APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1981, p. 1119-1122
0099-2240/81/121119-04$02.00/0

Vol. 42, No. 6

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Isolation, Enumeration, and Maintenance of Rumen Anaerobic Fungi in Roll Tubes

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Received 14 April 1981/Accepted 17 August 1981

Anaerobic phycomycetous fungi have been isolated from rumen fluid by using the Hungate roll tube technique. Cultures on solid substrates in roll tubes can be stored for long periods, and fungal zoospores can be enumerated in roll tubes.

Recently it has been found that anaerobic fungi are present in the gut of many herbivores (1, 2, 4, 8). These phycomycetous fungi have a motile zoospore stage previously thought to be a flagellated protozoa, and several species have been isolated by enrichment culture of rumen contents (4-6; T. Bauchop, personal communication). These organisms are believed to be important in fiber digestion (1, 2).

This paper reports that the Hungate roll tube technique (3) for culturing bacterial anaerobes is ideally suited to studying these fungi. By using this technique, fungal zoospores can be enumerated, and fungi can be isolated directly from rumen fluid without enrichment. Cultures can be maintained for extended periods in roll tubes stored at 39°C.

The methods used for the preparation of prereduced medium and the culture techniques were as described by Hungate (3). Salts solutions A and B were those described by Hungate (3). The medium, modified from Bauchop (1), consisted of salts solution A (17 ml), salts solution B (17 ml), clarified rumen fluid (15 ml), $NaHCO_3$ (500 mg), cellobiose (200 mg), yeast extract (50 mg), Trypticase (BBL Microbiology Systems) (100 mg), 0.05% hemin in 1:1 ethanol-0.05 M NaOH (0.2 ml), distilled water (56 ml), 0.1% (wt/vol) resazurin (0.3 ml), and cysteine hydrochloride (30 mg). Rumen fluid from a rumen-fistulated sheep was clarified by centrifugation at 10,000 × *g* for 30 min and stored at −20°C. Where specified, cellobiose in the above medium was replaced by glucose (100 mg), carboxymethyl cellulose (100 mg), ball-milled Whatman No. 1 filter paper (300 mg), or pieces (5 to 10 mm) of sisal (*Agave sisalone* L) twine. Sisal (20 mg) was added to each tube before the addition of the reduced medium (Bauchop, personal communication). For roll tubes, medium (4.0 ml) containing 1.3% (wt/vol) agar (Davis) was placed in culture tubes (18 by 142 mm) (Bellco Glass, Inc.). Inocula (0.3 ml) were added to melted agar media at 43°C. Where specified, 0.4 ml of antibiotic solution (benzyl penicillin, 2×10^4 IU/ml and streptomycin sulfate, 2 mg/ml) was added to tubes (1).

Rumen fluid, used as a source of fungi, was freshly collected from a sheep fed lucerne chaff and filtered through a layer of muslin. All cultures were incubated under a CO_2 atmosphere at 39°C. Fungal thalli and colonies were photographed in situ in roll tubes, using either a dissecting or an inverted microscope. Live zoospores were photographed by bright-field microscopy.

The number of fungi in strained rumen fluid was determined by diluting samples from the stirred solution through roll tubes containing antibiotics. During incubation, the tubes were regularly inspected under a dissecting microscope. At 6 h after inoculation, discrete fungal thalli of the types shown in Fig. 1B, 2B, and 3B were detected. A count of the thalli present after 20 h indicated that the rumen fluid sample contained 2×10^4 viable zoospores per ml. This is in good agreement with the zoospore population density of 10^4 to 10^5/ml for the anaerobic phycomycetes *Neocallimastix frontalis*, *Sphaeromonas communis*, and *Piromonas communis* in the rumen as measured by a microscopic counting procedure (4-6). Also, zoospores were enumerated in roll tubes containing glucose or carboxymethyl cellulose. A comparison of the enumeration results from a sample of rumen liquor, using different carbon sources, showed that in glucose-containing tubes the number of zoospores detected was approximately twice that

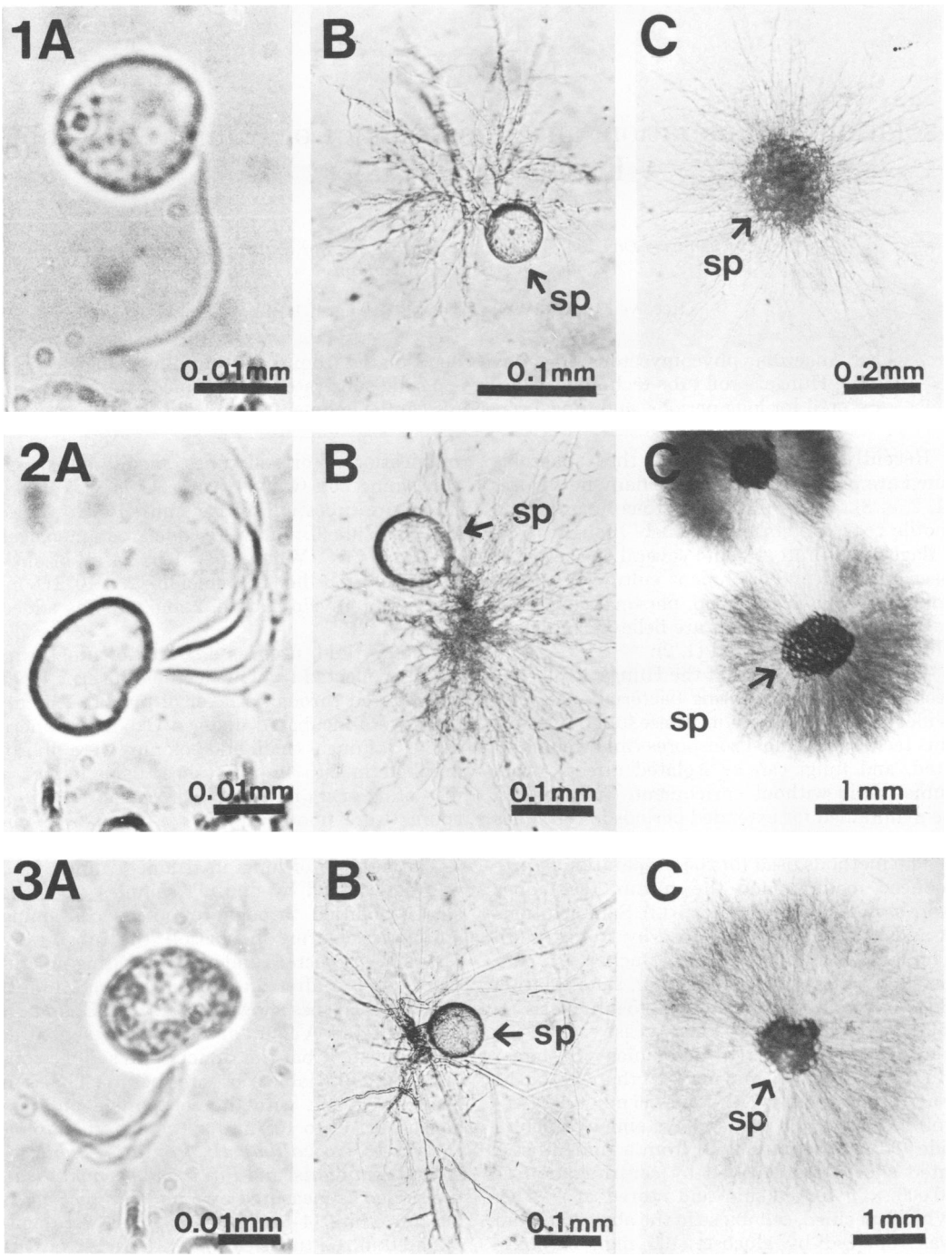


FIG. 1. *Growth stages of rumen phycomycete K1: A, live zoospore; B, fungal thallus in a cellobiose roll tube 24 h after inoculation; C, fungal colony in a cellobiose roll tube 48 h after inoculation. Sporangia (sp) are as indicated.*

FIG. 2. *Growth stages of rumen phycomycete K2. A, B, and C are as for Fig. 1.*

FIG. 3. *Growth stages of rumen phycomycete K3. A, B, and C are as for Fig. 1.*

found in tubes containing either cellobiose or carboxymethyl cellulose. Reproducible results were obtained from tubes containing a particular carbon source. It appears that some zoospores failed to develop on cellobiose or carboxymethyl cellulose. Although zoospores can be enumerated, it should be noted that the relationship between the number of zoospores in the liquid phase and the number of phycomycetes associated with the plant material in the rumen (1, 7) has yet to be established. Fungi were not detected in roll tubes inoculated with rumen fluid when antibiotics were omitted.

In the roll tubes, thalli increased in size until 20 to 24 h after inoculation, when zoospores were released from the sporangia. After their release, zoospores (visible under the dissecting microscope) maintained their motility for 30 to 60 min in the immediate vicinity of the ruptured sporangium but were unable to penetrate the agar. Because the zoospores could not migrate far, at 40 to 50 h after inoculation, a colony of fungal thalli developed at each site of zoospore release. The colonies consisted of a cluster of sporangia surrounded by the rhizoids which extended out into the agar (Fig. 1C, 2C, and 3C). Since each colony developed from a single sporangium, the number of zoospores released per sporangium could be estimated by counting the sporangia in some of the colonies. This revealed that the rumen phycomycetes in the roll tubes released 10 to 120 viable zoospores per sporangium.

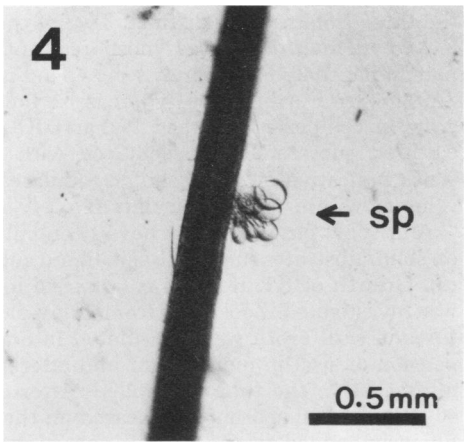
Over the next 2 days, further life cycles occurred within the colonies, but the individual colonies did not increase markedly in size. In an attempt to isolate specific fungi, three colonies (K1, K2, and K3), each of a different size (and appearance), were picked anaerobically and transferred into glucose broth (3). After 4 days, the cultures were transferred to sisal broth. To check the purity of isolates, zoospores from these cultures were inoculated into roll tubes. The differences in the size and the branching of the thalli which developed from K1, K2, and K3 (Fig. 1B, 2B, and 3B), respectively indicated that three different types had been obtained. This was confirmed by the distinct differences between the K1, K2, and K3 colonies which subsequently appeared (Fig. 1C, 2C, and 3C). Colonies in cultures of K1 were uniform in size and appearance as were colonies of K2. Roll tube cultures of K3 contained predominantly the large colonies (Fig. 3C) with a few smaller ones. Selection of either a small or a large K3 colony from a high-dilution tube continued to yield cultures producing large colonies with a few smaller ones. Examination of live zoospores, using bright-field microscopy, also confirmed that the three isolates were distinct. Two of the isolates had multiflagellated zoospores, and one had uniflagellated zoospore, as shown in Fig. 1A, 2A, and 3A. Furthermore, whereas K2 failed to grow in cellulose roll tubes, K1 and K3 were cultured successfully on cellulose with clear zones indicative of extracellular cellulases, developing within 40 h of inoculation.

Anaerobic phycomycetes have been cultured on solid substrates such as sisal in liquid cultures (2). Growth of K1, K2, and K3 on sisal in agar was investigated. Zoospores from 3-day-old cultures in sisal broth were inoculated into a suspension of sisal in melted agar and after 1 to 2 min at 43°C, the tube was rolled. After about 40 h, individual sporangia appeared on the sisal fibers imbedded in the agar. Upon rupture of the sporangia, many zoospores were seen in the agar, and, within 24 h, clusters of sporangia (Fig. 4) developed in the immediate vicinity of the invasion sites. (The generation time of 24 h for the life cycle of the phycomycetes is the same as that estimated for phycomycetes in the rumen [1].) The invasions remained very localized (Fig. 4) and did not spread along the fiber. Cultures on solid substrates in agar may thus prove useful as a source of samples for studies of fiber degradation by rumen phycomycetes.

A means of maintaining fungi without regular subculturing was sought, as anaerobic phycomycetes remain viable for only 2 to 5 days in glucose broth (5–7) and 7 to 10 days in sisal broth (Bauchop, personal communication). Cultures in broth or on agar with and without glycerol as a cryoprotectant failed to retain viability after storage at low temperature (−60°C). However, apparently senescent cultures on sisal agar which had been stored at 39°C could be reestablished by the addition of glucose. Glucose broth (5 ml) was added, and the tube was shaken vigorously to mix the agar and liquid phases. After 2 to 3 days of incubation, the culture was actively growing again. This provided a useful method of maintaining cultures of anaerobic fungi without the frequent subculturing previously required. Cultures of the three isolates K1, K2, and K3 remained viable in sisal roll tubes at 39°C for up to 7 months.

Nothing is known about the pattern of sexuality in anaerobic fungi, so it is noteworthy that, occasionally, thalli of K3, already bearing a sporangium, developed swellings at the tips of the hyphae (Fig. 5). If these structures are reproductive organs of the type found in some aquatic phycomycetes (9), it would mean that the rumen phycomycete K3 is homothallic.

I thank T. Bauchop for advice and encouragement and M. Broadhurst for valuable technical assistance.



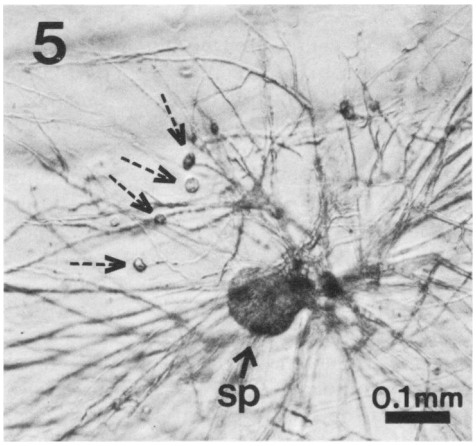


FIG. 4. *Rumen phycomycete K2 on a sisal fiber in a roll tube 60 h after inoculation. Sporangia (sp) are as indicated.*

FIG. 5. *Thallus of rumen phycomycete K2 in a cellobiose roll tube. Sporangia (sp) and swellings at the tips of the hyphae are as indicated.*

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