SHORT COMMUNICATIONS

The Occurrence of Chitin in the Cell Walls of the Rumen Organisms *Neocallimastix frontalis*, *Piromonas communis* and *Sphaeromonas communis*

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INTRODUCTION

The rumen organisms *Neocallimastix frontalis*, *Sphaeromonas communis* and *Piromonas communis* resemble phycomycete fungi in the morphology of their vegetative stages, reproduction and (with the exception of *Neocallimastix frontalis*) the motile flagellated stage (Orpin, 1975, 1976, 1977*b*). The flagellate of *N. frontalis* has up to 14 flagella, whereas not more than two were recorded by Sparrow (1960) for members of the Phycomycetes. Vavra & Joyon (1966), however, identified *Callimastix cyclopis*, a species with a flagellate stage possessing two to nine flagella, as a member of the phycomycete order Blastocladales. The flagellates of *N. frontalis* and *P. communis* invade plant tissues ingested by the host animal (Orpin 1977*a, b*) and the vegetative stage of both species grows at the expense of this tissue; they may also grow freely in the rumen fluid (Orpin, 1975).

After the liberation of the flagellates of *N. frontalis*, the sporangium wall and rhizoid walls remain intact in rumen fluid at 39 °C for 3 h *in vitro*, and for up to 24 h *in vivo*. This resistance to degradation, and the fact that many phycomycetes have chitin in their cell walls (Aronson & Preston, 1960) and many others may have cellulose (Parker, Preston & Fogg, 1963), prompted examination of the rumen organisms for the presence of chitin and cellulose. The finding of either compound would determine conclusively that these organisms were true fungi, since, amongst the non-photosynthetic micro-organisms, chitin and cellulose are found only in fungi (Rogers & Perkins, 1968).

METHODS

Organisms were those isolated by Orpin (1975, 1976, 1977*b*) and were cultured similarly. The volume of medium in cultures was increased to 100 ml contained in 8 oz (225 ml) 'medical flat' glass bottles. For *P. communis* culture medium II was used. After 48 h of growth (for *N. frontalis* and *P. communis*) or 96 h (for *S. communis*) the cultures were shaken vigorously by hand and centrifuged at 2500 *g* for 20 min. The pellet contained the vegetative growth together with adhering agar and flagellates.

Isolation of cell walls of vegetative growth. The pellet from 200 ml culture medium was suspended in 10 ml salts solution (Hofsten & Malmqvist, 1975) and incubated aerobically at 37 °C with the agarolytic bacterium NCMB1914 [0.1 g wet wt (ml pellet vol.)⁻¹] grown according to Hofsten & Malmqvist (1975). After 2 h incubation, the vegetative growth was removed by centrifuging (1000 *g* for 5 min at 4 °C) and the bacteria and flagellates were

removed by repeated centrifuging in 0.9 % (w/v) NaCl under the same conditions. The vegetative cells were ruptured by exposure to ultrasonic waves (MSE-Mullard ultrasonic disintegrator, 160 W) and the walls were sedimented at 1000 g for 30 min; the pellet was washed three times by centrifuging in distilled water, freeze-dried and stored at -20°C .

Cell walls were treated with aqueous KOH (saturated solution at 22°C) to remove the majority of the constituents, leaving any chitin or cellulose intact, by heating at 160°C for 20 min with 5 ml KOH (mg dry wt cell walls) $^{-1}$ (Rogers & Perkins, 1968). The undissolved material was sedimented at 2500 g for 5 min, and washed by centrifuging three times in distilled water.

Determination of chitin and cellulose. Chitin and cellulose were determined qualitatively by their iodine staining reactions in sulphuric acid (Rogers & Perkins, 1968). Freeze-dried vegetative growth was reconstituted in distilled water and stained with Lugol's iodine; chitin is stained brown, whereas cellulose is not stained. The supernatant fluid was removed by pipette, and replaced with 1 % (v/v) H_2SO_4 ; chitin is stained red-violet under these conditions, cellulose remains unstained. The supernatant was again removed and replaced with 75 % (v/v) H_2SO_4 ; under these conditions chitin dissolves, and cellulose is stained blue and swells.

Chitin was determined quantitatively using the method of Tracey (1955) by estimating *N*-acetylglucosamine released from the walls by chitinase (from *Streptomyces griseus*; Sigma).

Cellulose was determined quantitatively using cellulase from *Aspergillus niger*, following the methods of Pettersson & Porath (1966).

Sodium hypochlorite treatment was conducted according to Aronson & Preston (1960); this completely solubilizes chitin, but not cellulose.

RESULTS AND DISCUSSION

The qualitative tests (Table 1) indicated that the walls of all three species did not contain cellulose, but did contain chitin. The walls of *N. frontalis* showed the most intense response, staining deeply with iodine and losing a considerable amount of refractivity on treatment with 75 % H_2SO_4 . Under the same conditions, the walls of *P. communis* also lost refractivity, with complete dissolution at the junction of the rhizoid and sporangium. In *S. communis* no dissolution of the walls in 75 % H_2SO_4 was seen even though a positive iodine staining reaction occurred. Sodium hypochlorite treatment completely dissolved the alkali-extracted walls of *N. frontalis* within 2 min; those of *P. communis* showed partial dissolution with the sporangium becoming detached from the rhizoid. No significant decrease in the refractivity of *S. communis* walls was observed.

Treatment of wall preparations from all three species with chitinase resulted in a loss of weight and release of *N*-acetylglucosamine; most was released from alkali-treated material (Table 1). Treatment of wall preparations with cellulase did not cause release of soluble reducing compounds or change of weight, suggesting that cellulose was absent. Walls were treated with chitinase followed by cellulase in case chitin was protecting any cellulose from the action of cellulase. However, no cellulose was detected using this procedure. In addition, the walls of *N. frontalis* and *P. communis* showed great changes in refractivity after chitinase but not cellulase treatment.

These results provide conclusive evidence for the presence of chitin in the walls of *N. frontalis*, *P. communis* and *S. communis*. It is not surprising that cellulose was not detected, since the rumen is a highly cellulolytic environment (Hungate, 1966). The presence of chitin in

Table 1. *Qualitative and quantitative determinations of chitin and cellulose in the walls of the vegetative stages of N. frontalis, P. communis and S. communis*

Treatment	<i>N. frontalis</i>	<i>P. communis</i>	<i>S. communis</i>
Staining with Lugol's iodine (1)*	B	B	B
plus 1 % H ₂ SO ₄	DB	B	B
plus 75 % H ₂ SO ₄	±, YB	±, YB	—, YB
Alkaline sodium hypochlorite (1)	+	±	—
Weight of wall released as <i>N</i> -acetylglucosamine during chitinase treatment (%) (1)	86	23	18
(2)	61	19	10
Weight of wall released as glucose during cellulase treatment (%) (1)	3	—3	2
(2)	4	2	3

B, Brown; DB, dark brown; YB, yellow-brown. +, Dissolution; ±, partial dissolution; —, no apparent dissolution.

* (1) Alkali-extracted walls derived from (2) walls isolated after ultrasonic disruption.

these organisms confirms previous suggestions (Orpin, 1975, 1976, 1977a) based on morphological criteria and antibiotic sensitivities, that they are true fungi despite their ability to grow under conditions of low redox potential and in the absence of oxygen. Fungi with morphological characteristics and life-histories similar to *N. frontalis*, *S. communis* and *P. communis* have been classed as aquatic Phycomycetes (Sparrow, 1960); the flagellates released from the sporangia being correctly termed zoospores. Within this group, provision has been made only for species with zoospores possessing not more than two flagella. The zoospore of *N. frontalis* possesses up to 14 flagella but its vegetative stage strongly resembles that of members of the Chytridiales which have uniflagellated zoospores. *Piromonas communis* exhibits characteristic chytridiale morphology in both the vegetative and flagellate stages. *Sphaeromonas communis* has a more limited rhizoidal system, but its possession of posterior-uniflagellated zoospores suggests that it, too, may be a member of the Chytridiales. The walls of the Chytridiales frequently contain chitin (Sparrow, 1960).

Chitin is extremely resistant to degradation by micro-organisms, but may be attacked aerobically by certain bacteria including *Achromobacter* sp. (Bergey's *Manual of Determinative Bacteriology*, 1974) and fungi (Sparrow, 1960). It is not known to be digested by ruminants, and to the author's knowledge, no organism capable of chitin degradation has been isolated from the rumen. If chitin is not digested in the rumen, chitin synthesis must result in a net loss of energy to the host animal. It has not yet proved possible to determine the total synthesis of chitin in rumen fluid enzymically due to interference by the food particles, but the overall effect on rumen metabolism is probably not great due to the relatively low population density (up to 3.6×10^4 ml⁻¹) of the chitin synthesizing vegetative stages of the three organisms.

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