHODS

Organisms and culture media. The type strain, C94, of *R. flavefaciens* was obtained from M. P. Bryant, Department of Dairy Science, University of

Illinois, Urbana. Other strains were isolated from 10^7 or 10^6 dilutions of rumen fluid; strains 66 and 67 were isolated from bovine rumen fluid by using a broth medium containing strips of Whatman no. 1 filter paper as substrate (20), and strain R123 was isolated from the bovine cecum by using roll tubes of the nonselective medium, M10 (8). These strains were strictly anaerobic, gram-positive, chain-forming cocci that actively digested cellulose in the form of Whatman no. 1 filter paper with the production of a bright-yellow pigment. Succinate, acetate, formate, CO_2 , and H_2 were produced from the fermentation of cellobiose. The strains were accordingly identified as *R. flavefaciens*.

The organisms were grown in a medium containing cellobiose or cellulose as energy source, using the Hungate technique for the culture of strict anaerobes as described by Bryant (7). The medium, basal RUM10, contained (per 100 ml): Trypticase (Baltimore Biological Laboratory, Cockeysville, Md.), 0.3 g; yeast extract (Difco Laboratories, Detroit, Mich.), 0.2 g; resazurin solution (0.1%), 0.1 ml; mineral solutions 1 and 2, 7.5 ml each; volatile fatty acid mixture, 0.31 ml; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ solution (0.01%), 1.0 ml; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.01%), 1.0 ml; cysteine-hydrochloride solution (2.5%), 2.0 ml; and Na_2CO_3 solution (8.0%), 5.0 ml. Mineral solution 1 contained K_2HPO_4 , 0.6%. Mineral solution 2 contained: KH_2PO_4 , 0.6%; $(\text{NH}_4)_2\text{SO}_4$, 1.2%; NaCl , 1.2%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25%; and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.16%. The volatile fatty acid mixture comprised a mixture of the following concentrated acids: acetic, 17 ml; propionic, 6 ml; *n*-butyric, 4 ml; isobutyric, DL- α -methyl-*n*-butyric, *n*-valeric, and isovaleric, 1 ml each; and phenylacetic, 1 g. In complete media, cellobiose (0.3%) or cellulose (0.2%) was included as an energy source. The cellulose was in the form of Whatman no. 1 filter paper that had been hammer-milled through a 0.5-mm screen. Media were prepared with 100% oxygen-free CO_2 as the gas phase, and cultures were incubated at 39°C.

TEM. For conventional morphological study, cells from exponential-phase cultures in cellobiose RUM10 medium were harvested by centrifugation at $5,000 \times g$ for 10 min and fixed at room temperature for 1 h in 3% glutaraldehyde in 0.1 M cacodylate-hydrochloride buffer (pH 7.2). The cells were concentrated on the surface of a membrane filter (0.22- μm pore size, Millipore Corp., Bedford, Mass.) by drawing liquid from beneath the filter with absorbent paper. A thin layer of molten 2% agar was applied to the surface of the filter and allowed to solidify to prevent the loss of bacteria during processing. The filter was cut into strips, and the bacteria were postfixed for 1 h at room temperature in 1% osmium tetroxide in 0.1 M cacodylate-hydrochloride buffer (pH 7.2). Specimens were washed in distilled water, immersed in 1% aqueous uranyl acetate for 0.5 h, dehydrated in acidified dimethoxypropane (26), and embedded in Araldite (EMSCOPE, London). Sections were cut on a Reichert Om U2 ultramicrotome, stained with lead citrate and uranyl acetate, and examined in a Hitachi HU-11E electron microscope.

Periodate-reactive carbohydrates were localized cytochemically by using conventionally fixed and embedded material by the periodic acid-thiosemicarbazide-

silver proteinate method described by Thiéry (31). Control sections were treated similarly, but aldehyde groups were blocked either before or after periodic acid oxidation by immersion in an aqueous 0.1% (wt/vol) solution of sodium borohydride for 0.5 h at room temperature, as recommended by Craig (12). Experimental and control sections were examined by electron microscopy, without additional staining, in lead and uranium salts.

Two methods were used to demonstrate carbohydrate on the surface of fixed cells before embedment.

In the colloidal iron hydroxide method, a stock solution of colloidal iron hydroxide was prepared by the method given by Mowry (25). After primary fixation in glutaraldehyde as described above, bacteria were suspended for 1 h at pH 1.8 in a mixture of colloidal iron (10 volumes), glacial acetic acid (12 volumes), and distilled water (18 volumes). Cells were rinsed twice in 30% (vol/vol) acetic acid and postfixed for 1 h in osmium tetroxide in 0.1 M cacodylate-hydrochloride buffer (pH 7.2). After washing in distilled water, bacteria were dehydrated and embedded in Araldite as described above.

In the concanavalin A (ConA) method, the ability of ConA to bind to carbohydrate in the surface layers of strain 67 was tested by using a method based on that described by Martin and Spicer (24). Control cells treated with ConA were exposed to 0.2 M α -methyl-D-mannoside before addition of iron-dextran.

Digestion and extraction of bacterial coat. Various reagents were tested for their effect on the surface coat of strain 67. Cells from 15-h cultures in cellobiose RUM10 broth were harvested by centrifugation at $5,000 \times g$ for 10 min, washed once in 0.85% NaCl solution, and suspended in the appropriate buffer (given below) to an optical density of 0.15 at 580 nm, using a Lumitron colorimeter, model 401 (Photovolt Corp., New York, N.Y.). To separate portions of these cell suspensions were added 2 volumes of the following reagents: (i) 0.2 M sodium metaperiodate in 0.1 M acetate buffer, pH 4.0; (ii) pepsin, 0.2 mg/ml in 0.045 M citrate/0.01 M phosphate buffer, pH 2.6; (iii) trypsin, 2.0 mg/ml in 0.1 M phosphate buffer, pH 8.0; (iv) protease, 0.2 mg/ml in 0.1 M phosphate buffer, pH 7.5; (v) α -glucosidase, 3.0 mg/ml in 0.1 M phosphate buffer, pH 6.8; (vi) β -glucosidase, 3.0 mg/ml in 0.1 M acetate buffer, pH 5.0. The suspensions, together with control cell suspensions in buffer only, were incubated at 37°C with intermittent shaking for 30 min. After incubation, the cells were harvested by centrifugation, suspended in phosphate-buffered saline, and fixed by the addition of an equal volume of 6% glutaraldehyde. Cells were postfixed, dehydrated, and embedded in Araldite as described above and examined by TEM.

Three methods were used to extract coat material from cells of strains 67 and C94. Cells from 15-h cultures in 10 liters of cellobiose RUM10 broth were harvested by continuous-flow centrifugation at 18,000 rpm, using an MSE 18 centrifuge. They were washed twice in chilled distilled water and resuspended in 300 ml of distilled water. Portions of this cell suspension were then each treated with equal volumes of one of the following reagents under the specified conditions: (a) 0.2 M acetate buffer, pH 4.0, at 80°C for 25 min; (b) 0.1 M HCl at 80°C for 25 min; (c) pepsin, 0.2

mg/ml in 0.045 M citrate-0.01 M phosphate buffer, pH 2.6, and (d) citrate-phosphate buffer alone, 37°C for 1 h; (e) 0.2 M borate buffer, pH 8.0, 37°C for 1 h. After centrifugation at $15,000 \times g$ for 30 min, the clear supernatant was decanted into 4 volumes of cold absolute ethanol and allowed to precipitate at 4°C overnight. The ethanol precipitates were dried in vacuo and chemically analyzed for the presence of total carbohydrate (13), reducing sugar (16), protein (22), phosphorus (9), and ash. The presence of lipid in extracts c and d and their ethanol precipitates was determined gravimetrically after making Folch extracts, and long-chain fatty acids in these extracts were determined as their methyl esters by gas-liquid chromatography (21).

Preparation of antisera and serological tests. Antisera to whole cells of each strain of *R. flavefaciens* were raised in rabbits by intravenous inoculations of live cell suspensions in sterile 0.85% NaCl solution. Clear supernatants of extracts a, b, d, and e above were neutralized and tested against the antisera by the tube precipitin method.

Adhesion studies. Plants of *L. perenne* cultivar S24 (perennial ryegrass) were grown singly in pots containing perlite in a controlled environment. Lighting was provided by warm white fluorescent tubes supplemented by incandescent lamps to give a light intensity of 130 W/m^2 (400 to 700 nm) at plant level with an infrared-to-total radiation ratio of approximately 25%. The day temperature was 23°C, and the night temperature was 18°C. The photoperiod and the thermoperiod were both 16 h. The plants were watered daily, and nutrients were supplied as a modified Hoagland solution (29) on alternate days.

The lamina of the seventh leaf on the main stem was excised when this was the youngest fully expanded leaf lamina. There was no evidence of floral initiation when the apical meristems of 10 plants at this growth stage were examined (14). Transverse sections (70 μm thick) were cut under water from midway along this lamina with a Vibratome (Oxford Laboratories Inc., San Mateo, Calif.). The sections were placed in water and kept overnight at 4°C before use. Lignified cell walls were detected in the sections by light microscopy, using the phloroglucinol-hydrochloride and chlorine-sulfite reactions (32), and by ultraviolet fluorescence (17).

Overnight cultures of *R. flavefaciens* 67 in cellobiose RUM10 were harvested by centrifugation at approximately $5,000 \times g$ for 20 min and suspended in basal RUM10 to an optical density of approximately 1.65 at 580 nm. Direct microscopic counts of bacteria in these suspensions were in the range of 1×10^8 to 3×10^9 /ml. The bacterial suspension was distributed aseptically under CO_2 in 10-ml portions to sterile tubes (0.5 by 6 inches [ca. 1.3 by 15.2 cm]) fitted with neoprene stoppers, and the leaf sections were transferred to the suspensions by means of a flame-sterilized wire loop. Sections were also placed in uninoculated cellobiose RUM10 and basal RUM10. Several sections were removed from each tube (0 h), transferred to 10 ml of sterile physiological saline, and swirled to wash. The tubes containing the bacterial suspension and remaining plant sections were incubated at 39°C. The saline supernatant from the washed sections was re-

moved by suction, and the sections were fixed for electron microscopy by suspension in 5 ml of 3% glutaraldehyde in 0.1 M cacodylate-hydrochloride buffer, pH 7.2. Further sections were removed at 0.5- or 1-h intervals during the incubation and processed similarly. Strict anaerobiosis was maintained during the preparation of the cell suspension and during the transfer or removal of leaf sections. At intervals throughout the incubation, sections were examined by phase-contrast microscopy.

Adhesion to cotton cellulose was also examined, using strain 67 grown for 24 h in cellulose RUM10 broth. The sediment, consisting of cellulose fibers and associated bacteria, was fixed for 1 h in 3% glutaraldehyde in 0.1 M cacodylate-hydrochloride buffer (pH 7.2).

The washed and fixed leaf sections of *L. perenne* and the final preparation of bacteria on cotton fibers were embedded in 2% agar and then postfixed, dehydrated, embedded in Araldite, and sectioned for TEM as described above. Thin sections of the *L. perenne* material were cut parallel to the longitudinal axis of the leaf.

Scanning electron microscopy. Control and experimentally treated leaf sections of *L. perenne* were fixed for 1 h at room temperature in 3% glutaraldehyde in 0.1 M cacodylate-hydrochloride buffer at pH 7.2 and dehydrated in acidified dimethoxypropane. They were washed several times in acetone and dried by the critical-point method of Anderson (4). Specimens were mounted on stubs, coated with gold in a Polaron sputter coater, and examined in a Cambridge Stereoscan Mk II.

RESULTS

Properties of the bacterial coat. Cells of *R. flavefaciens* frequently stained gram-variable as cultures in cellobiose broth medium entered stationary phase. However, the cell wall always appeared as a triple-layered structure by TEM, identical to that normally associated with gram-positive organisms (15). A prominent coat of surface material (65 to 80 nm thick) completely enveloped the cell wall but was separated from it by an electron-lucent layer 6 to 8 nm thick. This uniform cell coat was differentiated into two poorly defined layers. The outer of these was more electron dense than the inner one, and, in tangential section, the outer layer was reticular in appearance. Occasionally, cells were encountered that had apparently made chance contact and were joined by filaments extending from the outer layers of their respective coats. This phenomenon was more commonly observed when bacteria were attached to a cellulose substrate (see below). Moreover, the coats of bacteria that had been brought into close contact during their collection on membrane filters (Millipore) had apparently fused, since the cell walls of adjacent organisms were either in contact or very closely apposed. Thus, large aggregates of bacteria were sometimes seen in

which the coat material formed a continuous surface layer. A thin outer layer of the coat stained intensely when it was treated with colloidal iron hydroxide at low pH (Fig. 1). Sections stained with periodic acid-thiosemicarbazide-silver proteinate showed that cytoplasmic storage granules and the outer layer of the cell coat contained periodate-reactive carbohydrate. The outer layer of the cell coat contained some granules of reaction product, but these were thinly scattered. Control sections were totally free of reaction product.

Treatment of live cells with periodate resulted in disruption of the cell coat so that only fragments remained on the cell surface. This was in contrast to the intact coat of cells treated with the same buffer system at the same pH. Other treatments, using α - and β -glucosidases, trypsin, and protease, had no effect on the coat. Treatment with pepsin in citrate-phosphate buffer, pH 2.6, was also effective in removing the coat from cells, but, since buffer alone also had some effect, the role of pepsin remained equivocal.

Suspensions of bacteria treated with ConA did not agglutinate, and surface-bound lectin could not be demonstrated by using the iron-dextran labeling technique. However, neutralized acid extracts of coat material gave a strong precipitin reaction with ConA.

Extracts of coat material from all strains prepared by treatment of whole cells with HCl or acid or alkaline buffers gave a strong precipitin reaction when tested with an antiserum to whole cells of strains 67, 66, and R123, indicating the presence of antigenically similar components in each extract. The antiserum to strain C94 was weak and gave correspondingly poor precipitin reactions. The strain 67 antiserum also reacted with the homologous cell-free culture supernatant and a dialyzed ammonium sulfate precipitate of strain 67 culture supernatant containing cellulase and hemicellulase enzymes (G. L. Pettipher and M. J. Latham, unpublished data). The coat material extracted from strain 67 by treatment with acetate buffer contained approximately equal amounts of total carbohydrate and protein (30% each) and 11% ash. The composition of the coat carbohydrate has not been fully elucidated, but preliminary results indicate that it contains rhamnose, glucose, and galactose (9.6, 5.3, and 1.5%, respectively) together with smaller amounts of ribose and an amino sugar. Phosphorus, presumably as phosphate, was a major component of the ash. Lipid, which may account for some of the unidentified material in these extracts, was found to be present and to contain C_{15:0} iso, C_{15:1} br, C_{12:0}, and C_{14:0} iso as the predominant fatty acids. Extracts prepared by HCl or citrate-phosphate buffer with or with-

out pepsin contained up to 50% carbohydrate.

Adhesion to cotton cellulose. All strains of *R. flavefaciens* readily adhered to and digested cotton cellulose in the form of Whatman no. 1 filter paper. Heavy colonization of fractured ends of the fibers could be seen by light microscopy, but elsewhere the bacteria were concentrated between ridges running parallel to the longitudinal axis of the fiber. TEM showed that each bacterial cell was surrounded by an extracellular coat, which fused to form a continuous layer where cells were closely apposed (Fig. 2). An irregular plaque, consisting of bacteria embedded in coat material, developed in areas of heavy colonization. The coat was ill defined and diffuse at the interface between cells and substrate, whereas it was more clearly defined on cells furthest from the substrate. Adjacent cells in the plaque were frequently connected by irregular filaments of coat material. Digestion of the cellulose by the bacteria created large pits in the fiber, and large numbers of intracellular granules, presumably glycogen, were observed in cells close to the substrate.

Adhesion to plant cell walls in *L. perenne* leaves. Incubation of cell suspensions of *R. flavefaciens* with leaf lamina sections of *L. perenne* resulted in bacteria binding to most types of plant cell wall. The incubations were carried out under anaerobic conditions, but it was noted in a preliminary experiment that adhesion of the bacteria to the plant cell walls also occurred when the suspending basal medium was allowed to oxidize. No attempt was made to determine if the degree of anaerobiosis influenced adhesion.

The major sites for adhesion were the cut or damaged surfaces of the plant cell walls. Large numbers of ruminococci adhered to these sites, whereas very few were observed on undamaged surfaces until the leaf sections were in an advanced state of digestion. Scanning electron micrographs of epidermal and mesophyll cells (Fig. 3) after exposure of the leaf sections to the ruminococci for 1.5 h revealed the presence of many bacteria aligned in chains along the epidermal cell wall contiguous with the cuticle. Fewer bacteria were attached to the other epidermal cell walls, and fewer still were attached to the cell walls of the mesophyll. Occasionally, bacteria were observed lying on the intact faces of the plant cell walls, but these were nearly always terminal organisms in chains that were, for most part, aligned along the cut edges of plant cell walls. Towards the end of the incubation, before disintegration of the leaf section (9 h), bacteria were observed digesting cavities in the surface of the mesophyll (Fig. 4) and epidermal cells.

The numbers of bacteria that attached to

FIG. 1. Thin sections of *R. flavefaciens* 67 stained with colloidal iron. Electron-dense outer layer of surface coat (arrow) indicates presence of acidic polysaccharide. TEM; bar = 0.2 μm

FIG. 2. Thin section of *R. flavefaciens* 67 attached to cotton cellulose (hammer-milled Whatman no. 1 filter paper). Bacterial coat is ill defined both between cells and between cells and substrate (arrows). TEM; bar = 1 μm

FIG. 3. Perennial ryegrass leaf section after 1.5 h of incubation with *R. flavefaciens* 67. Many ruminococci are attached to the cut edges of epidermal cell walls (E), but relatively few are attached to mesophyll cell walls (M). Scanning electron micrograph; bar = 5 μ m

FIG. 4. Mesophyll cell walls in sections of perennial ryegrass after 9 h of incubation with *R. flavefaciens* 67. Ruminococci are attached to and digesting the surface of mesophyll cell walls. Scanning electron micrograph; Bar = 2 μ m

these damaged cell wall sites varied greatly with plant cell type. Examination of scanning and transmission electron micrographs and light microscope observations of the leaf sections taken at different times during incubation of the sections with the ruminococci enabled the bacterial population associated with various types of plant cell wall to be assessed but not accurately quantified (Table 1). No adhering bacteria were observed on sections that had been exposed to the bacterial suspension for only a few minutes (0 h), but adhesion to both the abaxial and adaxial epidermis was observed after 0.5 h and to the mesophyll, phloem, and sclerenchyma cell walls 1 h later. After 3 h, the epidermal cell walls were heavily encrusted with bacteria, and bacteria covered the walls of the subepidermal sclerenchymatous fiber cells. Relatively few bacteria were associated with the mesophyll, phloem, outer and inner bundle sheath cells, and metaxylem. The small size of the inner bundle sheath cells made observation of adhering bacteria difficult. Colonization of the epidermal, mesophyll, phloem, and sclerenchyma cell walls was extensive after 6 h of incubation, and by 9 h the structural integrity of the sections was nearly destroyed. Although bacteria were associated with the outer bundle sheath cells and metaxylem vessels, their numbers did not increase after 3 h of incubation. Adhesion did not occur to the cuticle or to the walls of the protoxylem vessels. The chloroplasts hypertrophied and lost

their contents during incubation, and adhesion to the residual membrane was not observed. As the numbers of bacteria adhering to a particular site increased, a complex reticulum of extracellular filaments was formed between adjacent chains of bacteria (Fig. 5). These filaments were most prominent after 6 h of incubation and appeared to be extensions of the bacterial coat.

The configuration of the bacterial coat at the site of adhesion, as shown by TEM, was similar to that observed with cotton cellulose irrespective of the plant cell type. Large pits, where digestion was taking place, were particularly evident in the outer wall of the epidermis (Fig. 6). The cuticle remained intact, and there were large numbers of granules within the bacterial cells. Adhesion of the ruminococci to the walls of the phloem was particularly striking (Fig. 7), since the diameter of the bacteria was much greater than the thickness of the phloem cell wall, and adhesion occurred by means of a very narrow region of diffuse coat material. No bacteria were detected inside the phloem cells adhering to the undamaged walls.

The extent and speed with which plant cell walls were digested differed between the plant cell types. The cell walls of the epidermis and phloem were the most digestible (Table 1), being extensively degraded after 6 h of incubation. The loss of structural integrity of the leaf section was almost entirely due to the digestion of the epidermis. Mesophyll cell walls were more re-

TABLE 1. Adhesion to and digestion of walls of different cell types in leaf laminae of *L. perenne* by *R. flavefaciens* 67^a

Cell wall or structure	Time of incubation									
	0 h		1.5 h		3 h		6 h		9 h	
	Adhe- sion ^b	Diges- tion ^c	Adhe- sion	Diges- tion	Adhe- sion	Diges- tion	Adhe- sion	Diges- tion	Adhe- sion	Diges- tion
Damaged edges:										
Epidermis (outer wall)	—	nd	+++	d	+++	d	+++	D	+++	D
Epidermis (other walls)	—	nd	++	d	++	d	+++	D	+++	D
Mesophyll	—	nd	+	nd	+	nd	++	d	++	D
Phloem	—	nd	+	nd	+	d	++	D	++	D
Sclerenchyma	—	nd	++	nd	++	nd	+++	nd	+++	d
Outer bundle sheath	—	nd	—	nd	+	nd	+	d	+	d
Inner bundle sheath	—	nd	—	nd	+	nd	+	nd	+	nd
Metaxylem vessels	—	nd	—	nd	+	nd	+	nd	+	nd
Protoxylem vessels	—	nd	—	nd	—	nd	—	nd	—	nd
Undamaged surfaces:										
Epidermis	—	nd	—	nd	—	nd	—	nd	+	nd
Mesophyll	—	nd	—	nd	—	nd	+	nd	+	d
Cuticle	—	nd	—	nd	—	nd	—	nd	—	nd
Chloroplasts	-- ^d	--	--	--	--	--	--	--	--	nd

^a Composite observations from light microscopy, TEM and scanning electron microscopy.

^b +++, Complex plaque of bacteria with matrix of extracellular filaments; ++, single cells, diplococci, or chains of bacteria; +, occasional bacteria; —, no bacteria.

^c D, extensive digestion, pitting or deformation of plant cell wall; d, slight digestion; nd, no digestion.

^d --, Not examined.

FIG. 6. Epidermal cell wall after 6 h of incubation with *R. flavefaciens* 67. Ruminococci have digested large cavities in epidermal cell wall (E), but cuticle (C) remains undigested. TEM; bar = 2 μ m

FIG. 7. Phloem cell wall after 6 h of incubation with *R. flavefaciens* 67. Ruminococci have attached to cut edge of phloem cell wall (P) by means of a narrow portion of coat material. TEM; bar = 0.5 μ m Inset: Ruminococci similarly attached to other phloem cell walls. TEM; bar = 2 μ m.

sistant, requiring 9 h of incubation before they were digested to any significant extent, and only slight digestion, revealed by shallow pitting in the immediate vicinity of bacterial cells, was observed with the outer bundle sheath cells and

sclerenchyma. In contrast, cell walls of the metaxylem, protoxylem, and inner bundle sheath cells, the cuticle, and the chloroplast membrane did not appear to have been attacked after 9 h of incubation. The middle lamella of the phloem

tissue also appeared to be resistant to digestion, as observed by TEM, whereas the middle lamella at the epidermis-mesophyll junction was easily digested.

Lignin, as indicated by phloroglucinol-hydrochloride and chlorine-sulfite color reactions and by ultraviolet fluorescence, was absent from the epidermis (except over the abaxial sclerenchyma), outer bundle sheath, mesophyll, and phloem cells but present in the cell walls of the inner bundle sheath, metaxylem, protoxylem, and sclerenchyma.

DISCUSSION

Adhesion of bacteria to surfaces is a major ecological determinant in many environments, and the involvement of an extracellular carbohydrate coat in the adhesion process, in the form of either glycoprotein or glycolipid, is now well recognized. Chemical analysis and serological cross-reactivity of coat material extracted from *R. flavefaciens* indicated that all the various strains tested synthesized an antigenically similar glycoprotein coat. An outer layer of acidic carbohydrate was detected cytochemically by treatment with colloidal iron at low pH. Removal of the coat from whole cells by treatment with sodium metaperiodate, which produces oxidative cleavage of C-C bonds in α -glycols, confirmed the presence of carbohydrate, which was of structural importance to the integrity of the coat, but the inability to agglutinate whole cells with ConA indicated that α -pyranosyl-D-glucose residues were absent from the surface layers. Although tests for the presence of uronic acids were not made, the presence of rhamnose and galactose suggests some similarity to streptococcal capsular polysaccharide (30). The small amount of lipid in the extracts contained long-chain fatty acids characteristic of the ruminococci (19) and suggested that the extraction procedure may have removed some deep-seated material from the cell membrane. Preliminary results indicate that the high content of phosphorus was not related to the presence of a glycerol-teichoic acid backbone.

Extracellular material surrounds many of the bacteria in rumen fluid and some gram-negative rumen bacteria grown in pure culture (10, 11). The present study on *R. flavefaciens* together with the recent work of Patterson et al. (27) on *R. albus* has now shown that extensive surface coats are synthesized by both species of the gram-positive cellulolytic cocci when they are grown on soluble carbohydrate or insoluble cellulose. Costerton et al. (11) suggested that extracellular coats provided some protection to the cells in the rumen environment by acting as ion-exchange and molecular sieving agents, but for

bacteria that obtain their energy from the degradation of insoluble substrates, such as cellulose, extracellular coats serve the additional function of firmly binding the organisms to their substrate. By this means, localized high concentrations of degradative enzymes produced by the organism are brought into close contact with the substrate. The importance to cellulolytic bacteria of maintaining a relatively constant spatial relationship with their insoluble substrate is illustrated by the observation of Berg et al. (6) that *Sporocystophaga myxococcoides* is able to adhere to and digest any part of cotton fibers, whereas the highly motile cellulolytic bacterium *Cellvibrio fulvus* can only digest cotton fibers from within the lumen, where it becomes physically trapped.

Adhesion by *R. flavefaciens* to cotton fibers or to plant cell walls of *L. perenne* leaf laminae was dependent to a large extent on the availability of cut or otherwise damaged plant cell walls. As most of the cellulase and hemicellulase produced by growing cultures of *R. flavefaciens* are associated with the surface of intact bacterial cells (Pettipher and Latham, unpublished data), exposure of substrates in the plant cell wall to these enzymes may be a major factor in the adhesion process. Another factor may be the surface charge of the plant cell wall, since, for some marine bacteria, adhesion has been shown to be a two-stage process, comprising a so-called reversible electrostatic phase and an "irreversible" time-dependent phase in which adhesion is mediated by extracellular polymeric material (23). There is no reason to suppose that this two-stage process is peculiar to marine organisms. Thus, a charge that favors adhesion may only be generated on the plant cell wall as a result of damage. However, even with damaged cell walls, the presence of lignin appeared to partially or completely inhibit adhesion and digestion. A notable exception was the cell walls of the sclerenchyma, which became densely colonized even though the extent to which they were degraded was relatively small.

Mixed rumen bacteria seem capable of digesting some plant cell walls, such as those of mesophyll and phloem cells, without prior attachment of the bacteria (2). Digestion with the pure culture of *R. flavefaciens* occurred only in the immediate vicinity of the bacterial cell, but incubations of leaf sections with crude preparations containing *R. flavefaciens* cellulase and hemicellulase, though failing to produce visible degradation of any plant cell wall type, resulted in release of reducing sugars (Pettipher and Latham, unpublished data). The dense plaques of *R. flavefaciens* with their complex fibrillar reticulae that developed in pure culture, particularly

on the sclerenchyma, were not evident in the mixed culture systems described by Akin and Amos (2). However, these authors observed that individual cocci were attached to the cell walls of the epidermis by a few short extracellular fibers. It appears from the present work that *R. flavefaciens* diverts a considerable amount of substrate carbon into the synthesis of extracellular coat material. The reason why this material does not accumulate in mixed culture systems may be related to the constant scavenging by other bacteria of soluble polysaccharides released during the degradation of plant cell walls or of metabolic products formed by the rumenococci.

The results reported here indicate that, with leaf laminae of perennial ryegrass, the major degradative activity of *R. flavefaciens* is directed towards the epidermal cell wall. Since the ability of *R. flavefaciens* to adhere to and attack plant cell walls will be seriously hindered by the lack of damaged surfaces, the comminution of plant tissue by physical or chemical means must be a prerequisite for efficient digestion of plant cell walls by this organism.

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