JOURNAL OF BACTERIOLOGY. Mar. 1976, p. 1156-1162

Vol. 125, No. 3
Printed in U.S.A.

Ultrastructure of Rigid and Lignified Forage Tissue Degradation by a Filamentous Rumen Microorganism

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Received for publication 11 December 1975

A small (less than 1 μm)-filamentous, branching microorganism was observed in Gram-stained smears of the rumen microflora and was found to degrade tissues in forage samples incubated in vitro and in vivo with rumen fluid and observed by scanning and transmission electron microscopy. The microbe had prokaryotic cytoplasmic features and a gram-positive type of cell wall structure. Round to oval bodies apparently attached to hyphae resembled the sporulation pattern reported for *Micromonospora*. Filaments and rod and coccal forms of the microbe degraded rigid forage cell walls and lignified, thick-walled sclerenchymal cells. Location of the microbe at a slight distance from the degraded zones suggested the action of extracellular enzymes. The presence of a microbe with the capability of degrading lignified tissue represents an important and unique function in the rumen ecosystem.

The complexity of the rumen microbial ecosystem has been shown by the diverse genera and species of bacteria and protozoa described by Hungate (10). Additionally, workers have found in the rumen a morphologically novel, cellulolytic bacterium (13), occasionally yeasts and fungi (14), a mycoplasma (20), bacteriophages (1), and the cellulolytic actinomycete *Micromonospora* (9, 16).

Transmission and scanning electron microscopes (TEM and SEM, respectively) have been used in conjunction to delineate the microflora of particular ecological niches (18). Previously, we used the TEM and SEM to investigate the in vitro rumen microbial degradation of forage cell walls (2). Additionally, the TEM has been useful in revealing the ultrastructure of certain rumen bacteria (5, 16, 23).

The objectives of this report are (i) to demonstrate and describe a small-filamentous microorganism associated with the rumen microflora in in vitro and in vivo digested forage samples and (ii) to reveal the microbe's degrading of intact cell walls, including the lignified sclerenchyma, using the TEM and SEM.

MATERIALS AND METHODS

Microbial inoculum. For in vitro studies, rumen digesta was obtained from a cannulated steer and immediately squeezed through multilayered cheesecloth into a warmed vacuum bottle. At the laboratory, 1 part rumen fluid was mixed with 1 or 2 parts McDougall's buffer (15) previously bubbled 30 to 60 min with CO_2. The microbial-buffer suspension was used as inoculum for most studies. Additionally, the sediment obtained after separation of rumen fluid in a separatory funnel at 39 C was mixed 1:10 with McDougall's buffer and used as an inoculum. With the exception of fewer bacteria on plant cell walls in the 1:10 diluted inoculum, no differences were found between inocula, so the results are reported without reference to inoculum.

For in vivo studies, leaf blades were separated from rumen digesta removed from the cannulated steer, and the microbe-plant relationship was studied in situ.

Substrate. For in vitro studies, leaf blades of the forage grasses Coastal bermudagrass [*Cynodon dactylon* (L.) Pers.] and Kentucky-31 tall fescue (*Festuca arundinacea* Schreb.) were cut into sections 2 to 5 mm long. Substrate and inoculum were placed in flasks and incubated at 39 C with continuous bubbling of CO_2. For in vivo studies, leaf tissue included Coastal bermudagrass and other grasses present in the bermudagrass hay removed from the rumen.

Light microscopy. Gram-stained smears (4) were made of the microorganisms in rumen fluid. Control leaf blade sections from subsamples of the grasses used for in vitro studies were sectioned in a cryostat at 10 to 16 μm thickness and stained specifically for fungi with lactophenol cotton blue and acid fuchsin-lactophenol (21). Leaf blade sections of Coastal bermudagrass from the control samples were cut in a cryostat at 10 to 16 μm thickness and stained specifically for lignified tissue with acid phloroglucinol or with calcium hypochlorite-sodium sulfite (11).

SEM. Leaf blade sections of in vitro and in vivo digested samples were fixed in 4% glutaraldehyde (buffered at pH 7.2 in 0.1 M cacodylate buffer) for 12 to 24 h and postfixed in buffered 1.5% osmium tetroxide for about 4 h. Samples were then dehydrated

in graded ethanol-water washes (25 through 100%, vol/vol). A portion of the in vivo digested leaf blades was cryofractured in frozen ethanol to investigate degradation deeper then the leaf edge according to the procedure of Humphreys et al. (8). All samples were removed from 100% ethanol and critically point dried in liquid CO_2 without transitional solvent.

TEM. In vitro and in vivo digested samples were prepared as described previously (3), omitting the buffer wash steps.

RESULTS

Light microscopy. Gram-stained smears of microorganisms from rumen fluid revealed small (diameter, about 0.5 μm), gram-negative filaments, with gram-positive structures that appeared at times to be directly attached to the filaments. Occasionally, fungal hyphae were observed in the smears, but these septated hyphae were much larger (diameter, about 4 μm) than the small, gram-negative filaments observed with light microscopy and filaments in digested leaves observed with the TEM. Undigested subsamples of the same grass used for in vitro digestion studies stained with lactophenol cotton blue and acid fuchsin-lactophenol revealed no parasitic hyphae within the leaf, although occasionally fungal spores were observed on the cuticle.

Histochemical staining revealed a preferential staining of the sclerenchyma with calcium hypochlorite-sodium sulfite over acid phloroglucinol, indicating the presence of a syringyl-type lignin (22).

SEM. Small-filamentous, branching microorganisms were observed in numerous areas of in vitro and in vivo rumen digested samples of critically point dried and cryofractured-critically point dried leaf blades. The diameter of the branching hyphae (Fig. 1, inset) was smaller in width than the nearby bacteria (Fig. 1, B). At times, structures resembling the sessile spores reported for *Micromonospora* spp. (9) were observed on the hyphae (Fig. 1, arrows).

TEM. TEM observations showed the presence of nonseptate, small-filamentous organisms in sections of degraded leaf blades (Fig. 2). Filament width averaged 0.49 μm with a standard deviation of 0.37 μm (N = 43 from in vitro and in vivo preparations). Thin sections, furthermore, revealed cytoplasmic features of prokaryotic cells (i.e., lack of mitochondria, nuclear membranes, and rough endoplasmic reticulum). Mesosome-like structures of the tubular-vesicular type (Fig. 3, M) and a gram-positive type of cell wall structure similar to those reported for actinomycetes were observed in filaments and rods and coccal forms.

The small-filamentous microbe appeared to degrade actively rigid, intact plant cell walls. Figure 4 shows the presence of the microbe in the degraded zones of thick-walled epidermal cells, whereas Fig. 5 illustrates the degradation of the bundle sheath cell wall. The zones of degradation were unsymmetrical and the microbes were at a slight distance from the degraded areas at times such that attachment to plant cell wall did not appear necessary for forage digestion (Fig. 5). These microbes, furthermore, appeared to degrade the normally undegraded, rigid, lignified sclerenchymal tissues. Figure 6 shows the presence of filaments (arrows) and other rod and coccal forms similar to those reported herein that were actively degrading the entire sclerenchyma. Numerous sclerenchymal cells were degraded with cleared zones near the microbes; cells in which the microbes were not present were intact and undegraded (Fig. 7). Bacteria other than the small-filamentous ones were near the degraded sclerenchyma (Fig. 6B), and at times some were attached to and were degrading the periphery of the sclerenchyma (Fig. 7, arrow).

DISCUSSION

TEM and SEM investigations of grasses degraded by the rumen microflora in this laboratory have shown the presence of the small-filamentous microorganism only recently, and changes in diet or location of the steer may have allowed inoculation of the microbe into the rumen. Actinomycetes with a filamentous morphology similar to those reported here have been found in the rumen by Hungate (9) and more recently by Maluszynska and Janota-Bassalik (16), but fungi also have been shown to grow anaerobically at 40 C (7) and small numbers have been found on occasion in the rumen (14). No parasitic fungi were observed in control leaf sections, and thin sections of control leaves revealed no filamentous microorganisms; the microbes appeared to be associated with the rumen microflora. Gram-stained smears revealed on occasion the presence of fungal hyphae, but the diameter was greater than the small-filamentous hyphae of the microbe involved in degrading forage tissue. Additionally, detailed examination at high magnification and resolution of thin sections showed no eukaryotic cell characteristics in these small hyphae. Additionally, filaments and shorter cells resembling those of the rumen actinomycete *Micromonospora ruminatium* sp. nov. (16) and mesosomes characteristic of actinomycetes (28) were seen. Thin sections of the filamentous structures resembled, in general, those for

other actinomycetes (19). However, in an examination of the microflora (including actinomycetes, other bacteria, and fungi) of sand dune grass roots, Old and Nicolson (18) identified filamentous structures similar to those reported here as fungi, but an examination of their micrographs did not show eukaryotic cellular organelles and the microbes could have been actinomycetes. Additionally, the small, round structures that appeared to be sessilely bound to the small hyphae (Fig. 1, arrows) resembled the sporulation pattern shown for *Micromonospora* by light microscopy (9) and SEM (27).

Whereas positive identification of the small-filamentous microorganism must await the isolation and characterization presently underway in our laboratory, observations of the degradation of plant cell walls, in addition to morphological and ultrastructural characteristics, are compatible with those for actinomycetes. Many actinomycetes have been reported to degrade plant cell wall constituents such as cellulose and hemicellulose, and possibly lignin (25). TEM observations in the present study revealed the apparent degradation of the normally slowly digested bundle sheath and epidermal plant cell walls (3) by the small-filamentous microorganisms.

The observation of lignified tissue degraded by the small-filamentous microorganisms (not reported in the earlier reports of rumen actinomycetes) revealed the presence of a unique and important biochemical activity in a rumen ecosystem. Waksman and Hutchings (26) reported that certain *Actinomyces* spp. possibly degrade lignins from alfalfa, corn stalks, and oat straw aerobically, but Lacey (12) stated that this claim was unconfirmed. Observations reported here indicated that the filamentous microbe degraded the lignified sclerenchymal tissue. Many references can be found to the fact that lignin limits the rumen microbial digestion of plant wall polysaccharides (e.g., 24). Lignin-carbohydrate complexes have been isolated from ryegrass using dimethyl sulfoxide (17). Forage sclerenchymal tissue has been found in ruminant feces (6), and TEM observations have shown the general lack of degradation of lignified tissue by rumen microbes (other than the small-filamentous microbes), although the periphery of the sclerenchyma was attacked (3). The filamentous microbe attacks the more central and peripheral areas of the sclerenchyma. Stafford (22) had shown by biochemical and histochemical means that sclerenchymal lignin was of the syringyl type in timothy (*Phleum pratense* L.); histochemical studies used in this work indicated similarly a syringyl-type lignin in the sclerenchyma as shown by the preferential staining with calcium hypochlorite-sodium sulfite.

Knowledge of the types of enzymes present in the filamentous microbes and extent of activity

FIG. 1. *Scanning electron micrograph of in vitro degraded fescue leaf tissue incubated with rumen fluid for 18 h. Small, branching filaments in addition to other bacteria are present. The branching nature of the hyphae, smaller in width than nearby bacteria (B), is shown at higher magnification in the inset. Sessile structures appear attached to filaments (arrows) and resemble the sporulation pattern on Micromonospora hyphae.*

FIG. 2. *Transmission electron micrograph of a thin section of in vivo degraded Coastal bermudagrass leaf tissue. Note the elongated, nonseptated hypha with prokaryotic cytoplasmic features. Bodies of undetermined nature are present inside, but these do not appear to be mitochondria and may be storage bodies. A microbe similar in internal nature is seen within a degraded portion of the plant cell wall (arrow).*

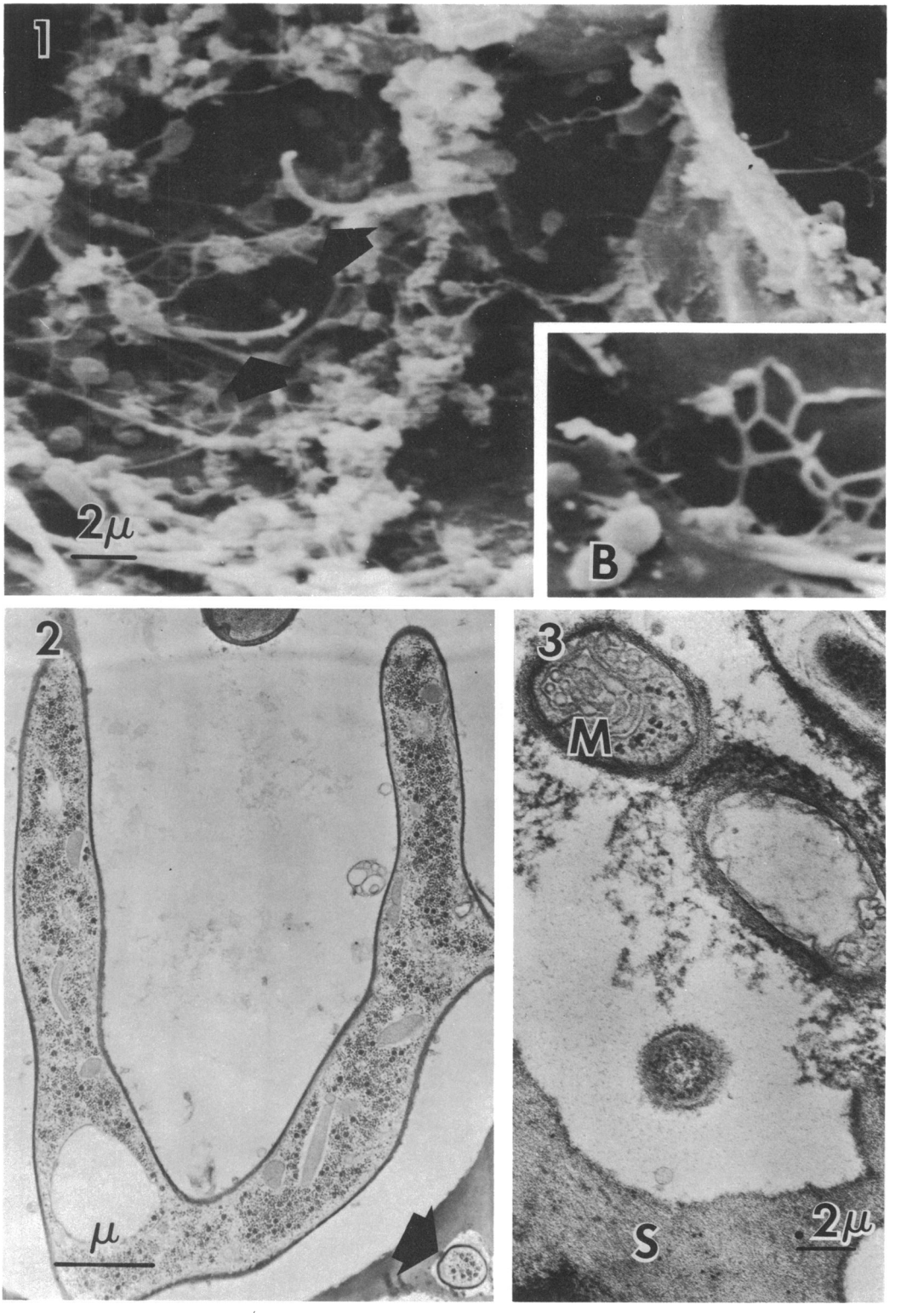
FIG. 3. *Transmission electron micrograph of a thin section of in vivo degraded Coastal bermudagrass leaf tissue. Mesosome-like structures (M) are seen within prokaryotic cells similar to those shown in the filamentous microbes. A sclerenchymal cell wall (S) is degraded to a large extent by the microbe.*

FIG. 4. *Transmission electron micrograph of a thin section of in vivo degraded Coastal bermudagrass leaf tissue. Elongated, small-filamentous structures (arrows) are present within the degraded epidermal cell wall (E).*

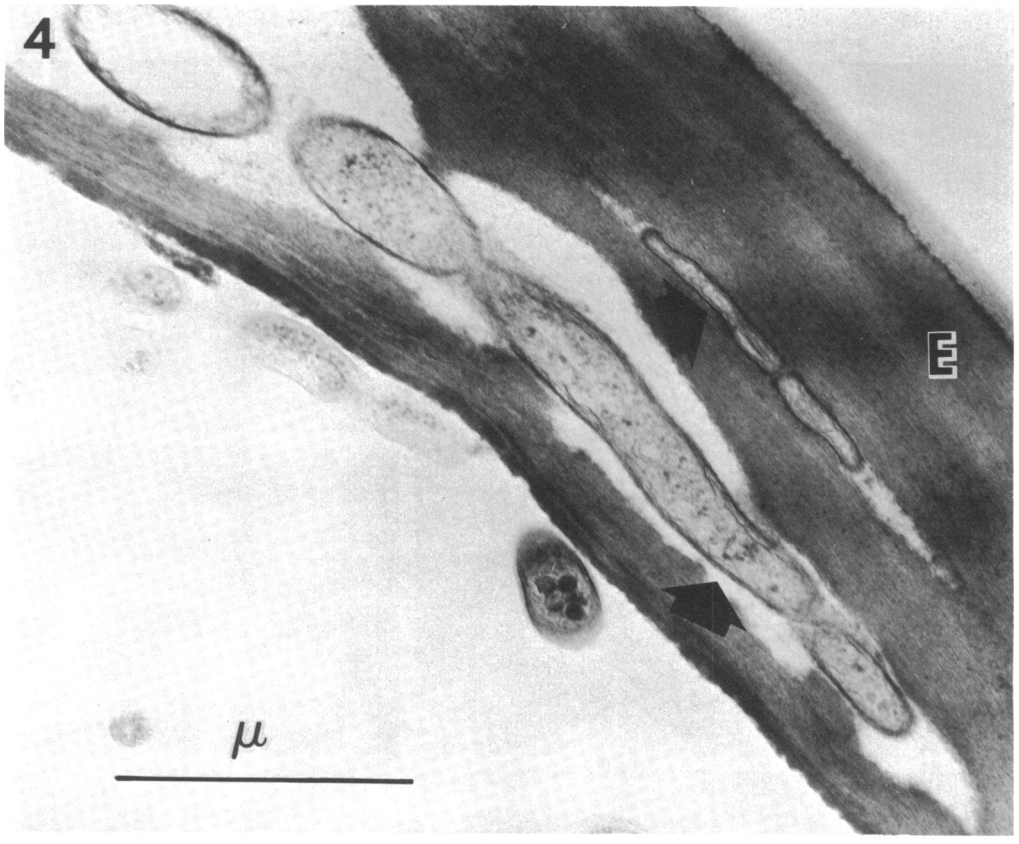
FIG. 5. *Transmission electron micrograph of a thin section of in vivo degraded Coastal bermudagrass leaf tissue. Small-filamentous microorganisms (arrows) apparently have partially degraded the bundle sheath cell wall (B). The cytoplasm is prokaryotic in nature with mesosome-like structures and numerous electron-dense particles, giving a granular appearance.*

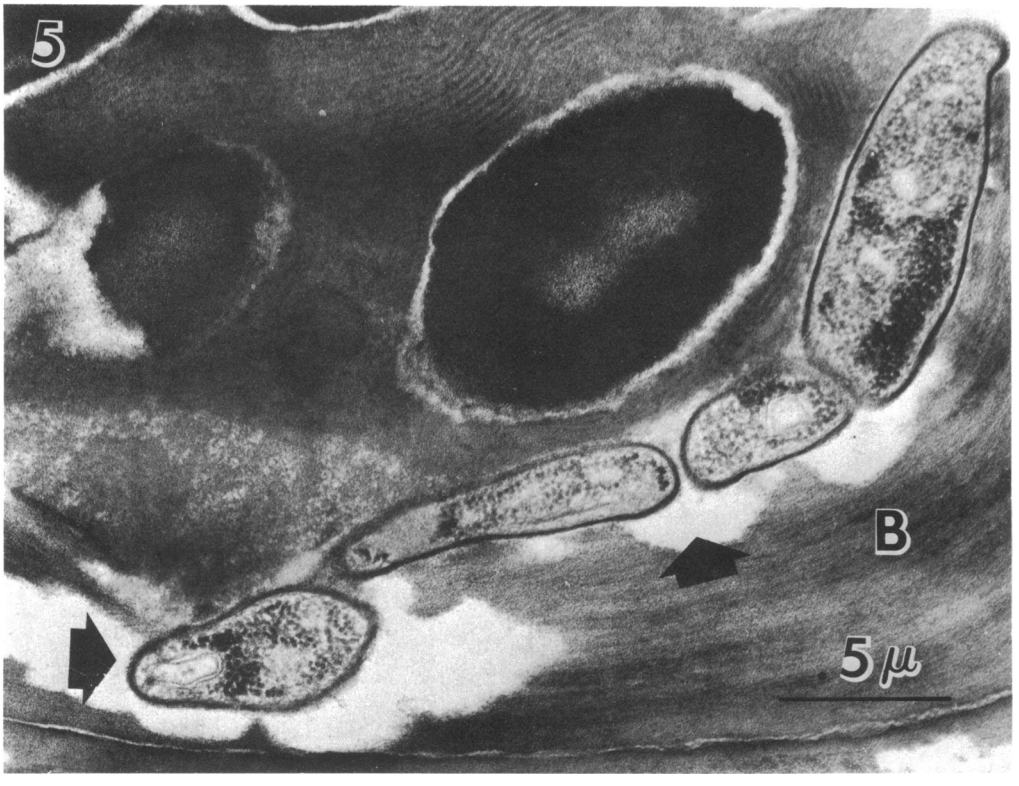
FIG. 6. *Transmission electron micrograph of a thin section of in vivo degraded Coastal bermudagrass leaf tissue. Small-filamentous microorganisms (arrows) have degraded a large part of the lignified sclerenchyma. Other coccal structures similar cytoplasmically to the filamentous microbes seen in other figures are numerous in the degraded regions. Other types of bacteria (B) are seen on the periphery of the sclerenchyma but few within the degraded sclerenchymal cells.*

FIG. 7. *Transmission electron micrograph of a thin section of in vitro degraded Coastal bermudagrass leaf tissue. A filamentous microbe (F) is within the degraded portion of a sclerenchymal cell (S). Other bacteria such as the attached encapsulated coccus (arrow) are present on the periphery of the sclerenchyma.*

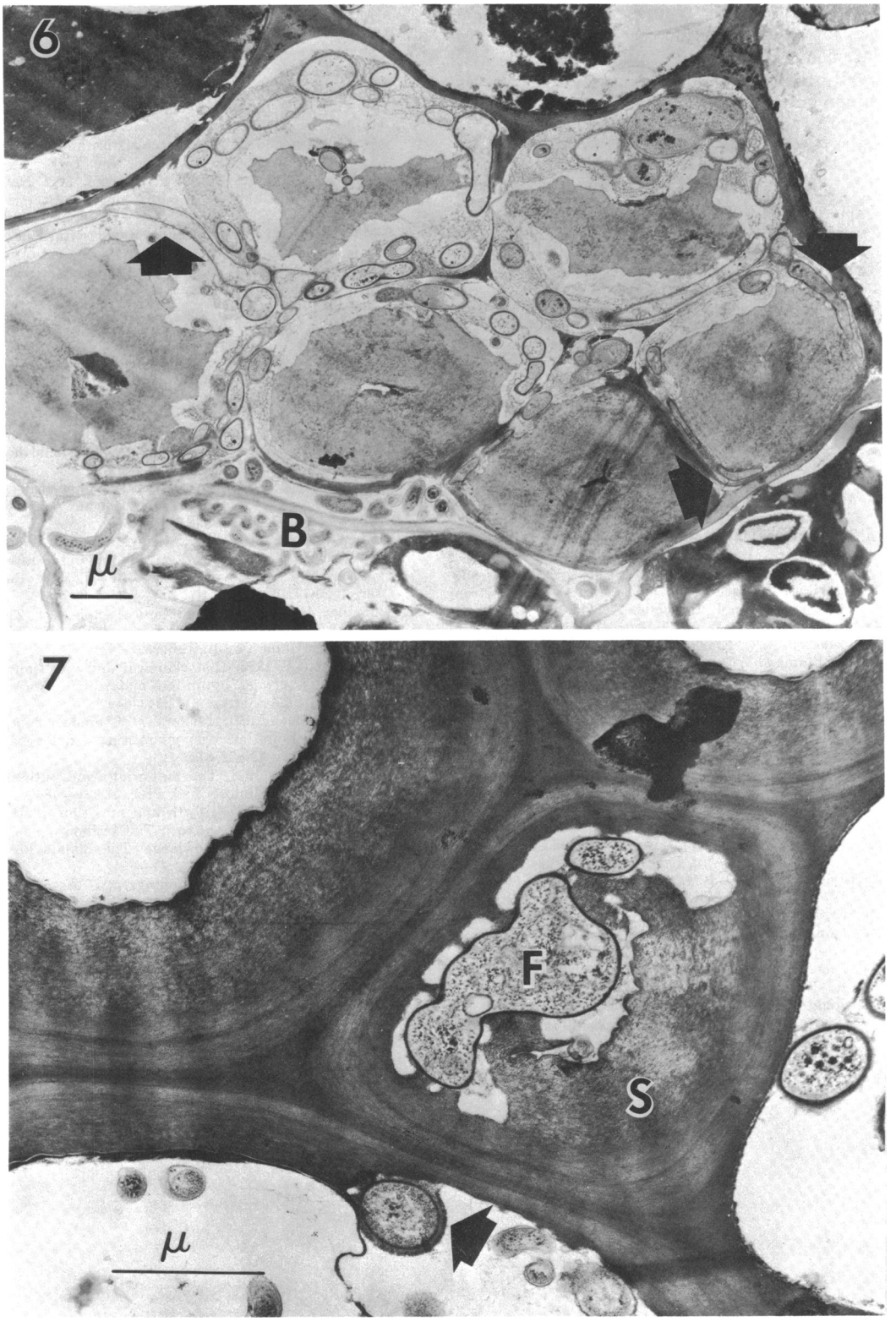


FIGS. 1-3





FIGS. 4–5



FIGS. 6–7

must await further studies. However, demonstration by electron microscopy of in situ degradation of rigid, lignified plant cell walls by a small-filamentous microbe associated with the rumen microflora suggests an important biochemical function heretofore believed to be nonexistent or limited in the rumen ecosystem.

ACKNOWLEDGMENTS

Appreciation is expressed to R. C. Buckner, Agricultural Research Service, U.S. Department of Agriculture, University of Kentucky, and Billy D. Nelson, Southeast Louisiana Experiment Station, Louisiana State University, Franklinton, La., for samples of the forage grasses used in in vitro studies, and to Henry E. Amos, Field Crops Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Athens, Ga., for assistance in collecting and preparing the rumen inocula.

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