Isolation of Cellulolytic Phycomycete Fungi from the Caecum of the Horse

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Microscopic examination of horse caecum contents revealed vegetative growth of phycomycete fungi on particles of digesta, and uniflagellated cells similar to fungal zoospores in the liquid phase. Three morphologically distinct isolates of strictly anaerobic phycomycete fungi were obtained from the caecum contents and cultured in vitro. Two of the isolates were able to utilize a wide range of plant carbohydrates for growth, including α-cellulose, xylan and particulate starch, and extensively digested water-insoluble plant tissues.

INTRODUCTION

In the horse, the caecum and colon are much enlarged relative to the other parts of the alimentary tract, and in them occurs an extensive fermentation of cellulose and other materials by bacteria and protozoa (Hungate, 1966; Elsden et al., 1946) with the subsequent production of short-chain fatty acids which are absorbed by the host animal (Barcroft et al., 1944). Little is known concerning the micro-organisms inhabiting the horse caecum and colon, and although many of the protozoa have been characterized (Hsuing, 1930; Ozeki et al., 1973) other organisms have been neglected. Some other herbivorous non-ruminant mammals, such as the guinea-pig and rabbit, also have an enlarged caecum and large intestine containing large numbers of bacteria and flagellate protozoa which perform a similar function to those in the horse caecum.

Fungi are normally regarded as obligate aerobes (Cochrane, 1958) unable to survive for extended periods in the absence of oxygen unless capable of facultative fermentation. This occurs in some yeast species such as Saccharomyces cerevisiae (Marchant & Smith, 1968), and some aquatic phycomycetes such as Aqualinderella fermentans (Emerson & Held, 1969) and Blastocladiella spp. (Emerson & Cantino, 1948; Held et al., 1969) which are obligately fermentative but not killed by exposure to oxygen. The discovery that three species of oxygen-sensitive aquatic phycomycete fungi live under strictly anaerobic, low-redox-potential conditions in the ovine rumen (Orpin, 1975, 1976, 1977a, b) prompted a search for other anaerobic species occurring in similar anoxic environments rich in organic matter, such as the caecum of the horse. In this organ earlier workers have identified uniflagellated single-cellular organisms morphologically similar to the zoospores of the anaerobic rumen phycomycetes Sphaeromonas communis and Piromonas communis (Liebetanz, 1910), which are partially saprophytic on the plant tissue in the rumen digesta. In addition, a multiflagellated organism similar to the rumen phycomycete Neocallimastix frontalis was observed and named Callimastix equi (Hsuing, 1930), later Neocallimastix equi (Vavra & Joyon, 1966). Hsuing (1930) believed both morphological types to be flagellate protozoa, but in view of the recent information concerning the rumen phycomycetes a re-examination of the horse flagellates was indicated.

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Table 1. Media used to culture anaerobic phycomycetes from caecum contents of the horse

All quantities are given in g per 100 ml final volume, except calf serum, autoclaved centrifuged rumen fluid (CRF) and autoclaved centrifuged horse caecum fluid (CHCF), which are in ml per 100 ml final volume. All the media also contained (per 100 ml): agar, 0.1 g; L-cysteine hydrochloride, 0.02 g; streptomycin sulphate, 0.1 g; sodium benzylpenicillin, 10000 I.U.; resazurin 0.001 g. They were dispensed and used under CO₂.

<table>
<thead>
<tr>
<th>Component</th>
<th>C1</th>
<th>C2</th>
<th>S1</th>
<th>S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>2.0</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.0</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Calf serum (inactivated)</td>
<td>10.0</td>
<td>10.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CRF</td>
<td>10.0</td>
<td></td>
<td>10.0</td>
<td>-</td>
</tr>
<tr>
<td>CHCF</td>
<td>-</td>
<td>10.0</td>
<td>-</td>
<td>10.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>-</td>
<td>-</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>-</td>
<td>-</td>
<td>0.65</td>
<td>0.65</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>-</td>
<td>-</td>
<td>0.64</td>
<td>0.64</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>-</td>
<td>-</td>
<td>0.64</td>
<td>0.64</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>-</td>
<td>-</td>
<td>0.006</td>
<td>0.006</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>-</td>
<td>-</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

METHODS

Horse caecum contents. Contents of three horse caeca were removed as soon as possible after the animals were killed by captive bolt, and placed in separate vacuum flasks for transport to the laboratory. Each sample was filtered through one layer of muslin and maintained at 39 °C under CO₂. Some of the filtered caecum fluid was then centrifuged at 38000 g for 30 min; the supernatant fluid was autoclaved (115 °C, 20 min) and designated 'centrifuged horse caecum fluid'.

Rumen fluid. Ovine rumen fluid was taken by aspiration through a permanent rumen cannula 19 h after the sheep was fed a diet of 1 kg hay and 100 g rolled oats. It was filtered, centrifuged and autoclaved as described for the horse caecum fluid, and designated 'centrifuged rumen fluid'.

Culture media. Four different anaerobic semi-solid media were employed, each supplemented as required with barley (Hordeum distichon) awns or leaf tissue. The media were based on those employed for the culture of rumen phycomycetes (Orpin, 1975, 1976) and incorporated either autoclaved centrifuged ovine rumen fluid or autoclaved centrifuged horse caecum fluid. The composition of the media is shown in Table 1. All media were prepared as described for the rumen fluid-containing media (Orpin, 1977a) and dispensed aseptically in 12.5 x 1.2 cm rimless test tubes under CO₂. Liquid media were prepared as above, with the omission of the agar.

Chitin and cellulose determination. Chitin and cellulose were determined qualitatively by their staining reactions with iodine in sulphuric acid (Rogers & Perkins, 1968) and by dissolving chitin in sodium hypochlorite (Aronson & Preston, 1960).

Digestion of plant tissues by fungal isolates. Leaf tissues taken from a mixed grass sward were dried (80 °C for 24 h) and milled. A temperature of 80 °C was employed to provide millable material without interfering too much with the digestibility of the tissues. The fraction passing through a 2 mm sieve but not a 1 mm sieve was retained. A weighed portion was dried at 106 °C and the dry weight was determined. Quantities of the milled grass (50 mg dry wt) were placed in weighed culture tubes and 9 ml liquid medium S₁, minus glucose, was added. Each tube was inoculated with 1 ml supernatant fluid from a 48 h culture of the isolate, grown on the same plant particles, and was incubated under CO₂ at 39 °C for 93 h. The supernatant was removed and the remaining plant tissue was washed three times with distilled water, dried at 106 °C for 24 h, and weighed. The tissue was analysed by the method of Thornber & Northcote (1961), and its composition was compared with that of control tissue incubated in uninoculated culture tubes.

Determination of growth. Growth was determined by correlation of lipid phosphorus with dry weight (Rouser et al., 1970; Bucholtz & Bergen, 1973; Ohki, 1972).

RESULTS

Microscopic examination of horse caecum fluid

Direct microscopic examination of whole caecum fluid was impossible due to the large quantities of food particles present. Examination of caecum fluid after straining through one layer of muslin was possible, and the fluid appeared normal, as judged by the intactness and motility of the ciliate protozoa present.
Uniflagellated cells similar to those described as *Oikomonas equi* (Hsuing, 1930) were present in all samples of the caecum fluid at population densities in the range $3.2 \times 10^4$ to $4.7 \times 10^4$ ml$^{-1}$. These cells were more refractile than would be expected for flagellate protozoa, and their motility was reminiscent of zoospores of phycomycete fungi. No *Neocallimastix equi* (Hsuing, 1930; Vavra & Joyon, 1966) were seen, but plant tissues showing probable phycomycete vegetative growth (Fig. 1, 2) were abundant.

**Isolation of phycomycetes from horse caecum fluid**

Caecum fluid filtered through muslin (0.3 ml) was overlaid on media C1, C2, S1 and S2 and the head spaces in the tubes were gassed with CO$_2$. The inoculum was mixed, by gentle agitation of the tube, into the top 1 cm of medium, and the inoculated tubes were incubated at 39 °C. Growth of phycomycetes morphologically similar to *Piromonas communis* (Orpin, 1977a) occurred in media C1 and C2, and vegetative growth similar to that of *Sphaeromonas communis* (Orpin, 1976) was visible within 24 h in all the culture tubes, together with many uniflagellated zoospores. By successive transfer to fresh culture medium containing barley awn or barley leaf tissues, three morphologically distinct isolates, free of other flagellates, were obtained; these were designated H1, H2 and H3. Successive transfers through fresh medium resulted in axenic cultures. No phycomycete growth was detected in isolation tubes inoculated with the diet of the host animal.

**Isolate H1.** This isolate grew well in all four media and it invaded and grew upon barley leaf tissue suspended in the same media. Little invasion of barley awns occurred under the same conditions. If the glucose was omitted from medium S1 or S2, growth was maintained on the barley leaf tissue, with no vegetative growth free in the surrounding medium. In the absence of plant tissue, no growth occurred if glucose or centrifuged rumen or horse caecum fluid was omitted.
Table 2. Dimensions and characteristics of phycomycete fungi isolated from the horse caecum compared with those of Piromonas communis and Sphaeromonas communis from the ovine rumen

The dimensions of the flagellate stages are the average of at least 50 measurements. The data for *P. communis* and *S. communis* are from Orpin (1976, 1977a).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Cell</th>
<th>Flagellum</th>
<th>Maximum dimensions of sporangium (µm)</th>
<th>Resting spores formed</th>
<th>Spherical bodies formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>7.3 x 14.9</td>
<td>32.6</td>
<td>78 x 95</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>H2</td>
<td>9.4 x 21.9</td>
<td>36.4</td>
<td>115 x 108</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H3</td>
<td>Diam. 6.9</td>
<td>27.5</td>
<td>90 x 73</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>P. communis</em></td>
<td>7.1 x 14.6</td>
<td>28.8</td>
<td>95 x 49</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. communis</em></td>
<td>Diam. 7.95</td>
<td>24.9</td>
<td>95 x 64</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

All the photographs were taken under phase-contrast illumination. The bar markers represent 20 µm in Figs 3 and 4 and 50 µm in Figs 5, 6 and 7. F, Flagellum; S, sporangium; R, rhizoid; G, germling.

Fig. 3. Flagellate stage of isolate H1. Living preparation.

Fig. 4. Flagellate stage of isolate H1, showing amoeboid movement. The flagellum is not in the same plane as the cell. Living preparation.

Fig. 5. Spherical bodies, possibly resting cysts, on the wall of the culture tube containing isolate H1. Living preparation.

Fig. 6. Normal vegetative stage of isolate H1. Fixed with 2% formaldehyde.

Fig. 7. Germination of encysted zoospore of isolate H2. Fixed with 2% formaldehyde.
The zoospores of H1 were spherical when resting (Fig. 3, Table 2) but during periods of motility became ovoid to elliptical and were similar to the uniflagellated cells observed in the filtered caecum fluid. They were frequently observed undergoing amoeboid movement (Fig. 4). After 2 to 3 d in culture, highly refractile spherical bodies, 13.6 to 20.2 μm in diameter (average 17.7 μm; n = 20) (Fig. 5), were found on the walls of the culture tube. Since the cultures contained H1 as the sole phycomycete and were free of other eukaryotes, it was deduced that these bodies developed from the motile zoospores, forming an encysted resting state.

The vegetative stage of H1 consisted of a single sporangium, ovoid to elongate but sometimes irregular in shape (Fig. 6, Table 2) borne on a single, much-branched rhizoid. When mature, the sporangium released the zoospores into the surrounding medium, where they subsequently germinated. Growth of the vegetative stage on barley leaves occurred with the penetration of the plant tissue by the rhizoids and a concomitant decrease in optical density in the tissue. After 3 d growth, disintegration of the plant tissue occurred.

Since growth on plant tissue resulted in its disintegration, growth was tested in medium S1 with the glucose replaced (at 0.1%, w/v) by one of a range of soluble and insoluble plant carbohydrates. Growth occurred on fructose, galactose, sucrose, maltose, cellobiose, soluble starch, particulate rice starch, α-cellulose, xylan and pectin.

Isolate H2. This isolate grew only in media C1 and C2. The vegetative stage occurred free in the medium and invasion of plant tissue was not demonstrated. Growth was weak and maintained for only 6 weeks, and did not occur if serum, centrifuged rumen or horse caecum fluid, yeast extract, or L-cysteine was omitted.

The posteriorly uniflagellated zoospore was ovoid to spherical, the cell being nearly twice the size of the zoospore of H1 (Table 2). No amoeboid movement was observed. The zoospore encysted and germinated (Fig. 7) with the growth of a single much-branched, non-septate rhizoid. The encysted zoospore developed into a nearly spherical sporangium, that was often obscured by the rhizoid, and was larger and more robust than that of H1 (Fig. 8). When mature the zoospores were liberated into the medium (Fig. 9).

Isolate H3. This isolate grew poorly in media C1 and C2, and better in media S1 and S2. Growth was strong on both barley awn and leaf tissue suspended in media S1 and S2.

The flagellate stage was similar to Sphaeromonas communis (Table 2) in size, morphology and amoeboid movement. Germination of the flagellate in liquid medium resulted in the formation of a short thallus on which developed a single sporangium (Fig. 10) and up to ten approximately spherical bodies up to 120 μm in diameter. These bodies readily collapsed under the coverslip during microscopic examination; their function is unknown.

No growth occurred in medium S1 or S2 if the glucose was omitted, but growth did occur in the presence of sucrose, fructose, cellobiose, maltose, xylose, soluble starch and particulate rice starch, xylan, pectin, gum tragacanth and α-cellulose.

Growth on barley awn tissue proceeded with the development of a clump of vegetative growth (Fig. 11, 12). As the growth aged, the spherical bodies became obvious and were extruded into the surrounding medium. Invasion of the awn tissue was principally near the lateral spikes, and where the epidermis was damaged. Considerable disintegration of the tissue occurred after 2 to 4 d growth (Fig. 13). No growth occurred if centrifuged rumen fluid or L-cysteine was omitted from the culture media.

Tests for chitin and cellulose

The walls of all the strains showed a positive result for chitin and a negative result for cellulose. Isolates H1 and H2 showed the deepest staining with iodine, staining deep brown. Isolate H3 stained yellow–brown but showed partial dissolution in sodium hypochlorite (positive for chitin at low concentrations), a result similar to that obtained for Sphaeromonas communis (Orpin, 1977b).
Figures 8, 9 and 10 were taken under phase-contrast, and Figs 11, 12 and 13 under bright-field illumination. Except where stated otherwise, the preparations were fixed with 2% formaldehyde. The bar markers represent 100 μm. S, Sporangium; R, rhizoid; Z, zoospores; B, spherical body.

Fig. 8. Vegetative stage of isolate H2.

Fig. 9. Zoospore liberation from the sporangium of isolate H2. The sporangium itself is obscured by the liberated zoospores. Living preparation.

Fig. 10. Vegetative stage of isolate H3. Note how the spherical bodies have collapsed under the coverslip. Living preparation.

Fig. 11. Barley awn tissue colonized by isolate H3.

Fig. 12. Clump of isolate H3 on barley awn tissue, showing the sporangia and spherical bodies and the decrease in density (arrowed) of the particle in the region of the growth.

Fig. 13. Barley awn tissue after growth of isolate H3 for 4 d.
Fig. 14. Digestion of mixed grass particles by isolates HI (●) and H3 (▲). Duplicate tubes containing milled dried grass (50 mg dry wt) in glucose-free medium S1 (9 ml) were each inoculated with 1 ml of a culture of H1 or H3 and incubated under CO₂ for up to 93 h at 39 °C. The particulate material was analysed at intervals during the incubation. The results are the average of three experiments.

Table 3. Digestion of components of plant tissues by isolates H1 and H3 in vitro

For each isolate, 20 culture tubes containing milled dried grass (50 mg dry wt) in glucose-free medium S1 (9 ml) were each inoculated with 1 ml of a well-grown culture and incubated under CO₂ for 93 h at 39 °C. The particles were then bulked and analysed, and their composition was compared with control material incubated under the same conditions but not inoculated. The results are the average of three experiments.

<table>
<thead>
<tr>
<th>Tissue component</th>
<th>Present in control (mg)</th>
<th>Digested by H1 (%)</th>
<th>Digested by H3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectin</td>
<td>101</td>
<td>48·5</td>
<td>57·4</td>
</tr>
<tr>
<td>Xylan</td>
<td>64</td>
<td>96·9</td>
<td>89·1</td>
</tr>
<tr>
<td>Lignin</td>
<td>96</td>
<td>11·5</td>
<td>16·7</td>
</tr>
<tr>
<td>Hemicellulose I</td>
<td>264</td>
<td>53·0</td>
<td>42·4</td>
</tr>
<tr>
<td>Hemicellulose II</td>
<td>32</td>
<td>68·5</td>
<td>62·5</td>
</tr>
<tr>
<td>Cellulose</td>
<td>260</td>
<td>38·5</td>
<td>33·1</td>
</tr>
</tbody>
</table>

Culture experiments

Effect of inhibitors. Complete inhibition of growth of all isolates occurred when puromycin or cycloheximide was incorporated into culture media at 10⁻⁴ M. No inhibition occurred with sodium selenate (10⁻³ M), rotenone (10⁻⁴ M) or 2-n-heptyl-4-hydroxyquinoline-N-oxide (10⁻⁴ M). These results confirm that the organisms are eukaryotic (cycloheximide inhibition) and that respiration by sulphate or nitrate reduction is not operative.

Growth in liquid media. Isolates H1 and H3 both grew in liquid media provided barley leaf tissue (H1) or awn tissue (H3) was present. In both isolates the vegetative growth was principally on the plant tissue and a little on the walls of the culture tube. Attempts were made to substitute plant tissue extracts or nylon or cotton mesh for the plant tissue without success.

Digestion of plant tissues by isolates H1 and H3. Both isolates were capable of extensive degradation of milled grass particles, including degradation of the major structural polysaccharides, cellulose and hemicellulose. The loss of dry weight of the insoluble plant tissues is shown in Fig. 14; it was slightly greater with H1 than H3. The plant particles were analysed at the end of the experiment, and the results are shown in Table 3. Both isolates digested pectin, xylan, hemicelluloses and cellulose in the tissues. H1 was more effective than H3 in digesting the major plant tissue components hemicellulose I and cellulose.
Oxygen relations. No phycomycete vegetative growth or flagellates were observed in any of the media inoculated with horse caecum fluid through which air had been bubbled for 5 min. If the culture media were allowed to oxidize (using resazurin as a redox indicator) or if L-cysteine was omitted, no growth of any of the isolates occurred after inoculation with a thriving culture, and if actively growing cultures were exposed to air at 39 °C until the resazurin was oxidized, death of the cultures ensued.

Vegetative growth on leaf tissue (H1) or awn tissue (H3) was killed by immersion in air-saturated L-cysteine-free culture medium for 5 min, as demonstrated by the inability of the organisms to grow after being returned to fresh L-cysteine-containing medium in which the resazurin was reduced. If 0.01% L-cystine was added to cultures, to simulate the oxidation of L-cysteine, the cultures continued to grow well, indicating that L-cystine itself was not toxic to the organisms. No growth occurred if the L-cysteine was replaced by 0.02% (w/v) L-methionine, 0.02% (w/v) sodium thioglycollate or 0.02% sodium thioglycollate and 0.02% methionine, suggesting that the L-cysteine was required to maintain a low redox potential as well as being a source of sulphur.

DISCUSSION

The morphology, cycloheximide sensitivity and presence of chitin in the cell walls of all the isolates confirms that they are true fungi, despite their inability to grow in oxidized environments or in reduced environments after a short exposure to air. On a morphological basis, all are probably members of the fungal class Phycomycetes (Sparrow, 1960) which possess similar life histories alternating between the motile flagellated (zoospore) stage and the vegetative reproductive stage. All the isolates had a flagellate stage which possessed a single, posterior, flagellum, and monocentric, holocarpic vegetative stages, suggesting that they are members of the Chytridiales. However, further nutritional and structural examinations need to be completed before their taxonomic positions can be determined precisely.

Isolate H1 was indistinguishable in the flagellate and vegetative stages from the rumen phycomycete 

*Piromonas communis*, but the preference of H1 flagellates for invasion of leaf tissue contrasted with the preference of *P. communis* flagellates for inflorescence tissues (Orpin, 1977a). The mechanism of tissue selection in H1 is not known. It seems unlikely that it is related to soluble carbohydrate levels in the different tissues [which controls substratum selection in the rumen phycomycete *Neocallimastix frontalis* (Orpin & Bountiff, 1978)], for little soluble carbohydrate is likely to be present in the tissues after transit through the stomach, abomasum and small intestine of the host animal. Tissue selection in H3 may also be by a mechanism other than chemotaxis to soluble carbohydrates. Isolate H1 also differed from *P. communis* by the production *in vitro* of what appeared to be an encysted resting stage. The status of these ‘cysts’ is not known; their germination has not yet been initiated *in vitro*, it has not been possible to conduct the experiment of infecting horses with them, and it is not known if they are formed from a single zoospore or after the fusion of two (or more) zoospores. Adam (1951) did not observe encysted organisms in the faeces of horses, and concluded that transfer from animal to animal was by way of viable cells in the faeces. The cysts of H1 are, however, small relative to the cells of ciliate protozoa and may have been unnoticed in her experiments.

None of the multiflagellated *Neocallimastix equi* observed by Hsuing (1930) and Vavra & Joyon (1966) were seen in the horse caecum fluid or in the primary cultures. No conclusions can therefore be made regarding the taxonomic status of this flagellate, which is similar to the flagellates of *N. frontalis* and *Callimastix cyclops* (Vavra & Joyon, 1966).

Since no anaerobic phycomycetes could be isolated from the diet of the host animals, and apparently similar flagellates were present in the caecum fluid of the host animal, it is likely that all the isolates are true caecum inhabitants. This view is strengthened by the presence of
typical phycomycete growth on plant particles in the horse caecum, and their requirement for strict anaerobiosis.

The demonstration that at least two species of caecal phycomycete (isolates H1 and H3) are capable of digesting plant cellulose and hemicellulose indicates that the digestion of plant cell walls is their primary niche in the caecum. Although they are both capable of utilizing soluble carbohydrates, these are unlikely to be present in caecal contents in high concentration after the diet has passed through the abomasum and small intestine of the host animal. The quantitative significance of these organisms in the nutrition of the host animal remains to be elucidated.

Since anaerobic phycomycetes have now been demonstrated to occur in the rumen and in the caecum of the horse, it is likely that a search of other anoxic, low-redox-potential environments rich in organic matter would yield more species. Such environments include the forestomachs of marsupials (Moir et al., 1956), the caeca of rodents, lagomorphs and elephants (Hungate, 1966) and the hind gut of termites.

I thank Dr P. Kemp for cannulating the sheep used as a source of rumen fluid.

REFERENCES


