APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1979, p. 148-158
0099-2240/79/07-0148/11$02.00/0

Vol. 38, No. 1

Rumen Anaerobic Fungi of Cattle and Sheep

T. BAUCHOP

Applied Biochemistry Division, Department of Scientific and Industrial Research, Palmerston North, New Zealand

Received for publication 9 April 1979

Plant fragments obtained from natural rumen digesta of fistulated cattle and sheep were examined by scanning electron microscopy. Various plant materials suspended in the rumen for different times were examined likewise. By 2 h large numbers of phycomycetous fungal zoospores were found attached to fibrous plant fragments, particularly vascular tissues. The subsequent development of these fungi resulted in production of thalli with extensive rhizoids and with sporangia up to 175 μm long. Scanning electron microscope examination of plant fragments randomly selected from natural rumen contents of both cattle and sheep demonstrated widespread colonization by large populations of these anaerobic fungi. Furthermore, all plant fragments suspended in nylon bags in the rumen were also extensively colonized. These findings demonstrate that plant fragments in the rumen are the sites of colonization and development by the anaerobic phycomycetous fungi. In addition, the results suggest that these fungi may form a significant part of the rumen microbiota in cattle and sheep fed on fibrous diets and suggest that they may be important in fiber digestion.

Orpin was the first to demonstrate (4, 5, 7) that some of the sheep rumen microorganisms believed to be protozoan flagellates were in fact zoospores of anaerobic phycomycetous fungi. These fungi, however, remain classified as protozoa (4, 5, 7).

Initially Orpin (4) isolated a multiflagellated organism, which he named *Neocallimastix frontalis* and which possessed a life cycle alternating between a motile flagellated form and a "nonmotile vegetative, reproductive form." Possession of a rhizoid by the reproductive body led to the suggestion (4) that the organism might be a fungus rather than a protozoan. Additional "flagellates", named *Sphaeromonas communis* (5) and *Piromonas communis* (7), were isolated later, and a resemblance to aquatic phycomycetous fungi was noted (5). After this, *N. frontalis* was described as a rumen phycomycete for the first time (6), and invasion of plant materials suspended in nylon bags in the sheep rumen was described. Orpin found that when zoospores did attach, they showed a preference for inflorescence tissue from feed materials (6, 7, 9). However, *N. frontalis* was found only rarely attached to food particles in filtered rumen contents (6), and it was not possible to determine whether sporangia present in whole rumen contents were attached to particles (6). Our understanding of the relationships of these fungi to rumen digesta plant fragments has remained uncertain, as indicated by the recent statement (9) that "zoospores may germinate upon and grow at the expense of plant tissue present in the rumen."

Because zoospores of the rumen phycomycetes had been found to be present in low numbers in strained rumen fluid (4, 5, 7), they were believed to be unimportant members of the rumen microbiota. Sporangia of these fungi had been found mainly free in strained rumen fluid, and it was believed that they could exist in that state in rumen contents (4, 5, 7, 8). However, in view of the low count of sporangia (up to 3.6×10^4/ml) in strained rumen fluid, it was concluded that the overall effect of these fungi on rumen metabolism was probably not great (8).

During a scanning electron microscope (SEM) investigation of microbial digestion of plant materials suspended in the sheep rumen, large numbers of spherical to ovoid bodies (6 to 10 μm) were found to attach rapidly to exposed vascular cylinders of partially degraded lucerne stems. Their identity was revealed by the occasional finding on the plant pieces of similar but flagellated forms that corresponded to the descriptions of some of the zoospores of the rumen anaerobic phycomycetous fungi given by Orpin (4, 5, 7). The attached nonflagellated forms were then identified as an early developmental stage in the life cycle of these fungi. These findings suggested a central role for fibrous plant materials in the life cycle of these fungi in the rumen and prompted a search for them on natural rumen digesta of cattle and sheep.

In the present study, fibrous plant fragments were shown to be the principal sites of colonization by the rumen anaerobic fungi. The rapid attachment of large numbers of zoospores, together with the demonstration of extensive populations of thalli attached to plant fragments, suggests that the rumen phycomycetes may form an important part of the rumen microbiota.

MATERIALS AND METHODS

Animals. Romney Marsh wether sheep (*Ovis aries*), each fitted with a rumen cannula, were kept in metabolism crates and fed once daily. Three sheep received pelleted lucerne (*Medicago sativa* L.), and a second group of three received chaffed lucerne. Four cattle (*Bos taurus*), steers and cows, each fitted with a rumen cannula, were maintained outdoors on a sawdust loafing pad with free access to water and salt. During experiments they were taken indoors for feeding and received meadow hay ad libidum between 0900 and 1500 h.

Natural rumen digests. Samples of whole rumen contents, usually obtained after overnight fasting, were fixed with an equal volume of 4% (wt/vol) unbuffered formaldehyde. Stem or leaf samples were removed by using forceps, washed thoroughly by agitating in normal saline, and then transferred to 4% (wt/vol) unbuffered formaldehyde to await further processing for SEM or light microscopy.

Plant fragments. Stem vascular cylinder tissue of fresh or chaffed lucerne was prepared by gently shaving off the outer tissues of the stem with a razor blade. Sisal (vascular bundles surrounded by schlerenchymatous sheaths from leaves of *Agave sisalona* L.) was commercially available as twine. Pieces of other plant materials approximately 20 mm long were obtained from chaffed lucerne, meadow hay, and wheat straw and from fresh plants.

Pieces of plant material suspended in a nylon bag in the rumen were removed at timed intervals, rinsed in normal saline, and fixed in 4% (wt/vol) unbuffered formaldehyde.

SEM. After fixation, stem samples from the rumen were rinsed three times with distilled water, blotted dry, and freeze dried (1). Other details of sample preparation for SEM examination were as described previously (1).

Light microscopy. Pieces of stem or stem vascular cylinder from the rumen were sliced longitudinally with a razor blade. Thin slices were stained with 0.1% (wt/vol) trypan blue in lactophenol. Leaf samples were stained without slicing. Stained samples were examined by bright-field illumination.

Culture of fungi. Fungi were grown anaerobically at 39°C in tubes of medium prepared by the method of Hungate (3). The gas phase was CO_2.

Salt solutions (2) were used in medium preparation. Solution A contained (percent, wt/vol): KH_2PO_4, 0.3; NaCl, 0.6; $(NH_4)_2SO_4$, 0.3; $CaCl_2$, 0.03; and $MgSO_4$, 0.03. Solution B contained 0.3% (wt/vol) K_2HPO_4.

The medium used was modified from Orpin (4) and had the following composition: solution A, 16.5 ml; solution B, 16.5 ml; cell-free ovine rumen fluid, 17 ml; distilled water, 50 ml; $NaHCO_3$, 0.5 g; yeast extract (Difco), 0.1 g; peptone (Difco), 0.1 g; glucose, 0.3 g; agar, 0.1 g; cysteine-HCl, 0.02 g; and resazurin, 0.0001 g. Medium (7 ml) was tubed in Hungate tubes (Bellco Glass, Inc.) and sterilized by autoclaving. On cooling to room temperature, 0.35 ml of antibiotic solution (benzylpenicillin, 2×10^4 IU/ml and streptomycin sulfate, 2 mg/ml) was added.

Inoculum was 0.35 ml of fresh rumen contents, strained through cheesecloth.

RESULTS

Ovine rumen fungi. In each study where animals consumed appreciable amounts of fiber (pelleted or chaffed lucerne), large numbers of fungal sporangia were found attached to stem fragments selected at random from rumen contents of sheep before morning feeding (Fig. 1a through c). Highest numbers were found with the chaffed lucerne diet, which contained more stalky material than the pelleted lucerne diet.

The presence of fungi at different stages of development on the same piece of plant material (Fig. 1a and b) demonstrated that fungal infection could occur at widely differing times of the day. Fig. 1c shows a heavy population of fungi at an early stage of development. In each of these samples (Fig. 1a through c) the fungi were attached to the vascular cylinder of the lucerne stem; the outer, thin-walled tissues of the stem were already removed by this stage of digestion. Several examples were also obtained where portions of fungal rhizoids ("mycelium") remained attached, even when exposed (Fig. 2a and b) after digestion of stem thin-walled tissues.

Lucerne leaves in the chaffed diet were extensively digested after 24 h in the rumen, but fragments also had fungi attached (Fig. 3), mainly to the stalks and ribs.

Zoospores were always present in rumen contents of sheep receiving pelleted or chaffed lucerne diets, and inoculation of fresh rumen fluid into anaerobic medium produced rich cultures of characteristic (4) thalli within 48 h.

Phycomycetous fungal sporangia or zoospores were not detected microscopically in rumen contents of sheep on diets low in fiber, e.g. continuously grazed pasture that did not have the opportunity to develop stalks or seedheads. Attempts to culture anaerobic fungi from rumen contents of such sheep were also unsuccessful. These rumen contents contained only soft leafy material, and after starvation of sheep for 24 h, contents were very liquid and contained little fibrous material.

Bovine rumen fungi. The meadow hay diet used in this study contained large amounts of stems, mainly of mixed grasses but with small quantities of red clover (*Trifolium pratense* L.).

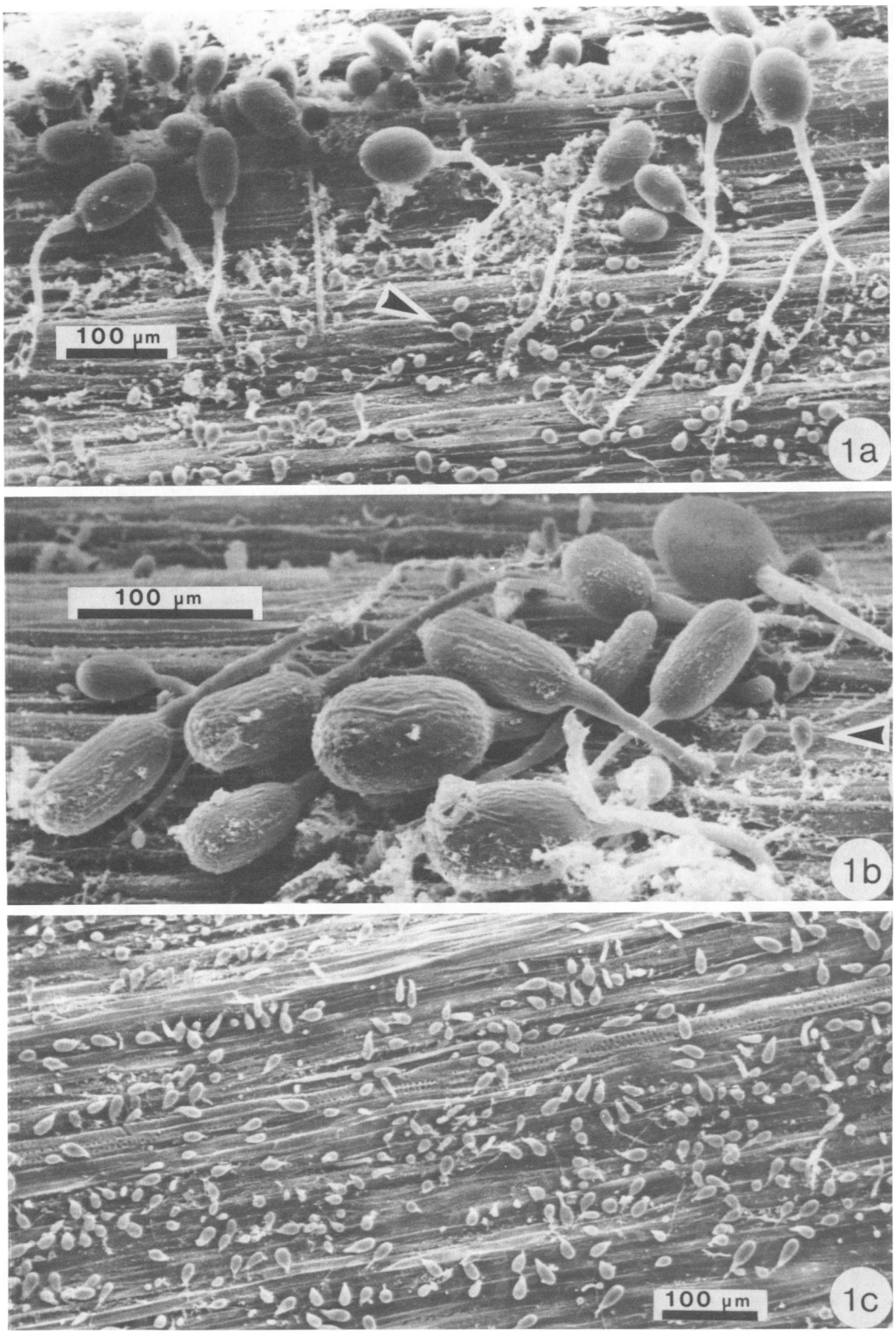


FIG. 1. *Sporangia of rumen phycomycetes attached to vascular cylinder of lucerne stem from natural rumen digesta of sheep. Samples were obtained before feeding in the morning. (a) and (b) Sporangia at early (arrows) and late stages of development. (c) A large population at an early stage in development of sporangia.*

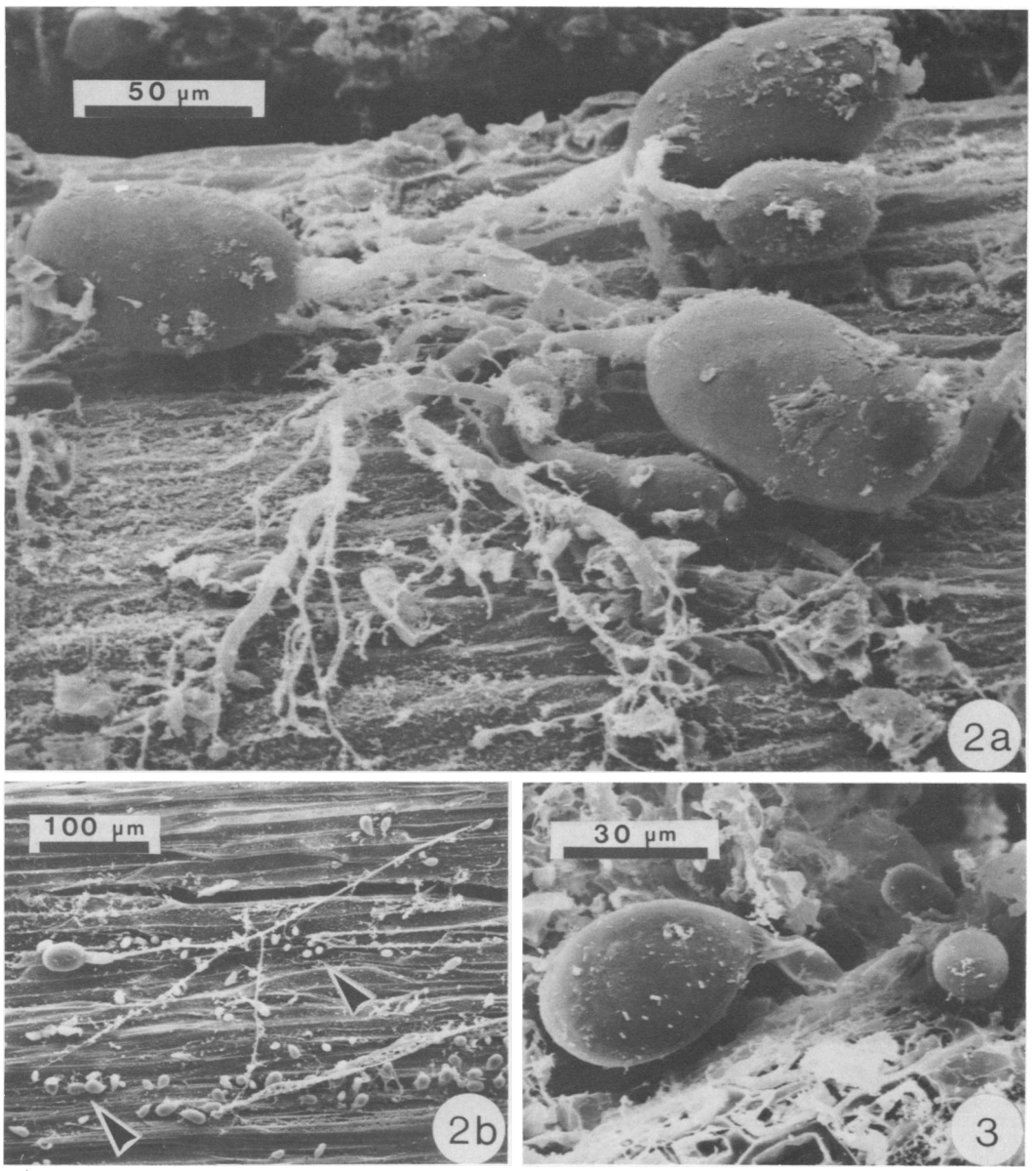
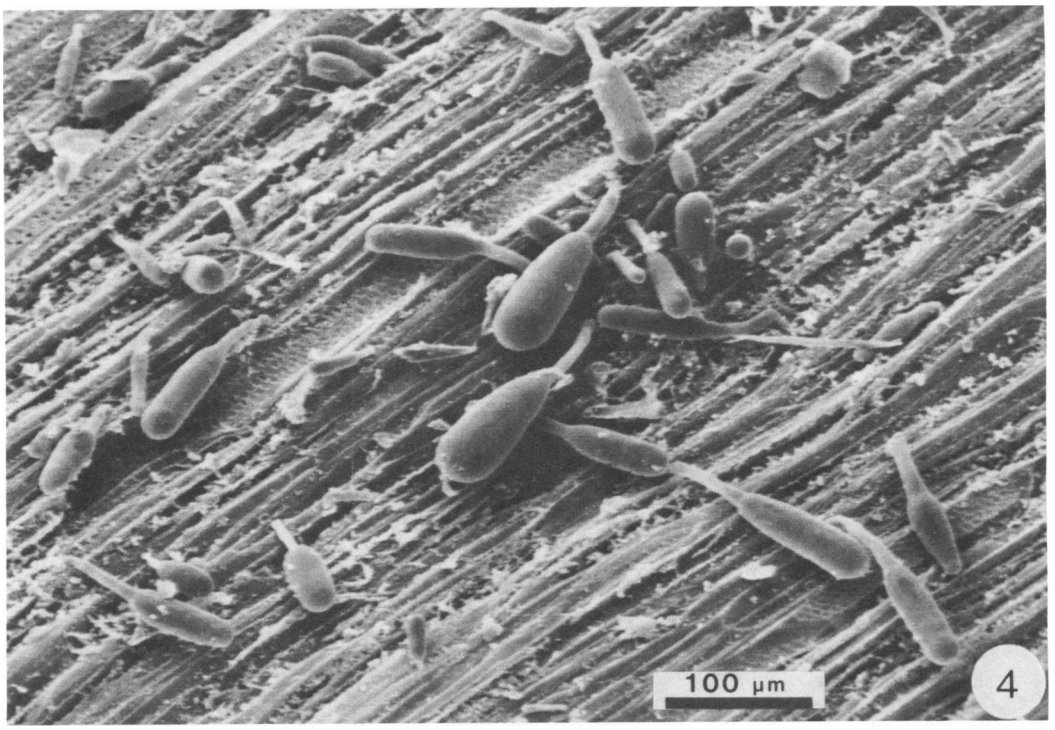


FIG. 2. *Thalli of rumen phycomycetes with part of rhizoids remaining attached to lucerne stem vascular cylinder after soft stem tissues have been digested. (b) also shows many sporangia (arrows) at different stages in development from zoospores. Samples were from natural rumen digesta of a sheep and were obtained before feeding in the morning.*

FIG. 3. *Sporangia of rumen phycomycetes attached to the main rib of a leaf from lucerne chaff in the natural rumen digesta of a sheep. The sample was obtained before feeding in the morning. Mesophyll tissue was extensively digested by this time.*

Red clover stem digesta fragments, removed from rumen contents before feeding in the morning, had large numbers of fungi attached to them (Fig. 4). As with lucerne stem in sheep, the softer, outer tissues were digested away by this stage, and the fungi remained attached to the more resistant vascular cylinders.

Fungal colonization on grass stems in meadow hay possessed several features not observed with other feed materials. The highest fungal populations were always found on the inner surface of the hollow stem (Fig. 5). A range of sizes of sporangia was present, indicating different times of fungal colonization, but on the basis of spor-



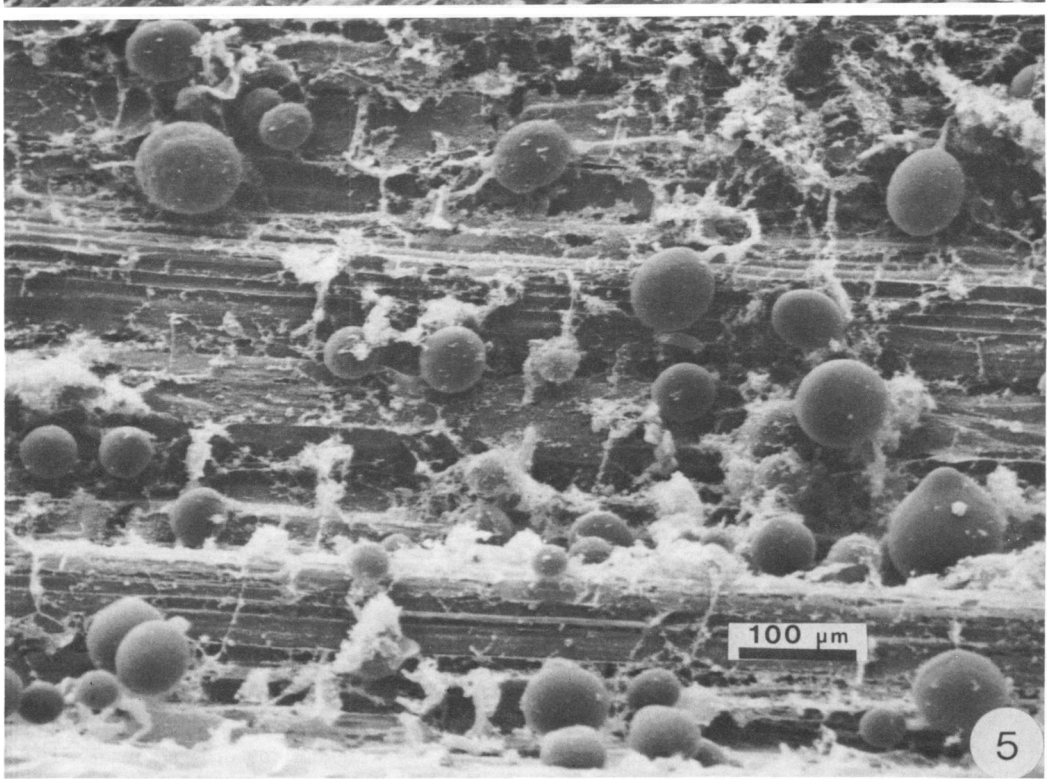


FIG. 4. *Sporangia of rumen phycomycetes attached to the vascular cylinder of a red clover stem from the natural rumen digesta of a steer before feeding in the morning.*

FIG. 5. *Sporangia of rumen phycomycetes attached to the inside surface of the hollow stem of a grass from the natural rumen digesta of a cow. The sample was obtained before feeding in the morning.*

angial morphology few types appeared to be present in this niche. In contrast, although few fungi colonized the outer surface of grass stems, there was a greater variety of sporangia present. The long cylindrical form (Fig. 6a and b) may be identical to that present on red clover stem (Fig. 4). Sharply pointed sporangia (Fig. 6c) and long thin sporangia (Fig. 6d) have been found only on the outer surfaces of grass stems in bovine rumens.

Fungal colonization of plant materials suspended in the rumen. Pieces of lucerne stem in nylon bags were suspended in the rumens of sheep, and samples were removed at intervals for examination by SEM. At 2 h large numbers of spherical to ovoid bodies (6 to 10 μm) (Fig. 7a and b) were found attached to exposed vascular cylinders. A few similar but flagellated forms were also found on stem vascular cylinders (Fig. 8). On the basis of morphology, these were identified as zoospores of rumen anaerobic phycomycetous fungi. The attached unflagellated forms were then identified as an early developmental stage in the life cycle of these fungi. Apparently flagellae separated soon after attachment of the zoospores to plant tissues. Extensive zoospore attachment was rapid, and colonization in high numbers was indicated by the count of 10^5 sporangial early developmental forms per cm^2 (counted on an area of 0.14 mm^2) (Fig. 9) on the surface of the stem vascular cylinder.

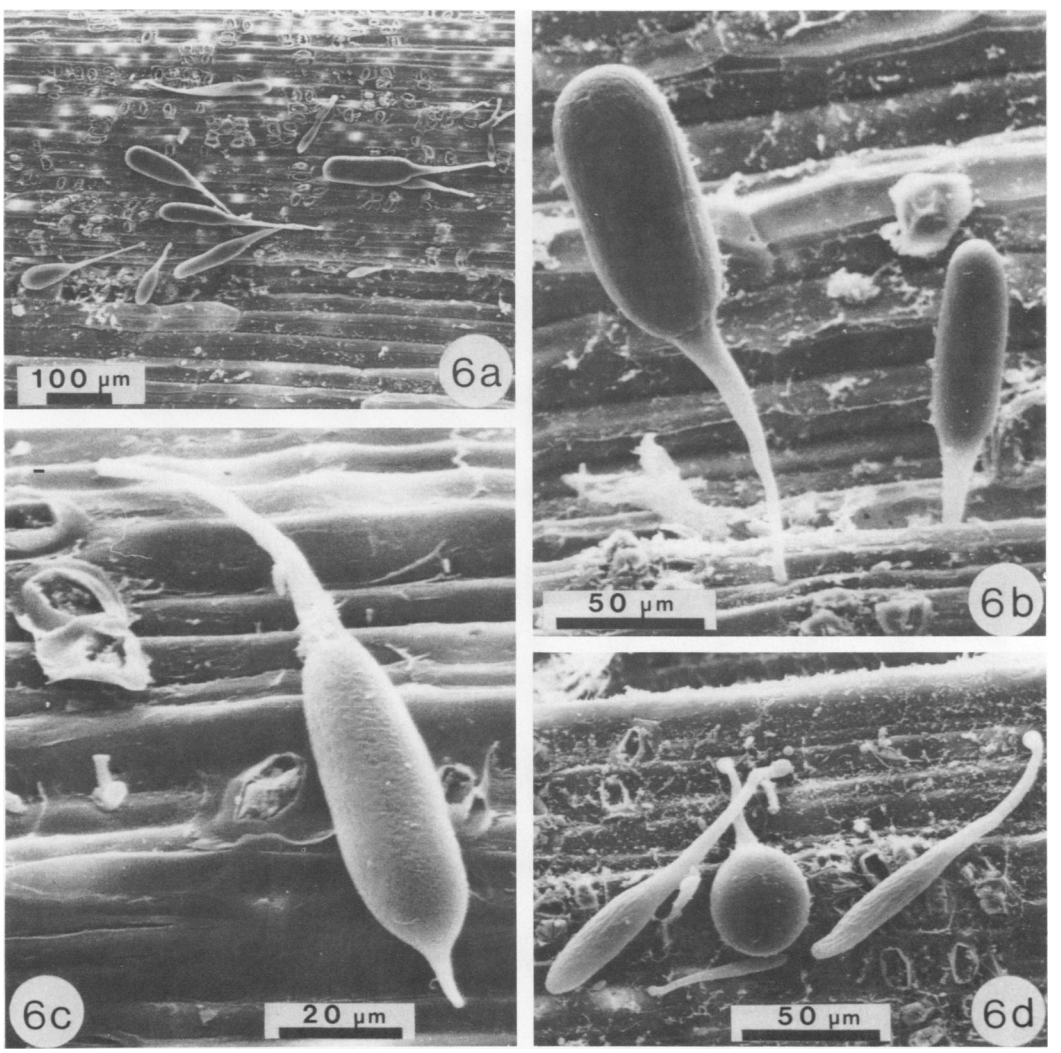


FIG. 6. *Different sporangial forms of rumen phycomycetes on the epidermis of a grass stem from the natural rumen digesta of a cow. Samples were obtained before feeding in the morning.*

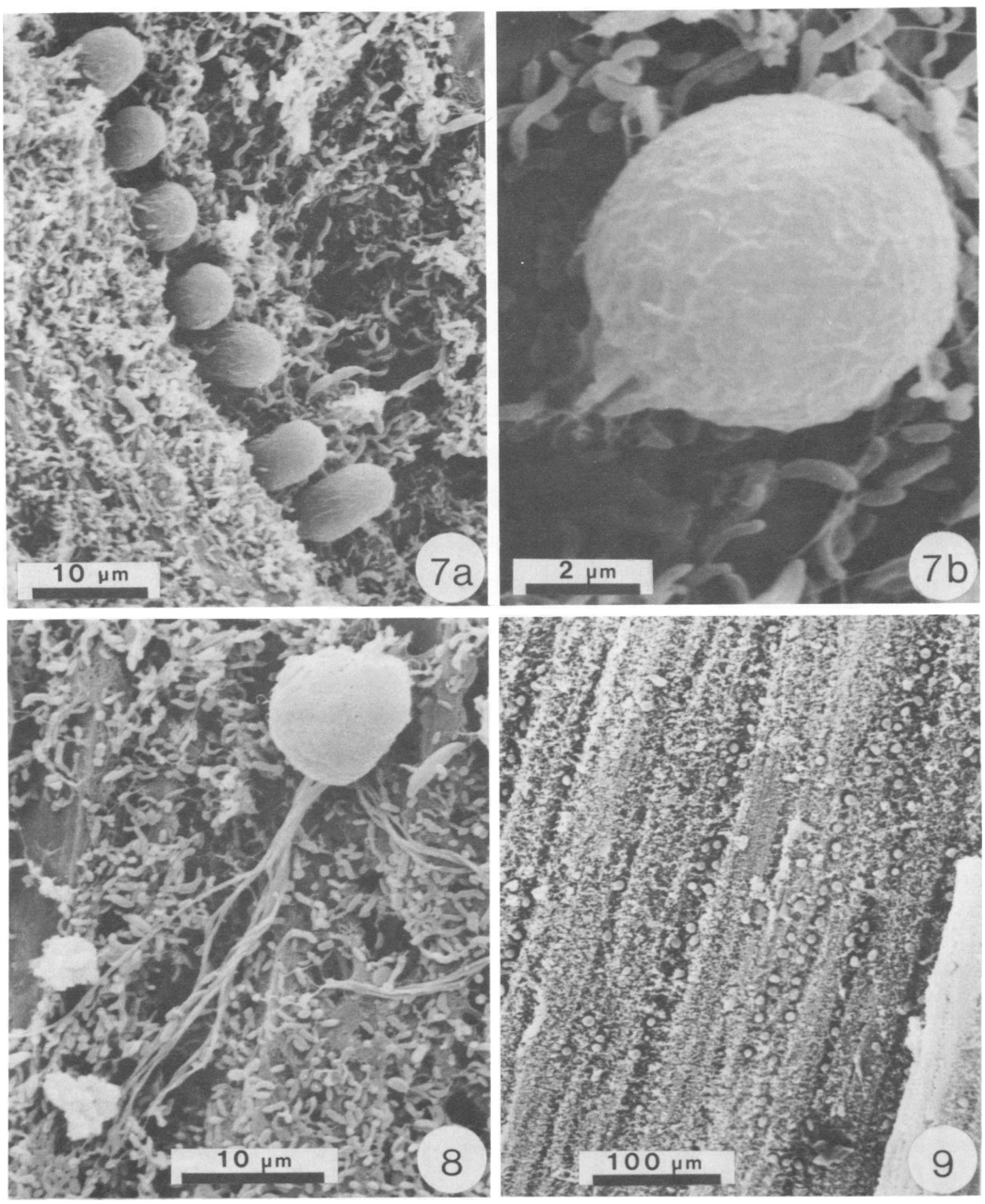


FIG. 7. *Early stage in the development of sporangia from zoospores of rumen phycomycetes. Attached to the vascular cylinder of a lucerne stem suspended in a sheep rumen for 2 h.*

FIG. 8. *Zoospore of rumen phycomycete on the vascular cylinder of a lucerne stem suspended in a sheep rumen for 2 h.*

FIG. 9. *Colonization by rumen phycomycetes on the vascular cylinder of a lucerne stem suspended in a sheep rumen for 2 h. Early stage of sporangial development from zoospores.*

The presence of fungal sporangia or rhizoids after rapid colonization by zoospores was observed with several different plant materials suspended in the rumens of cattle and sheep for different time intervals. Plant materials studied in sheep included sisal fiber, stems of fresh lucerne, chaffed lucerne and wheat straw, vascular cylinders from stems of fresh and chaffed lu-

cerne, and leaves from chaffed lucerne and wheat straw. In cattle, plant materials investigated included stems of red clover and grass in meadow grass hay and leaves of wheat straw and grass in meadow grass hay. The vascular cylinders of stems and the vascular bundles and adjacent mesophyll tissue in leaves were the principal regions of colonization. Grass stems were colonized mainly on the inside surface of the hollow stem.

Rumen incubation periods of 24 to 48 h were required for development of large sporangia on sisal fiber (Fig. 10a and b). With wheat straw stems, fungal colonization was low, and large sporangia developed even more slowly (2 to 4 days). Every piece of all of the other plant materials suspended in the rumen at different times of day was extensively colonized by fungi, and by 24 h many large sporangia were always found attached to the surfaces (Fig. 11a through c). These experiments were repeated many times in both cattle and sheep with several pieces of individual plant material on each occasion. With every piece of these plant fragments suspended in the rumens extensive fungal colonization was obtained.

Fungi in feed. The possibility that some of the fungi were derived from feed was examined. With plant fragments stained with trypan blue, fungal hyphae were frequently detected in the dried feeds studied, but sporangia similar to those found on plant fragments from the rumen were never observed. Attempts to culture rumen fungi from feed material proved negative also, although the anaerobic phycomycetes were readily isolated from rumen contents of both cattle and sheep. Furthermore, sterile (autoclaved) wheat straw leaves suspended in the rumen were rapidly colonized by the anaerobic phycomycetous fungi.

DISCUSSION

The rumen anaerobic phycomycetous fungi have now been shown to extensively colonize plant materials in rumen natural digesta. The present demonstration of rapid colonization and attachment by large numbers of zoospores to rumen fibrous plant fragments, with subsequent development of thalli, resolves much of the confusion surrounding the understanding of the life cycle of these fungi in the rumen. In major measure, this confusion has been the result of the practice, common in studies of rumen microbiology, of using strained rumen contents (4–9) and discarding the rumen solids. This paper has shown that the commonly discarded solids fraction contains large numbers of the vegetative and reproductive stages of the rumen phycomycetes.

Zoospores of phycomycetous fungi characteristically attach to fragments of biological materials, where development of rhizoids can occur in or on the substratum. The evidence presented in this paper demonstrates that this is the case also with the rumen phycomycetes. Stems of several species of pasture plants and leaves of graminaceous plants are the main substrates for fungi in the rumen. Ultimately the fungi were found closely associated with the more slowly digested fractions, the vascular tissues of stems and leaves.

Although the fungi found on stems were mainly attached to vascular cylinder tissue, this does not mean that hyphae do not invade softer

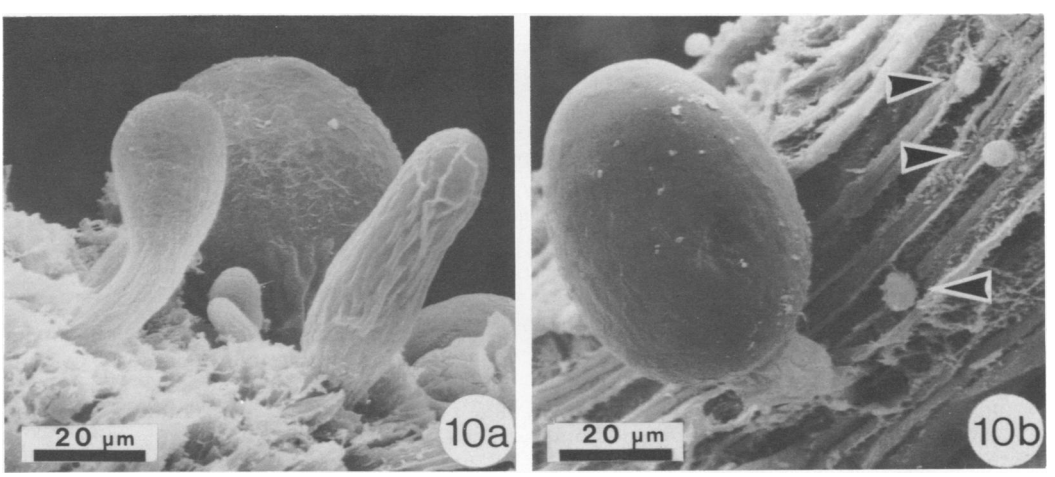


FIG. 10. *Sporangia of rumen phycomycetes attached to sisal twine (vascular tissue) suspended in a sheep rumen for 48 h. (a) Cut end. (b) Side of piece of twine with attached sporangia at early (arrows) and late stages of development.*

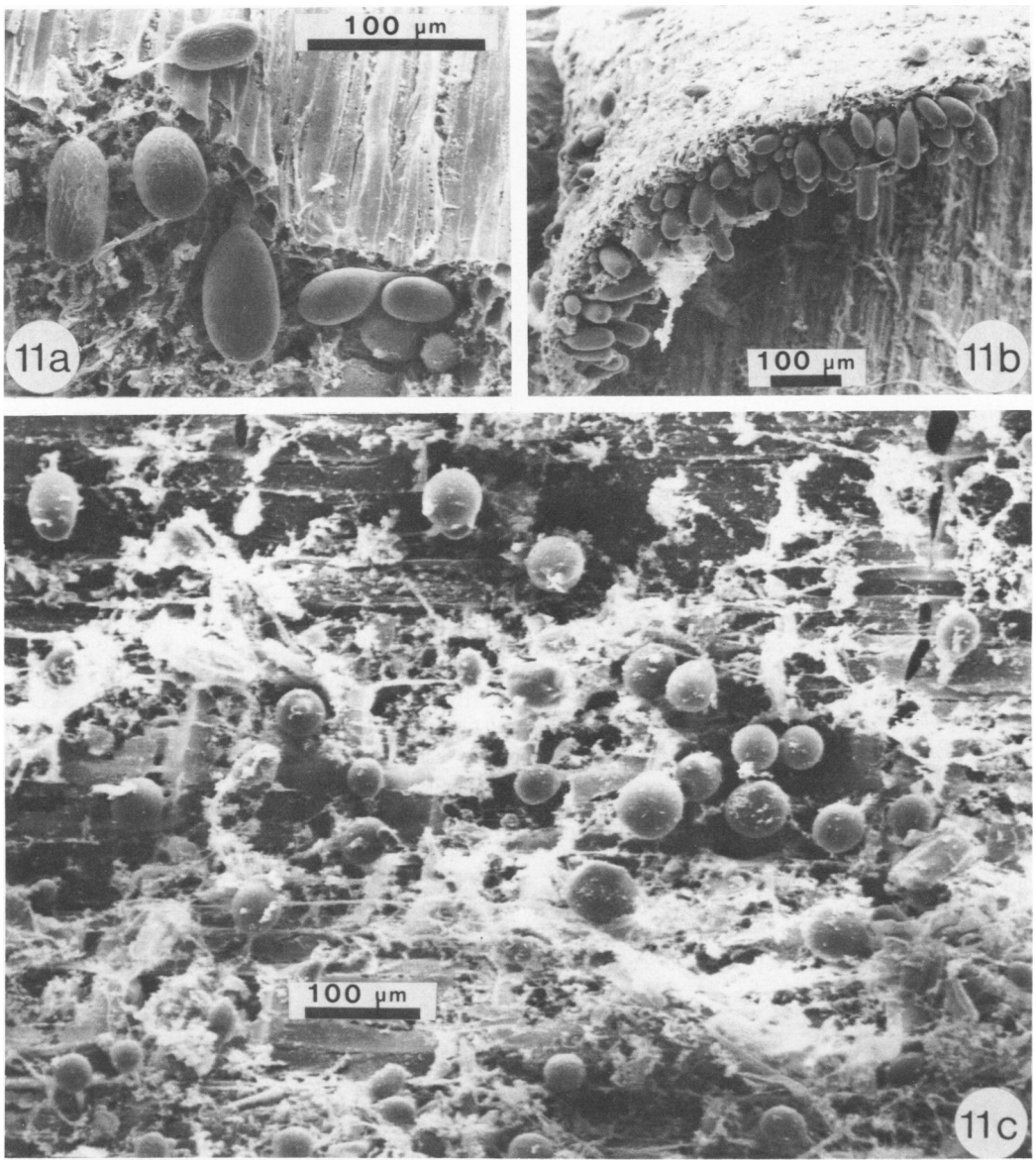


FIG. 11. *Sporangia of rumen phycomycetes attached to stem of food fragments suspended in a rumen for 24 h. (a) Cut end of lucerne stem from a sheep. (b) Cut end of red clover stem from a cow. (c) Inside surface of hollow stem of grass from a cow.*

tissues also. Exposure of fungal rhizoids on the outside of the lucerne vascular cylinder (Fig. 2a and b) indicates that thin-walled tissue, in this case cortex, is also invaded. This is indicated also in several examples (Fig. 1a and 4) where considerable lengths of hyphae were found outside the vascular cylinder, with sporangia thus appearing on stalks. The length of this exposed stalk may represent the thickness of thin-walled tissue through which the fungus has penetrated before reaching the vascular cylinder after initial zoospore colonization at the epidermal surface of the stem. In contrast, sporangia which are directly attached to the vascular cylinder probably represent colonization by zoospores at a later stage in the digestion of the plant fragments.

The major route of fungal invasion of all plant fragments studied was via damaged tissue. The main exception was in the case of grass stems,

where the inner surface of the hollow stem was the major site of colonization. But as with damaged tissue sites, this surface also is an exposed interior tissue. A few fungi were capable of invading grass stem epidermis where there was no obvious evidence of damage. However, these were numerically unimportant, and where grass stem epidermis was damaged, much heavier fungal colonization was found at the sites of damage.

In this investigation, stomata have been found to be a minor, relatively unimportant place of phycomycete invasion. Stomatal invasion was commonly observed only with leaves of graminaceous plants, and even there direct attachment to vascular bundles and mesophyll tissue was much more common. However, most of the leaves studied were derived from hay or straw, and it is possible that minor epidermal lesions of the dried materials may have provided greater access to fungal invasion, although lesions were not obvious.

An indication of the extent of plant tissue invasion by one of these fungi can be seen in Fig. 2b. In this case the length of rhizoids extended to over 450 μm, compared with the sporangium which was only 41 μm long. A thallus with a larger sporangium may colonize even more plant tissue.

The time scale of fungal development in the rumen is also of importance. Although development of the phycomycetes on rumen plant fragments remains to be investigated in detail, the results from experiments with plant fragments suspended in the rumen indicate that the life cycle covers approximately 24 h, by which time large sporangia up to 92 μm long (Fig. 11a and b) were produced. The retention time of plant fragments in the rumen is of a similar order (M. J. Ulyatt, personal communication).

The key to understanding the life cycle of the rumen fungi is the appreciation of the central role of plant fragments in zoospore attachment and fungal development. In the present work it has been uncommon to find sporangia free in strained rumen contents. This suggests that their frequently reported presence there (4, 5, 7, 8) may be a direct result of the manipulative procedures used in straining the rumen contents. It appears likely that free sporangia found previously, with or without rhizoid fragments attached, may have been broken off from the main portions of the rhizoids, which remained within the tissues of the rumen digesta plant fragments. In view of this and the large numbers of fungi now shown to be present on rumen plant fragments, it is clear that counts of these free sporangia in strained rumen fluid (4, 5, 7) do not give a true indication of the quantity of fungal tissue in the rumen.

The low numbers of zoospores previously found in strained rumen fluid (4, 7) have also appeared to indicate that these fungi are unimportant in the rumen microbiota. However, the rapidity of zoospore attachment to plant fragments suspended in the rumen at different times of day suggests that at any given time the free zoospore count is merely an expression of the zoospores being turned over between sporangial release and plant attachment. A primary function of zoospores is to attach to a suitable substratum, and the rapid attachment of high numbers of zoospores reported in this work indicates that they must be constantly replaced to maintain the zoospore count, albeit low, in rumen fluid. The argument for continuous turnover of zoospores is further supported by the finding of different stages of fungal development on the same plant fragments, a finding most readily explained by zoospore colonization at different times during the day.

It is concluded, therefore, that unlike the rumen bacteria or protozoa, the importance of the fungi cannot be assessed by enumeration of any of the stages of their life cycle. Zoospores cannot serve this purpose because of their rapid attachment to plant fragments. Although counts of sporangia on plant fragments might be more meaningful, quantitation would appear to be very difficult. Therefore, direct measurement of fungal mass will be required. It is the quantity of fungal vegetative rhizoid tissues and their enzymic activities within the tissues of digesta plant fragments that holds the key to assessing the importance of these fungi in the rumen fermentation. Quantitation of the fungal rhizoids present within plant tissues of the rumen will undoubtedly present a problem difficult to resolve. Nevertheless, the present results alone suggest that the rumen anaerobic fungi may be significant members of the rumen microbiota. The extent of attachment, colonization, and growth of the fungi on fibrous plant fragments suggests also that they may have a role in fiber digestion in the rumen, perhaps as initial colonizers in ligno-cellulose digestion.

ACKNOWLEDGMENTS

I thank D. Hopcroft for preparation of samples for SEM and for assistance with operation of the SEM. I am indebted to R. T. J. Clarke for discussion.

LITERATURE CITED

1. **Bauchop, T., and R. T. J. Clarke.** 1976. Attachment of the ciliate *Epidinium* Crawley to plant fragments in the sheep rumen. Appl. Environ. Microbiol. **32**:417-422.
2. **Hungate, R. E.** 1966. The rumen and its microbes. Academic Press, Inc., New York.
3. **Hungate, R. E.** 1969. A roll tube method for cultivation of strict anaerobes, p. 117-132. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology. Academic Press, Inc., London.

4. **Orpin, C. G.** 1975. Studies on the rumen flagellate, *Neocallimastix frontalis*. J. Gen. Microbiol. **91**:249–262.
5. **Orpin, C. G.** 1976. Studies on the rumen flagellate *Sphaeromonas communis*. J. Gen. Microbiol. **94**:270–280.
6. **Orpin, C. G.** 1977. Invasion of plant tissue in the rumen by the flagellate *Neocallimastix frontalis*. J. Gen. Microbiol. **98**:423–430.
7. **Orpin, C. G.** 1977. The rumen flagellate *Piromonas communis*: its life-history and invasion of plant material in the rumen. J. Gen. Microbiol. **99**:107–117.
8. **Orpin, C. G.** 1977. The occurrence of chitin in the cell walls of the rumen organisms *Neocallimastix frontalis*, *Piromonas communis* and *Sphaeromonas communis*. J. Gen. Microbiol. **99**:215–218.
9. **Orpin, C. G., and L. Bountiff.** 1978. Zoospore chemotaxis in the rumen phycomycete *Neocallimastix frontalis*. J. Gen. Microbiol. **104**:113–122.