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Attack on Lignified Grass Cell Walls by a Facultatively Anaerobic Bacterium

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A filamentous, facultatively anaerobic microorganism that attacked lignified tissue in forage grasses was isolated from rumen fluid with a Bermuda grass-containing anaerobic medium in roll tubes. The microbe, designated 7-1, demonstrated various colony and cellular morphologies under different growth conditions. Scanning electron microscopy revealed that 7-1 attacked lignified cell walls in aerobic and anaerobic culture. 7-1 predominately degraded tissues reacting positively for lignin with the chlorine-sulfite stain (i.e., sclerenchyma in leaf blades and parenchyma in stems) rather than the more resistant acid phloroglucinol-positive tissues (i.e., lignified vascular tissue and sclerenchyma ring in stems), although the latter tissues were occasionally attacked. Turbidimetric tests showed that 7-1 in anaerobic culture grew optimally at 39°C at a pH of 7.4 to 8.0. Tests for growth on plant cell wall carbohydrates showed that 7-1 grew on xylan and pectin slowly in aerobic cultures but not with pectin and only slightly with xylan in anaerobic culture. 7-1 was noncellulolytic as shown by filter paper tests. The microbe used the phenolic acids sinapic, ferulic, and *p*-coumaric acids as substrates for growth; the more highly methoxylated acids were used more effectively.

Microorganisms with the ability to attack lignocellulose have been reported. For example, *Thermomonospora fusca* can utilize pulping substrates with up to 18% lignin (10). However, the slow utilization of lignocellulose by thermophilic actinomycetes, mesophilic bacteria, and white-rot fungi has been reported to be a hindrance to the utilization of cellulosic waste, which requires chemical or physical pretreatment to expose the structural polysaccharides (4). Indeed, the biodegradation of lignin and lignocellulose has been reported to be a major problem in the commercial use of cellulosic wastes (5, 8). In general, anaerobic bacteria are not considered to have the ability to utilize lignin (4).

Lignin reduces the availability of structural carbohydrates in forage cell walls to rumen microorganisms (19). Chemical bonding between phenolic acids (*p*-coumaric and ferulic acids present presumably as lignin precursors) and β-glucans and β-xylans in ryegrass has been reported (18). Histochemical and biochemical studies (21) showed that lignified tissues varied in the type of lignin present in the cell walls, and these cell walls varied in their reaction to delignification with $KMnO_4$ (3). In electron microscopy studies (1), a filamentous microbe that attacked sclerenchyma (a lignified tissue) in leaf blades of the digesta removed from a cannulated steer was found, implying that attack on lignocellulose occurred under anaerobic conditions.

The objective of the present work is to report the isolation from rumen fluid of a filamentous, facultatively anaerobic bacterium capable of attacking lignified tissues in forage grasses.

MATERIALS AND METHODS

Isolation of 7-1. Rumen digesta from a permanently cannulated steer maintained predominately on Bermuda grass hay was strained through four layers of cheesecloth into a vacuum bottle and then transported to the laboratory. Tenfold dilutions of the strained rumen fluid were made with the anaerobic dilution solution of Bryant and Burkey (6). For isolation, 0.3 ml of the 10^3 to 10^5 dilutions was placed into test tubes (15 by 150 mm) with 5 ml of anaerobic medium. The isolation medium consisted of the basal medium of the rumen fluid-glucose-cellobiose agar of Bryant and Burkey (6) with reducing agents as modified by Bryant and Robinson (7). The medium was modified to include 1 to 3% (wt/vol) 8-week- old, freeze-dried and ground coastal Bermuda grass (CBG) as the sole substrate. This grass was chosen because of its high fiber content (68% neutral detergent fiber, 37% acid detergent fiber), high lignin content (6%), and low in vitro dry matter digestibility (52%) (F. E. Barton, personal communication). Therefore, this substrate would be conducive for selecting microbes that degrade lignified tissue.

Roll tubes were prepared by the Hungate method (15), and the inoculated, rolled tubes were incubated

at 35°C. Suspected filamentous, lignified tissue-degrading microbes were subcultured by excising entire colonies, suspending the cells in anaerobic dilution solution, and then inoculating roll tubes (containing 1% CBG and 0.2% glucose as substrates) with 1 ml of the suspension. After growth for 5 weeks, colonies were again selected for subculture on the basis of a filamentous nature and overgrowth of lignified fiber fragments in the substrate. Colonies were subcultured two more times, and Gram-stained smears were examined for purity. The isolate was designated 7-1 and further studied for degradation of lignified tissues. A culture has been deposited in the ARS Culture Collection, Northern Regional Research Center and assigned the accession number NRRL B-4370.

Microscopic studies of colony and cellular morphologies. For scanning electron microscopy (SEM) of colony morphology, 7-1 was grown in the anaerobic roll tube medium used for subculturing and also on aerobic medium. The aerobic medium was made as slants with 15% each of solutions 1 and 2 described by Bryant and Burkey (6), 65% distilled water, 1% 8-week-old CBG, 0.2% glucose, 0.5% yeast extract, and 2% agar. Tubes of each of the media having isolated colonies grown for 2 to 3 weeks were filled with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and fixed overnight at 5°C. Fixed colonies in agar blocks were excised and mounted on SEM stubs with conductive silver paint. Specimens were then postfixed over osmium tetroxide vapor, sputter-coated with gold-palladium alloy (60:40), and examined by SEM at 15 kV. Plant and microbial samples were prepared for transmission electron microscopy as described previously (2). For light microscope study of cellular morphology, Gram-strained smears of 7-1 growing in broth or on agar were examined.

Tests for optimal pH and temperature. The semisynthetic anaerobic basal medium of Caldwell and Bryant (9) was used with 0.2% cellobiose as sole carbon and energy source. (Earlier turbidity tests had shown that cellobiose supported good growth.) Working samples of the anaerobic medium were adjusted to pH 6, 7, 8, or 9, and 7 ml of broth was dispensed under CO_2 into tubes (6 by 125 mm) for each pH. These tubes were fitted with Hungate septa and caps (no. 2047; Bellco Biological Glassware and Equipment, Inc.). Media were autoclaved under fast exhaust. The tubes were matched for identical absorbance on a Bausch and Lomb Spectronic-20 spectrophotometer before use in growth studies. The tubes were then inoculated, using 25-gauge needles, with 0.1 ml of a 72-h CBG broth culture of 7-1. Duplicate tubes at each pH were then incubated at 25, 30, 35, 39, or 45°C for 72 h. Growth was evaluated by absorbance at 520 nm. Inoculated, matched tubes were read against blanks of uninoculated media. Confirmation tests were run for optimal pH using 0.2 ml of a 48-h broth culture and methods similar to that described above.

Tests for utilization of cell wall-type carbohydrates and phenolic acids. Anaerobic turbidimetric growth studies were carried out using the Caldwell and Bryant (9) basal broth medium adjusted before autoclaving to pH 6.7 (rumen pH) or 7.6 (optimal growth pH) in matched tubes fitted with Hungate septa. Aerobic growth studies were carried out on the basal broth for tryptone-yeast extract-glucose (TYG) medium (12), but without glucose, and adjusted to pH 7.6 in matched tubes with screw caps loosened one-quarter turn. Carbohydrates, at a level of 0.2%, included purified xylan (Koch-Light), pectin (purified by dialysis to remove contaminating sugars and freeze dried), cellulose (Solka Floc or ground Whatman no. 1 filter paper), cellobiose, and glucose. Basal medium without carbohydrates was also tested to ensure that growth was due to the carbohydrate tested.

Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid), and *p*-coumaric acid (*p*-hydroxycinnamic acid) at a level of 0.2% were tested for the ability of 7-1 to use these phenolic acids as the sole carbon and energy sources both aerobically and anaerobically. Since this amount of acid (0.2%) did not go into solution for all the acids, all media were filtered through a 0.45-μm filter before 7 ml was dispensed into each of the matched tubes. Basal medium with 0.2% glucose and basal medium without any added substrate were included as growth controls.

Duplicate matched tubes were then inoculated with 0.2 ml of an active culture of 7-1 in CBG broth medium, incubated at 39°C, and read for absorbance daily as described. Absorbance at 0 h was subtracted from the final absorbance to correct for initial differences in the inocula.

Isolate 7-1 was also tested for its ability to degrade filter paper both aerobically and anaerobically. Five milliliters of basal anaerobic or aerobic medium-containing strips (12.5 by 144 mm) of Whatman no. 541 filter paper was inoculated with 0.2 ml of an active culture of 7-1 and incubated at 39°C for 28 days.

Spectrophotometric (ultraviolet) analysis for loss of sinapic acid from broth cultures. Standards, uninoculated aerobic medium with and without sinapic acid, and inoculated aerobic medium with sinapic acid were analyzed with a Varian spectrophotometer (model 635). The standards were prepared in the same manner as the media, but with distilled water only. Samples were diluted 1:200 or 1:300 with distilled water, placed in 10-mm cuvettes, and scanned from 350 to 180 nm. Three trials were run, and spectra were compared for differences in peak heights at the absorbance maxima for sinapic acid.

Microscopic evaluation of fiber digestion by microorganisms. All microscopic investigations of the degradation of lignified tissue were carried out using blades and stem sections prepared from a single harvest of 5-week-old CBG. The central portion of the fourth internode and associated leaf blades were cut into 2- to 3-mm sections. Representative blade and stem samples were sectioned freehand for light microscopy, and the sections were stained for lignin with acid phloroglucinol and chlorine-sulfite (16).

Evaluation for microbial attack on lignified tissues was carried out by incubating the microbes (0.2 to 0.5 ml of an active culture) with 10 sections each of the blades and stems in 7 ml of basal anaerobic medium contained in tubes (16 by 125 mm) as described above. All tests were conducted with control blades and stems incubated in uninoculated medium. After incubation, blades and stems were fixed without washing for SEM as described (1).

Several tests were conducted to evaluate the attack

of 7-1 on the various types of lignified tissues in blades and stems in basal anaerobic medium or in basal medium supplemented with cellobiose. One test evaluated sections incubated for 28 days. Another test was included to compare the degradation of tissues by 7-1 after 7 days of incubation in aerobic and anaerobic media.

In another test, 20 sections each of blades and stems were incubated in uninoculated basal anaerobic medium or in medium inoculated with 0.5 ml of a broth culture of 7-1 to pretreat tissues for 72 h. The pretreated and control sections were then autoclaved to inhibit further action of 7-1, and the sections were washed with distilled water at 5°C for 3 days. Three of each of the pretreated and control blades and stems were prepared for SEM. Rumen fluid inoculum was prepared by straining digesta from a cannulated steer through 12 layers of cheesecloth, mixing 1 part of the strained fluid to 2 parts of McDougall carbonate buffer as described previously (1). Pretreated and control blades and stems were then placed into each of two 50-ml glass centrifuge tubes and inoculated with 30 ml of the rumen fluid suspension. The tubes were gassed with CO_2, capped with a one-way valve, and incubated at 39°C. Representative blades and stems from each of the tubes were removed after 6, 24, and 48 h of digestion and prepared for SEM, and freehand sections of stems were prepared for light microscopy.

Another test was conducted to compare the relative degradation of lignified tissues by the cellulolytic fungus *Trichoderma viride* QM6a (17); the lignocellulose-degrading, thermophilic actinomycete *T. fusca* 190Th (ATCC 27730) (10); strained rumen fluid; and 7-1. For this test, blade and stem sections were placed into matched tubes (16 by 125 mm) containing the optimal medium for cellulose or lignocellulose degradation or, for rumen fluid, the environmental conditions approximating those of the rumen.

For *T. viride*, the medium described by Mandels and Weber (17), but without cellulose, was used, and tubes were inoculated with a mat of mycelium and spores from a viable culture maintained on potato-dextrose-agar. Inoculated tubes were incubated at 28°C on a rotary shaker at 75 rpm. For *T. fusca*, the medium of Crawford et al. (12) was used, but without pulping fines, and tubes were inoculated with hyphae and spores from a 5-day-old culture maintained on TYG slants. Inoculated tubes were then incubated at 53°C on a reciprocal shaker at 75 rpm. For rumen microbial degradation of tissues, 0.2 ml of rumen digesta strained twice through cheesecloth was inoculated into Caldwell and Bryant's basal broth medium (9) in anaerobic tubes, and the tubes were incubated at 39°C. For 7-1, 0.2 ml of a 96-h broth culture was inoculated into the anaerobic broth medium (pH 7.5) with and without cellobiose, and tubes were incubated at 39°C. All tubes were read against medium blanks at 520 nm for absorbance after 24 and 48 h. The tubes were incubated for 8 days, and then blades and stems were prepared for SEM.

RESULTS

Colony morphology. Anaerobic colonies from roll tubes were flat, lacked aerial hyphae, and showed an irregular periphery; they often overgrew lignified fragments of CBG in the medium (Fig. 1a). The colony periphery at higher magnification showed extremely long (several micrometers), unbranched filaments often fragmenting into shorter forms; often coccoid forms and rods of a few micrometers were seen (Fig. 1b and inset). Conversely, colonies from aerobic culture demonstrated a different morphology. These colonies were raised and had entire edges (Fig. 1c). Cells of diverse lengths were present throughout the colony (Fig. 1d); however, in general, the aerobically cultured cells were markedly shorter than the filaments present in the anaerobic cultures.

Cellular morphology. Because of the extreme variations in filament lengths under various growth conditions, further studies were undertaken to assess colony purity and to evaluate the filamentation. Gram-stained smears of colonies subcultured for purity several times consistently showed this difference in filament length when grown in the anaerobic and aerobic media prepared in this manner. Tests for growth with CBG anaerobic broth at various pH levels showed that cells grown at pH near 7.5 had filaments that were markedly shorter than those of cells grown near pH 6.7. (Although the pH levels were adjusted to 7.5 or 6.7, the final levels after autoclaving were 7.3 and 6.4, respectively.) Further tests confirmed these observations (Fig. 2a and b). Further, cross-inoculation from CBG medium at pH 6.7 and 7.5 to the medium at pH 7.5 and 6.7, respectively, showed a reversal of the original morphology consistent with structures shown in Fig. 2a and b. After 48 h of incubation, 10 of the longest filaments grown at pH 7.5 averaged 12 ± 5 μm, whereas at pH 6.7 filaments averaged 67 ± 45 μm in length. These filaments were not necessarily the longest because of difficulty in finding the ends of the cells. Cells of 7-1 grown in aerobic broth (TYG, pH 7.5) or on TYG slants for up to 14 days revealed filaments of 5 to 10 μm (Fig. 3), but extremely long filaments were infrequently found. It was not ruled out that changes in redox potential per se affected filament length. However, the differences in the colony and cellular morphologies shown in Fig. 1 could also have been due to pH differences in the media. Gram-stained smears of both anaerobically and aerobically grown cultures revealed an array of cellular forms including gram-positive and -negative areas in filaments of various lengths (Fig. 2 and 3). Frequently, long, gram-negative filaments were present in the anaerobic broth. The relatively slow growth (e.g., 24 h for visible growth on TYG), the consistent change in filament length with pH, and the finding of similar structures

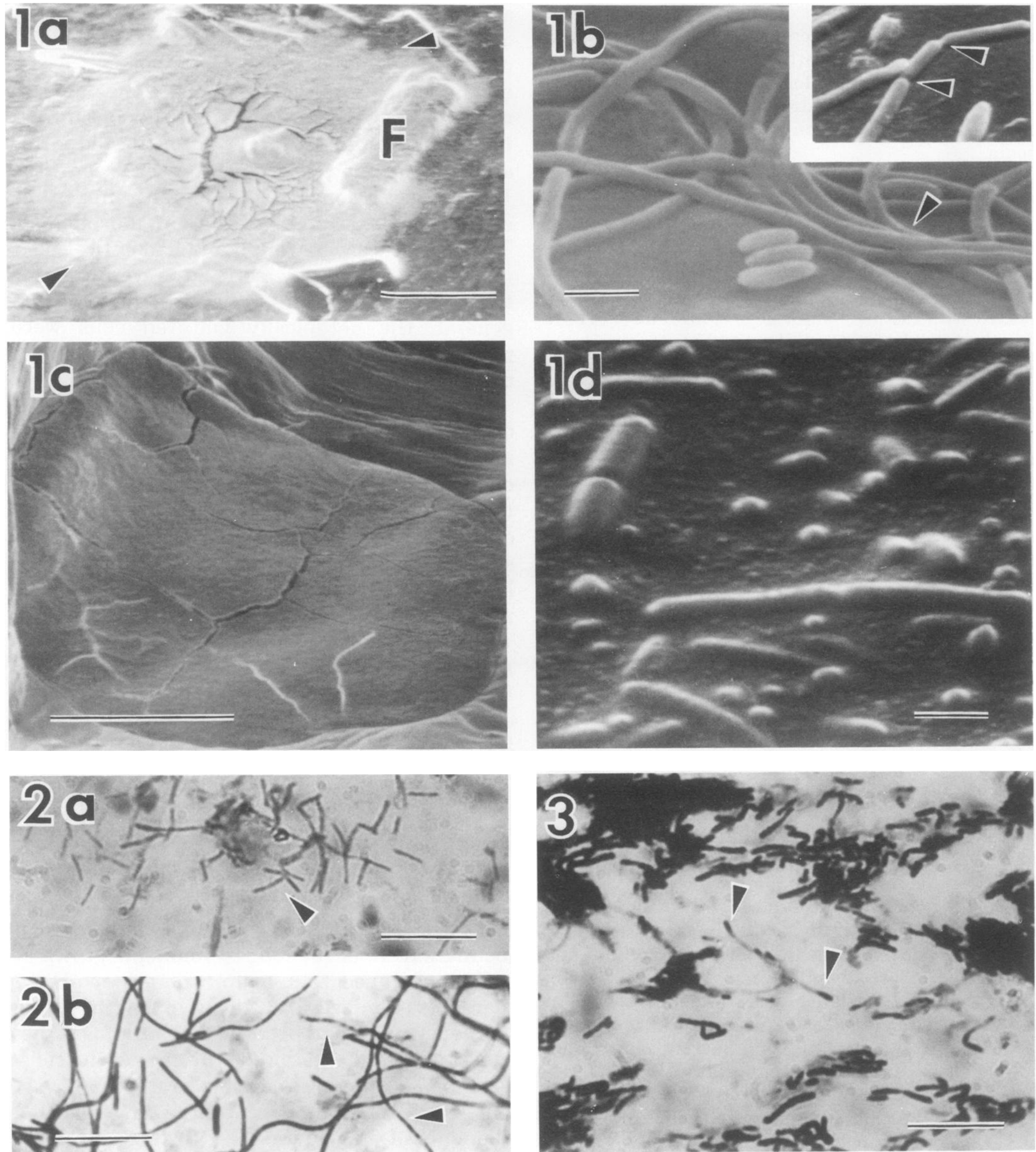


FIG. 1. *(a) SEM of 7-1 grown for 2 weeks in anaerobic roll tubes. The colony is flat, the center is cracked, and the periphery (arrows) is irregular. A fiber fragment (F) in the CBG substrate was overgrown by 7-1. Bar, 0.5 mm. (b) SEM of the periphery of an anaerobic colony of 7-1 showing long, unbranched filaments (arrow). The filaments fragmented into shorter cells of various lengths (inset, arrows). Bar, 2 μm. (c) SEM of 7-1 grown for 3 weeks on aerobic slants. The colony is raised from the agar and shows a definite edge. Bar, 0.5 mm. (d) SEM of an aerobic colony of 7-1 with cells of various lengths, but lacking long filaments. Bar, 2 μm.*

FIG. 2. *Gram-stained smears of 16-h cultures of 7-1 grown in anaerobic CBG broth at different pH levels. Bar, 10 μm. (a) pH 7.5; microbial cells are about 5 to 10 μm long, with some cells (arrow) having light and dark areas of staining. (b) pH 6.7; long filaments with gram-positive and -negative areas (arrows) are present.*

FIG. 3. *Gram-stained smear of 7-1 grown aerobically on TYG slants for 5 days. Cells of various lengths are present, but long filaments are lacking. Short, gram-negative filaments with gram-positive areas (arrows) are present. Bar, 10 μm.*

(i.e., gram-positive and -negative cells of various lengths) in both aerobic and anaerobic media indicate that isolate 7-1 apparently is a pure culture that demonstrates variable and unusual morphologies.

Tests for optimal anaerobic growth conditions. Turbidimetric tests for growth on 0.2% cellobiose broth revealed that optimal growth occurred at 39°C with a pH range of 7.4 to 8.0. Growth was not apparent below pH 6, with the exception of slight growth at 39°C after 74 h. Growth did not occur at 45°C.

Electron microscopy observations of lignified tissue degradation. Preliminary studies to differentiate types of lignin in cell walls of blades and stems were undertaken by light microscopy. Cell walls stained for lignin by using light microscopy are indicated by SEM in Fig. 4. In blades the inner bundle sheath and xylem tissues stained positively for lignin with acid phloroglucinol, whereas the sclerenchyma and to a lesser extent portions of the inner sheath gave a chlorine-sulfite-positive reaction (Fig. 4a). The parenchyma bundle sheath gave a positive but more transient reaction with chlorine-sulfite. In stems, the thick-walled sclerenchyma ring, the bundle sheath and xylem cells, and the epidermis were acid phloroglucinol positive, whereas chlorine-sulfite-lignin was present in the parenchyma cells, with the intensity of staining increasing from the center to outer part of the tissue (Fig. 4b). Blades and stems incubated in uninoculated medium (i.e., controls) for 8 days were intact and undegraded (Fig. 4a and b).

Transmission electron microscopy examination of a colony from a roll tube overgrowing a lignified fiber fragment in the medium such as shown in Fig. 1a revealed that 7-1 cleared the cell walls, especially the intercellular layer, of sclerenchyma cells, leaving only a small residue of electron-dense cell wall material (Fig. 5). These cells were easily identified as blade sclerenchyma by their cell wall thickness and arrangement within the tissue (see Fig. 6 of Akin [1]). Incubation of 7-1 with blades and stems at optimal growth conditions (pH 7.5, 39°C) in anaerobic broth (both with and without 0.2% cellobiose as an additional energy source) showed degradation of lignified tissue; occasionally even the most resistant lignified vascular tissue was degraded (Fig. 6). Similar tissues were degraded with or without cellobiose added, but evaluation for growth after 48 h did show increased turbidity with added cellobiose (absorbance at 520 nm of 0.56 versus 0.14).

No differences in tissue degradation were found in a study of anaerobically and aerobically grown cultures of 7-1 for 7 days. Short filaments attacked sclerenchyma in blades (Fig. 7). Infrequently, long filaments were associated with degraded parenchyma cells, indicating that these diverse filamentous forms could degrade lignified cell walls aerobically.

Because 7-1 demonstrated an ability to attack lignified tissues, a comparison of lignified tissue degradation was made by using various microorganisms reported to have the ability to degrade plant cell walls or cell wall components. *T. viride* did not degrade any of the blade tissues, not even the most easily digested phloem and mesophyll; however, hyphae were present on the plant sections (Fig. 8). *T. fusca* separated the inner bundle sheath of blades into individual cells, caused a layering of the sheath wall, and partially degraded the sclerenchyma cells (Fig. 9a); *T. fusca* showed only slight attack on parenchyma cells in the stems, and filaments did occasionally appear to associate with cells of the acid phloroglucinol-positive ring (Fig. 9b). Rumen microorganisms extensively removed the unlignified cell walls in blades, leaving a residue in which the inner bundle sheath cells, the xylem, most of the abaxial sclerenchyma, and the cuticle were present and maintained the structural organization (Fig. 10a); a large part of the parenchyma bundle sheath remained (not shown). In stems, the acid phloroglucinol-positive tissues and the most intensely staining parenchyma cells resisted degradation (Fig. 10b).

Isolate 7-1 did not extensively degrade the unlignified tissue in blades and left a residue with some mesophyll and parenchyma bundle sheath, but deformation of the inner sheath and separation of sclerenchyma into cells occurred (Fig. 11a). In stems, 7-1 degraded virtually all but the acid phloroglucinol-positive tissues (Fig. 11b); long filaments extended into cells of the ring (Fig. 11b, inset).

A comparison of pretreated and control stems incubated for 72 h and then digested with rumen bacteria for up to 48 h showed that pretreatment with 7-1 resulted in more digestion of the parenchyma cells, even after 6 h (Fig. 12a and b). Measurements of parenchyma tissue remaining in control and pretreated stem cross-sections, which had been sectioned for light microscopy, showed that about three times more tissue remained in the control stems after rumen microbial digestion for both 6 and 48 h.

One study was conducted to test the degradation of lignified tissues by 7-1 after 28 days of incubation in anaerobic broth. Both sclerenchyma and the inner bundle sheath in the blades showed degradation by cells of various lengths, and inner sheath cells were disrupted into layers (Fig. 13). Mesophyll and parenchyma bundle

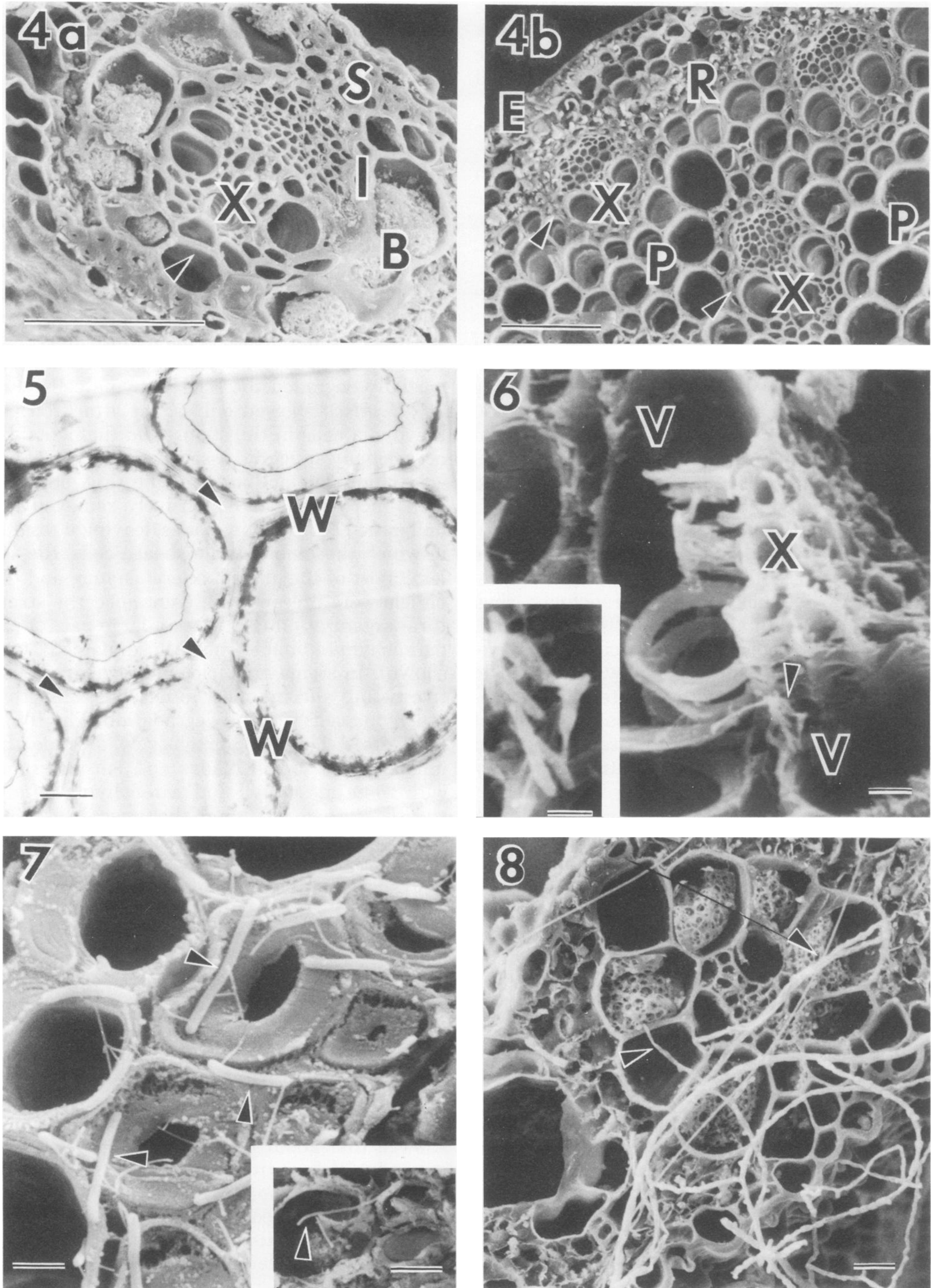


FIG. 4. *SEM of sections of CBG blade and stem incubated for 8 days in basal anaerobic medium without inoculation, showing the intact structure of control cell walls. Cell walls stained for lignin by light microscopy-histochemistry are indicated by SEM. Bar, 50 μm. (a) Blade. Chloride-sulfite-positive lignin was present primarily in the sclerenchyma (S), adaxial part of the inner bundle sheath (arrow), and to a lesser extent in*

sheath cells were distorted. In stems, digestion was primarily limited to parenchyma cells with digestion up to the first or second layers of cells adjacent to the ring. Most lignified vascular and ring tissues were not degraded.

Utilization of cell wall-type carbohydrates and phenolic acids. Anaerobic and aerobic media containing xylan, pectin, cellobiose, and Solka Floc or ground filter paper were tested for their ability to support growth (as measured by turbidity) of 7-1. The maximum absorbance and the day this maximum was reached are shown for aerobic growth after 28 days (Table 1). Growth on Solka Floc, which is insoluble and settles to the bottom, was evaluated by reading at 520 nm, swirling the tube to suspend the cellulose, and allowing the cellulose to settle totally before reading again. This method indicated no growth of 7-1 on cellulose. However, definite growth did occur on xylan and pectin, although growth on pectin was slow. Anaerobic growth studies at pH 7.5 using these same carbohydrates (except ground filter paper was substituted for Solka Floc) showed that only cellobiose supported growth. However, when cultured anaerobically at pH 6.7 on xylan, the bacterium showed slight but definite growth (absorbance at 520 nm = 0.10) in two tests; the bacterium did not grow on pectin. Tests for cellulose degradation using filter paper strips in aerobic and anaerobic media incubated for 28 days indicated that 7-1 was noncellulolytic.

Growth studies (measured by turbidity) using phenolic acids as sole carbon and energy sources for 7-1 are shown for aerobic and anaerobic media (Table 2). Basal medium and basal medium plus 0.2% glucose were included in these tests as negative and positive growth indicators, respectively. In aerobic tubes the sinapic acid broth changed from yellow to dark brown after a few days in both uninoculated controls and inoculated tubes. Growth as shown by the increased absorbance was confirmed by the presence of short gram-negative filaments with gram-positive areas. In anaerobic medium, although discoloration was not noted, the sinapic acid, and ferulic acid to some extent, precipitated and thus interfered with the absorbance due to bacterial growth. However, smears again showed the gram-negative filaments with gram-positive zones, indicating that 7-1 grew on ferulic acid.

Spectrophotometric (ultraviolet) spectra of sinapic acid. Tests for loss of sinapic and ferulic acids in anaerobic broth were not possible because of the precipitate. However, the aerobic medium with sinapic acid was analyzed. Sinapic acid standards showed absorbance maxima at 308, 244, and 200 nm. The 308-nm peak was markedly lower in tubes in which the brown discoloration was apparent and therefore was not included in comparison for loss of sinapic acid due to microbial growth. Absorbance maxima at 244 and 200 nm were present in uninoculated and inoculated media tubes. Peak heights of inoculated broth at 244 and 200 nm were reduced 7.5 and 7.0%, respectively, from those for uninoculated broth. This analysis, in conjunction with turbidimetric growth studies, indicated that growth of 7-1 was due to utilization (in some form) of sinapic acid.

DISCUSSION

Attempts have been made to classify 7-1, but the unusual morphology and the responses to certain biochemical and histological tests do not permit placing 7-1 into an established classification at present. Although 7-1 has not been identified, the various cellular morphologies (i.e., gram-positive and -negative filaments, frag-

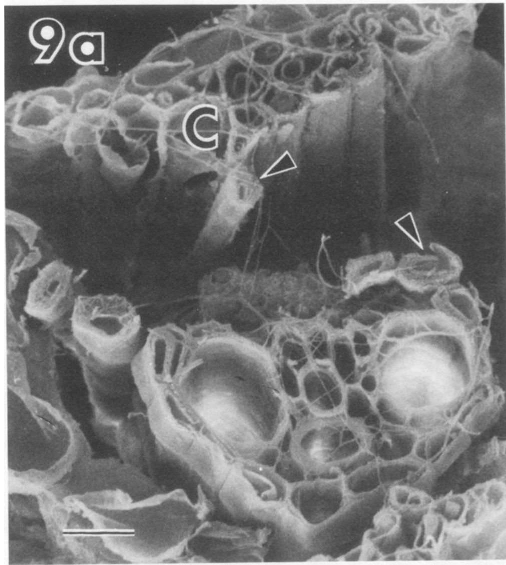
the rest of the inner sheath (I). The parenchyma bundle sheath (B) also gave a positive but more transient reaction with chlorine-sulfite. Acid phloroglucinol-positive lignin was present in the inner bundle sheath and xylem (X) tissues. (b) Stem. Chlorine-sulfite-positive lignin was present in the parenchyma cell walls (P), with the stain intensity increasing centrifugally from the center, and in the walls of cells underneath the epidermis. Acid phloroglucinol-positive tissues included the epidermis (E), sclerenchyma ring (R), and xylem (X); sheath cells (arrows) of the vascular bundles.

FIG. 5. *TEM of sclerenchyma cells in CBG blades from a fiber fragment overgrown with 7-1 in anaerobic roll tube medium similar to (F) in Fig. 1. Extensive clearing of cell walls indicates loss of wall material, especially in the intercellular areas (arrows). Electron-dense areas indicate remnants of remaining cell wall (W). Bar, 2 μm.*

FIG. 6. *SEM of lignified xylem (X) cells and metaxylem vessels (V) of stem vascular bundles degraded after anaerobic incubation for 4 days. The inset shows an enlargement of microorganisms at arrow associated with degraded xylem cells. Bar, 5 μm; inset bar, 2 μm.*

FIG. 7. *SEM of blade sclerenchyma degraded by 7-1 after 7 days of aerobic incubation. Rods and short filaments (arrows) with narrow threads associated with degraded zones in cell walls. The inset shows low magnification of a stem with a long filament (arrow) within degraded parenchyma cells. Bar, 2 μm; inset bar, 10 μm.*

FIG. 8. *SEM of blade incubated for 8 days with T. viride QM6a. Although hyphae (arrows) overgrew the blade, no plant tissues were degraded. Bar, 10 μm.*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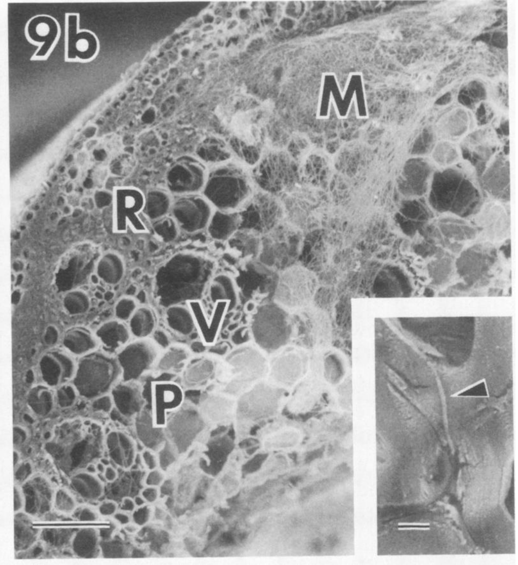
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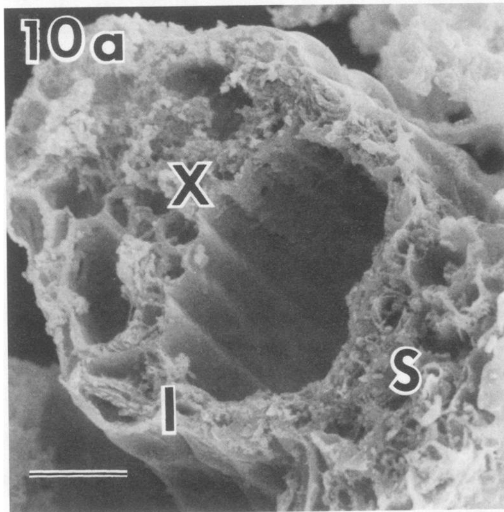
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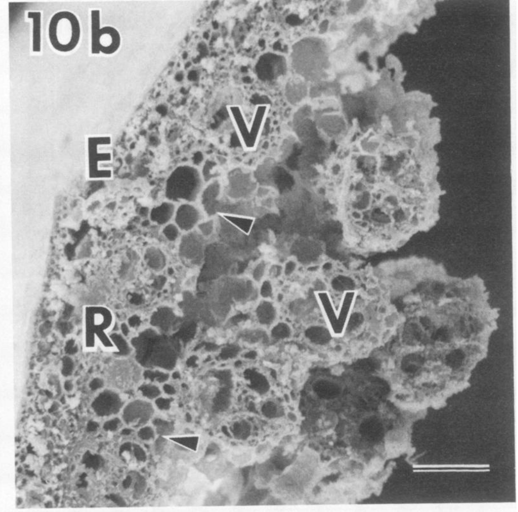
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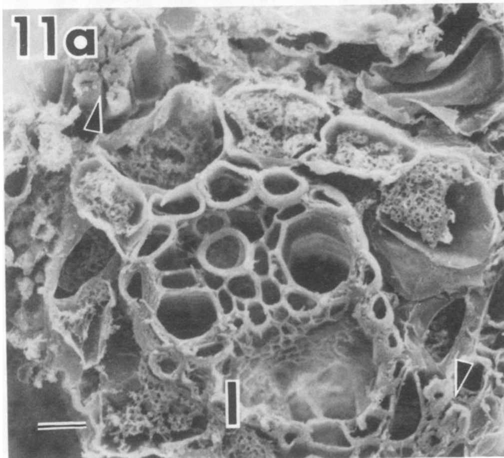
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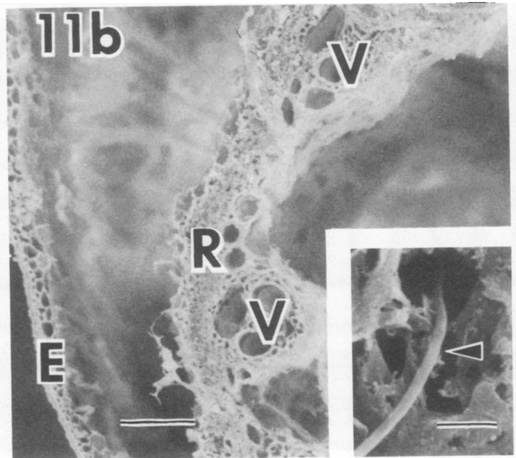
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TABLE 1. *Utilization of carbohydrates for aerobic growth (turbidity) by isolate 7-1*[a]

Carbohydrate	Maximum absorbance	Day of maximum absorbance
None (basal medium)	0.07	21
Cellobiose	0.76	4
Pectin	0.72	28
Xylan	0.32	6
Cellulose (Solka Floc)	0.06	7

[a] Medium was TYG (but without glucose) with carbohydrates at the 0.2% level, pH 7.5, 39°C. Turbidity was measured at 520 nm against a blank of uninoculated medium. Absorbance is that remaining after subtracting the absorbance at 0 h to correct for differences due to inoculum.

TABLE 2. *Utilization of phenolic acids for growth (turbidity) by isolate 7-1*[a]

Carbon source	Maximum absorbance	Day of maximum absorbance
Aerobic[b]		
None (basal medium)	0.07	21
Glucose	0.92	3
p-Coumaric acid	0.19	28
Ferulic acid	0.26	28
Sinapic acid	0.77	24
Anaerobic[c]		
None (basal medium)	0	
Glucose	0.48	3
p-Coumaric acid	0.05	6
Ferulic acid	0.45	24
Sinapic acid	0.23	17[d]

[a] Turbidity was measured at 520 nm against a blank of uninoculated medium. Absorbance is that remaining after subtracting the absorbance at 0 h to correct for differences due to inoculum.

[b] TYG basal medium, but without glucose, pH 7.5, 39°C.

[c] Basal medium of (9), pH 7.5, 39°C.

[d] May not reflect true absorbance because of precipitated acid.

ments of shorter filaments and cocco-rods) are representative of this culture. The filaments, although variable in length with changes in pH, were useful in tracing the microbial attack of cell walls by SEM. Isolate 7-1 grown under optimal conditions of pH (7.4 to 8.0) and temperature (39°C) degraded structurally intact, lignified cell walls that microbes in rumen fluid alone did not degrade. 7-1 was isolated from the rumen population, but the suboptimal pH (6.7) of the rumen for growth of 7-1 may have prevented the extensive degradation of lignified cell walls by rumen fluid detectable by SEM in this study. The data indicated that growth on hemicellulose-type constituents (i.e., xylan and pectin) was slow in aerobic culture and slight to lacking in anaerobic culture. This fact plus the fact that 7-1 was noncellulolytic by all methods tested herein indicated that much of the available (i.e., unlignified) constituents in forage cell walls would not be used by 7-1. Indeed, comparisons of digestion by SEM confirmed that rumen microorganisms were markedly more efficient in degrading unlignified tissues than was 7-1. Although degradation of tissues by 7-1 was not extensive, pretreatment with 7-1 before digestion with rumen microorganisms disrupted unlignified and some lignified tissues and made the cell walls more readily available to rumen microorganisms.

Although other bacteria (14) and notably a thermophilic actinomycete (10) have been reported to degrade lignocellulose, 7-1 degraded lignified cell walls of CBG in anaerobic and aerobic culture. The anaerobic degradation of intact lignified tissues indicates a unique role for this microbe. Comparison by SEM of the attack of CBG cell walls by the lignocellulolytic actinomycete *T. fusca* and 7-1 showed that both microorganisms degraded lignified tissues, although the degree of digestion was not extensive after 8 days. Notably, lignified tissues such as the inner bundle sheath and sclerenchyma were

FIG. 9. *SEM of blade and stem sections incubated for 8 days with T. fusca 190Th. (a) Blade. The inner bundle sheath is separated into cells which show a layered wall (arrows). Sclerenchyma cells (C) were degraded, and the actinomycete overgrew the vascular bundle. Bar, 10 μm. (b) Stem. T. fusca degraded a portion of the parenchyma cells (P), whereas the sclerenchyma ring (R) and vascular bundles (V) resisted degradation. The microbe formed a mycelial mat (M). The inset shows a hypha (arrow) in a hole between cells of the resistant ring cells. Bar, 50 μm; inset bar, 2 μm.*

FIG. 10. *SEM of blade and stem sections incubated for 8 days with rumen fluid. (a) Blade. The sclerenchyma (S), inner bundle sheath (I), and xylem (X) resisted digestion and appear intact for the most part. Bar, 10 μm. (b) Stem. The vascular bundles (V), sclerenchyma ring (R), epidermis (E), and the most intensely staining parenchyma cells (arrows) near the ring resisted digestion. Bar, 50 μm.*

FIG. 11. *SEM of blade and stem sections incubated for 8 days with 7-1. (a) Blade. The inner bundle sheath (I) is deformed whereas the sclerenchyma (arrows) is separated into individual cells. A large portion of unlignified tissue remains. Bar, 10 μm. (b) Stem. The epidermis (E), the sclerenchyma ring (R), and vascular bundles (V) resisted degradation. The inset shows a filament (arrow) in a hole in the ring cells. Bar, 50 μm; inset bar, 2 μm.*

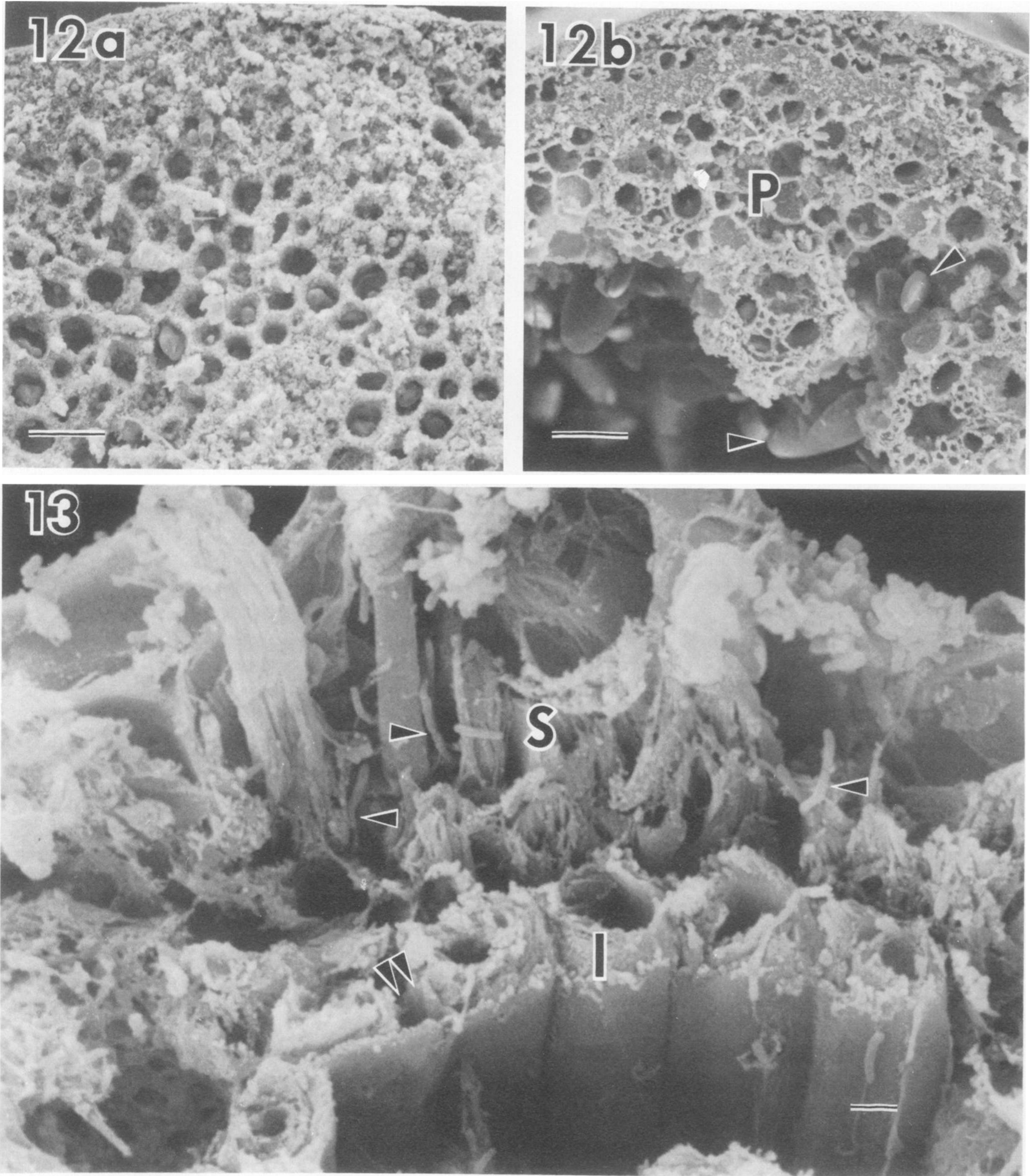

FIG. 12. *SEM of control and pretreated (with 7-1) stem incubated for 6 h with rumen microorganisms. Bar, 50 μm. (a) Control stem, incubated for 72 h in uninoculated anaerobic medium before rumen microbial digestion. A coating from the rumen fluid occurred on undegraded cell walls. (b) Stem pretreated for 72 h with 7-1 in anaerobic medium before rumen microbial digestion. The parenchyma tissue (P) was partially degraded, and many protozoa (arrows) were within the stem section.*

FIG. 13. *SEM of blade incubated anaerobically for 28 days with 7-1. The sclerenchyma tissue (S) was extensively degraded, and many rods and filaments (arrows) were present within disrupted cells. The inner bundle sheath (I) was partially degraded with the cells showing a layered wall (double arrows). Bar, 2 μm.*

often separated into individual cells, thus indicating that the intercellular substances were more available to enzymatic degradation by these microorganisms. *T. fusca* has been reported to use the cellulose but not the lignin moiety of lignocellulose pulps included as substrates (11). Anaerobic and aerobic culturing of 7-1 on cell wall-type carbohydrates and phenolic

acids representing lignin moieties suggested another mode of attack in which the acids supplied carbon and energy for growth. Ultraviolet spectrophotometry showed that the amount of acids decreased in the spent broth, indicating that sinapic acid was removed during growth. However, the mechanism of degradation of the phenolic acids was not studied. Gas-liquid chromatography of each of the phenolic acids used as substrates showed a purity of greater than 99% (M. E. Snook, personal communication), further indicating that growth was due to the phenylpropanoid unit per se and not a contaminating energy source.

SEM showed that 7-1 predominately attacked the chlorine-sulfite-positive tissues (i.e., sclerenchyma in blades, parenchyma in stems), whereas attack on the acid phloroglucinol-positive tissue (i.e., lignified vascular tissue and xylem) was markedly less. Chlorine-sulfite gives a positive reaction for syringyl-type lignin, i.e., 3,5-dimethoxypropane units (20). The primary attack on this type of lignified tissue agreed with aerobic culturing in which sinapic acid supported greater growth than phenolic acids with one or no methoxyl groups substituted on the aromatic ring. A similar trend was present in anaerobic growth studies of 7-1 in which the less methoxylated acids supported less microbial growth. Furthermore, chlorine-sulfite-positive tissues are more easily degraded into individual cells by oxidation with the delignifying agent $KMnO_4$ (3). The greater ease of delignification and the attack on chlorine-sulfite-positive tissues by 7-1 suggested that these tissues are less rigidly complexed than the acid phloroglucinol-positive tissues.

Filamentous bacteria in rumen fluid degrading sclerenchyma cells such as those reported (1) have been found by using transmission electron microscopy in six other grass species; further, sclerenchyma cells were essentially undegraded in the absence of these bacteria (unpublished data). Isolate 7-1 probably is able to exist in the rumen despite its low number and relatively slow growth rate because of its ability to use substrates other rumen bacteria cannot use. A soluble form of substrate may be available to 7-1 from lignin-carbohydrate-complexes reportedly solubilized from plant cell walls in rumen fluid (13).

Research is needed to test the potential of 7-1 to use lignocelluloses at higher rates. Furthermore, research with 7-1 and other microorganisms of known specificities for lignin-carbohydrate degradation could be useful in clarifying the role of the various types of lignin in limiting the availability of structural polysaccharides to microbial degradation.

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