Characterization of an anaerobic fungus from llama faeces

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An anaerobic fungus was isolated from llama faeces. Based on its morphological characteristics, polyflagellated zoospores, extensive rhizoid system and the formation of monocentric colonies, the fungus is assigned to the genus *Neocallimastix*. *Neocallimastix* sp. L2 is able to grow on several poly-, oligo- and monosaccharides. It differs from other *Neocallimastix* isolates in its inability to ferment inulin. *Neocallimastix* sp. L2 requires CO₂ for growth. In the presence of 100% CO₂ in the gas phase glucose is fermented to H₂, CO₂, formate, acetate, lactate, succinate and ethanol (33.8, 15.4, 74.3, 69.2, 26.7, 8.2, and 28.7 mmol per 100 mmol glucose, respectively). Reduced sulphur compounds can be used as sulphur source and ammonium or amino acids as nitrogen source. The temperature range for glucose fermentation is from 37 to 42 °C with an optimum of around 38 °C. The pH range for glucose fermentation is from pH 6 to pH 8 with a broad optimum between pH 6.5 and pH 7.5. The zoospores of *Neocallimastix* sp. L2 contain ribosomal 'globules' and hydrogenosomes. In the kinetosomes of the zoospores spurs, scoops and skirts are visible. In both the rhizoids and the sporangia 'crystal bodies' and hydrogenosomes are present. Mitochondria were not detected in either of these life stages.

Introduction

Anaerobic fungi inhabit certain regions of the alimentary tract of many herbivorous mammals (Orpin & Joblin, 1988; Teunissen *et al.*, 1991). The importance of anaerobic fungi in herbivore digestion remains unclear, but their capacity to produce large amounts of fibre-digesting enzymes (Williams & Orpin, 1987) and their ability to digest up to half of the dry weight of plant tissue *in vitro* (Lowe *et al.*, 1987) suggest that they contribute significantly to plant fibre digestion in herbivores.

A considerable number of anaerobic fungi have been isolated from the alimentary tract, saliva and faeces of many different herbivores. In dried faeces anaerobic fungi can survive for several months, possibly in some sort of resistant form (Milne *et al.*, 1989). All anaerobic fungi have been assigned to the family of the *Neocallimasticaceae* of the Spizellomycetales (Chytridiomycetes) (Barr, 1988). Within this family six genera have been recognized: *Neocallimastix* (Heath *et al.*, 1983; Orpin, 1975; Orpin & Munn, 1986), *Piromyces* (previously *Piromonas*) (Breton *et al.*, 1991; Li *et al.*, 1990; Orpin, 1977a), *Caecomycetes* (previously *Sphaeromonas*) (Orpin, 1976; Gold *et al.*, 1988), *Anaeromyces* (Breton *et al.*, 1990), *Orpinomyces* (Barr *et al.*, 1989) and *Ruminomyces*

(Ho *et al.*, 1990), which differ from each other primarily in the number of flagella attached to their zoospores and in the appearance of their thallus. Other morphological characteristics, such as shape and size of sporangia and zoospores, have been used in the characterization of anaerobic chytridiomycetes (Barr *et al.*, 1989; Breton *et al.*, 1989, 1990, 1991; Lowe *et al.*, 1987; Orpin, 1975, 1976, 1977a; Wubah *et al.*, 1991).

The ultrastructure of several species of the genera *Neocallimastix*, *Piromyces* and *Caecomycetes*, and one species of the genus *Orpinomyces* (Li *et al.*, 1991), has been studied. Differences were found in the structure of the implanting of the flagella (flagellar pole) and the distribution of organelles in the zoospore (Gold *et al.*, 1988; Li *et al.*, 1990, 1991; Munn *et al.*, 1988).

Carbohydrate utilization and fermentation products have been studied for many strains of different genera (Breton *et al.*, 1989, 1990, 1991; Kostyukovsky *et al.*, 1991; Mountfort & Asher, 1985; Orpin, 1975, 1976, 1977b, Orpin & Letcher, 1979; Phillips & Gordon, 1988; Theodorou *et al.*, 1988).

Although intergeneric differences have been found in carbohydrate utilization patterns, such differences cannot be used in distinguishing between strains. The fermentation products H₂, CO₂, formate, acetate, lactate and ethanol were reported for all strains tested; formate production by *Neocallimastix patriciarum* is doubtful

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(Marvin-Sikkema *et al.*, 1990). Many strains also produced succinate (Breton *et al.*, 1989, 1990, 1991; Marvin-Sikkema *et al.*, 1990; Orpin & Joblin, 1988). Less attention has been paid to other physiological characteristics. Detailed studies of the ultrastructure and physiology of the anaerobic fungi are necessary to improve our understanding of the role of these fungi in their natural environment and their relationship towards each other.

In this paper we describe both metabolic and morphological characteristics of a *Neocallimastix* strain recently isolated from llama faeces (Marvin-Sikkema *et al.*, 1990) and discuss its relationship to other described isolates of anaerobic fungi.

Methods

Isolation and cultivation. *Neocallimastix* sp. L2 was isolated as described by Marvin-Sikkema *et al.* (1990) from freshly collected faeces from a llama (*Lama guanicoe glama*) that was kept at the Rowett Research Institute, Aberdeen, UK, and was fed a diet containing approximately, 50% hay, 30% barley grain, 9% fish meal, 10% molasses and a supplement (1%) of vitamins and minerals. The fungi were grown in a basal medium at 39 °C with 10 mM-glucose as the carbon and energy source (unless stated otherwise) as described before (Marvin-Sikkema *et al.*, 1990). The organism was subcultured every 2–3 d in order to avoid cell death. Stock cultures were preserved as described by Teunissen *et al.* (1991).

Zoospore isolation. A 1.5% (w/v) agar layer, containing basal medium with 10 mM-glucose, was made and solidified in a 500 ml serum bottle with a butyl rubber stopper. The agar was inoculated with 5 ml of a 48 h culture of *Neocallimastix* sp. L2, and after 3–4 d at 39 °C, 20 ml of basal medium with 20 mM-glucose was added. After 2 h at 39 °C the liquid medium, containing free zoospores, was collected.

Microscopy. Phase-contrast microscopy was performed with a Carl Zeiss G42-110-e Axioscope. For ultrathin-section electron microscopy, cells were grown for 24–48 h in basal medium without agar. Mycelium was collected by centrifugation (10 min, 200 g), washed with 0.1 M-sodium-cacodylate buffer (pH 7.2) and fixed for 1 h in 3% (v/v) glutaraldehyde in 0.1 M-sodium-cacodylate buffer (pH 7.2) at 0 °C. The cells were postfixed for 1 h in a solution of 1% (w/v) OsO₄ and 2.5% (w/v) K₂CrO₇ in the same buffer at room temperature and subsequently poststained in 1% (w/v) uranyl acetate. After dehydration in a graded ethanol series (70–100%) the cells were embedded in Epon 812 resin, sectioned with a diamond knife on an LKB microtome and examined in a Philips CM10 electron microscope. Zoospores were collected as described above and prefixed with glutaraldehyde (0.1%, v/v, in the medium) for 1 h at 4 °C. The prefixed zoospores were concentrated by centrifugation (5 min, 700 g) and subsequently postfixed, poststained and embedded as described above.

Growth requirements. Substrates were added to a final concentration of 60 mM-carbon. When growth with nitrogen sources other than ammonium was tested the following nitrogen-containing compounds were added to a final concentration of 5 mM-nitrogen: L-arginine, L-aspartate, L-methionine, L-cysteine, L-isoleucine, L-valine, L-tryptophan, L-glutamine, L-glutamate, L-glycine, L-phenylalanine, L-proline, L-histidine, L-serine, a mixture containing equal concentrations of the dipeptides Ala-Ala, Arg-Glu, Glu-Glu, His-Leu, Leu-Leu, Leu-Pro, Pro-Met and D-Ala-D-Glu, a mixture containing equal concentrations

of the tripeptides Ala-Val-Leu, Gly-Gly-Gly, Gly-His-Gly, Gly-Pro-Ala, Gly-Ser-Ala, His-Gly-Gly, His-Pro-Val, Ile-Pro-Ile, Pro-Gly-Gly, Pro-His-Val, Pro-Pro-Gly and Pro-Val-Asp, a mixture containing equal concentrations of the tetrapeptides Ala-Ala-Ala-Ala and Arg-Pro-Gly-Gly, a mixture containing equal concentrations of the pentapeptides Met-Met-Met-Gly-Gly and met-enkephalin (all mixtures obtained from Bachem Feinchemikalien AG, Bubendorf, Switzerland), the proteins bovine serum albumin or casein (nach Hammarsten), nitrate, methylamine, choline, or betaine. In these incubations with different nitrogen sources (NH₄)₂SO₄ and cysteine.HCl were omitted from the medium and 0.5 mM-Na₂S was used as the reducing agent. In order to examine the range of sulphur sources which could be used by *Neocallimastix* sp. L2 the sulphur-containing compounds L-cysteine, L-methionine, Na₂S, Na₂SO₃, Na₂SO₄, Na₂S₂O₃, dithiothreitol or elemental sulphur were added to a final concentration of 1 or 10 mM-sulphur. In these incubations cysteine.HCl and (NH₄)₂SO₄ were omitted from the medium, (NH₄)₂SO₄ was replaced by NH₄Cl, the sulphates were replaced by their chlorides in the trace element solution and a titanium(III)-NTA reductant, prepared as described by Moench & Zeikus (1983), was used. In the tests for utilization of nitrogen and sulphur sources 10 mM-glucose was used as the carbon and energy source. All soluble additions were sterilized by membrane filtration (0.20 µm pore diameter). Insoluble additions were autoclaved separately (121 °C, 20 min) in suspensions which were added aseptically to the medium. The experiments as described above were performed in 16 ml capacity Hungate tubes containing 10 ml medium and fitted with butyl septum stoppers (Bellco Glass). The tubes were inoculated with 0.5 ml culture. The cultures were subcultured twice and after 7 d, growth was examined by measuring cell protein. Cultures without carbon, nitrogen or sulphur were used as controls.

Temperature and pH profiles were tested in 130 ml bottles, fitted with butyl rubber septa, containing 100 ml basal medium and 10 mM-glucose. After inoculation, samples were taken two or three times a day and were analysed for glucose and fermentation products (expressed as total organic carbon). The pH profile was tested under an atmosphere of O₂-free N₂ gas containing 5% (v/v) CO₂. Several buffers were used for various pH ranges: 30 mM-MES was used at pH 5–6, 30 mM-KH₂PO₄/K₂HPO₄ was used at pH 6–7.5 and a 30 mM-Tris/HCl was used at pH 7.5–8. NaHCO₃ was added in different amounts, to avoid changes in pH caused by the CO₂ in the gas phase (Umbreit, 1964).

Chemical analysis. Cell protein content was determined by the Lowry method, using harvested cells washed once with 5 ml 0.5% (w/v) NaCl. Glucose was determined according to Somogyi (1952). Total organic carbon and cell carbon were analysed with a Shimadzu TC-500 carbon analyser, with bipthalate as a standard. Alcohols (Laanbroek *et al.*, 1984) and short-chain fatty acids, both volatile and non-volatile (Nanninga *et al.*, 1986), were analysed by gas chromatography. Formate was determined by the method of Lang & Lang (1972). H₂ in the gas phase of the cultures was analysed as described by Gerritse *et al.* (1990).

To detect the presence of cytochromes, cells were grown for 72 h in the basal medium with 10-mM glucose without resazurin, harvested and washed three times with 20 mM-KH₂PO₄/K₂HPO₄ buffer, pH 7.4. The cells were disrupted with glass beads (1–2 mm diameter) in the same buffer with 5% (w/v) Triton X-100. Cytochromes were detected by recording a KF₃(CN)₆-oxidized/dithionite-reduced spectrum with an Aminco DW-2a UV/VIS spectrophotometer.

In order to measure maximum oxygen consumption rates, cells were cultured statically for 4 d in basal medium with 10 mM-glucose as carbon and energy source, with 2% O₂ in the gas phase to induce respiratory activity. Cells were harvested anaerobically and washed twice with a N₂-flushed buffer. This buffer, pH 6.8, contained the following (mM): KH₂PO₄/K₂HPO₄ 28, NaCl 15.4, (NH₄)₂SO₄ 3.4, CaCl₂ 0.6, MgSO₄ 0.4. Maximum oxygen consumption rates of washed

Fig. 1. Morphology and ultrastructure of *Neocallimastix* sp. L2. (a) Phase-contrast micrograph of sporangium with thallus. (b) Phase-contrast micrograph of zoospore. (c–k) Electron micrographs of thin sections of: (c) flagella; (d) flagellar pole, showing circumflagellar ring (cr), spur (sp), and microtubuli (m); (e) flagellar pole, showing spur (sp), scoop (sc), skirt (sk), and microtubules (m); (f) rhizoid, containing hydrogenosomes (h); (g) overview of a zoospore with hydrogenosomes (h), nucleus (n), and ribosomal aggregates [ribosomal globules (rg) and ribosomal helices (rh)]; (h) zoospore with hydrogenosomes (h), containing internal membranes and possessing branches; (i) zoospore with hydrogenosomes (h) with cytoplasmic regions; (j) hydrogenosome, containing internal membrane, surrounded by a single membrane; (k) rhizoid with 'crystal body' (cb). Bars represent 1 µm, unless indicated otherwise.

Fig. 2. Electron micrographs of serial sections (a–d) of hydrogenosomes in a zoospore of *Neocallimastix* sp. L2. Bar represents 1 μ m.

cell suspensions were measured polarographically in a YSI-Biological Oxygen Monitor, using the same buffer containing 200 mM-glucose, in the presence of low oxygen concentrations ($\leq 10 \mu$ M) as described in detail elsewhere (Gerritse *et al.*, 1992). All experiments were carried out in triplicate.

Results

Morphological characterization

In roll tubes *Neocallimastix* sp. L2 produces monocentric colonies. In liquid medium its thallus consists of a sporangium with an extensive rhizoid (Fig. 1a). The zoospores are polyflagellated (Fig. 1b). Electron micrographs of a series of thin sections of these zoospores showed that one zoospore generally possessed nine flagella. The flagella have a typical configuration of 9+2 axonema (Fig. 1c). A circumflagellar ring surrounds the flagellum near the entrance region of the zoospore (Fig. 1d). In the kinetosomes, spurs, scoops and skirts were visible (Fig. 1e). Struts could not be detected with certainty. Microtubules radiating from the spurs were clearly visible (Fig. 1d, e). In both mycelium and zoospores, microbodies were observed which appeared very similar to those identified as hydrogenosomes by Yarlett *et al.* (1986). In the sporocyst they were detected both in the sporangium and in the rhizoid and are regularly shaped, round to oval (Fig. 1f). In the zoospores they were located primarily in the vicinity of the kinetosome, mostly as extremely elongated, irregularly shaped organelles, becoming narrower in the direction of the kinetosome (Fig. 1g, h). In many hydrogenosomes electron-transparent lines and/or cytoplasmic regions were observed (Fig. 1g, h, i). These cytoplasmic regions formed tubular structures (Fig. 2). The hydrogenosomes were apparently surrounded by a single membrane (Fig. 1j). In the zoospores two forms of ribosomal aggregates (Munn *et al.*, 1988) were detected: helices and globules (Fig. 1g). These structures are located predominantly opposite the flagellar pole. Structures

very similar to those observed by Munn *et al.* (1988) and referred to as 'crystal bodies' were detected only in the sporocyst (Fig. 1k). Mitochondria were absent in all life stages.

Metabolism

Neocallimastix sp. L2 was able to grow in a CO₂-flushed medium only after addition of a reducing agent. When *Neocallimastix* sp. L2 was grown statically in reduced medium, O₂ levels up to 10% in the gas phase could be applied, without influencing the growth of the fungus. The apparent K_m value and the V_{max} of O₂ utilization of washed cell suspensions with glucose as substrate were $1.0 \pm 0.5 \mu$ M and $2.0 \pm 0.4 \text{ nmol O}_2 \cdot \text{min}^{-1} (\text{mg cell protein})^{-1}$, respectively (means $\pm$ SEM, $n = 3$). *Neocallimastix* sp. L2 was not able to use NO₃[−], SO₄^{2−}, S₂O₃^{2−}, Fe³⁺ or fumarate as electron acceptors and no cytochromes could be detected.

Neocallimastix sp. L2 was able to grow on wheat straw, sisal, xylan, soluble starch, amylose, filter-paper, Avicel-cellulose, cellulose CF11, raffinose, cellobiose, maltose, sucrose, lactose, glucose, fructose and xylose. No growth was observed on carboxymethylcellulose, inulin, pectin, polygalacturonate, galactose, fucose, ribose, galacturonate, sorbitol, mannitol, glycerol, citrate, pyruvate, malate, L-alanine, L-glutamate, 1-propanol or 2-propanol. In addition to ammonium, amino acids, but not peptides (see Methods for peptides tested), could serve as nitrogen sources. Only reduced sulphur compounds (Na₂S) and sulphur-containing amino acids (L-cysteine and L-methionine) could be used as sulphur sources. Growth on glucose yielded the following fermentation products (mmol per 100 mmol glucose fermented; means of three experiments $\pm$ SEM): H₂ (33.8 ± 2.0), CO₂ (estimated from acetate + ethanol – formate – succinate; 15.4 ± 2.5), formate (74.3 ± 3.4), acetate (69.2 ± 2.9), lactate (26.7 ± 1.5), succinate (8.2 ± 0.8) and ethanol (28.7 ± 0.16). The carbon in the cells was 125.0 ± 4.0

Fig. 3. Fermentation of glucose by *Neocallimastix* sp. L2. Cells were grown on glucose in basal medium as described in Methods. Δ , Glucose; $\blacksquare$, H₂; $\circ$, formate; $\blacktriangle$, acetate; $\bullet$, lactate; $\blacklozenge$, succinate; $\square$, ethanol; $\blacktriangledown$, cell protein.

mmol per 100 mmol glucose, the carbon recovery was $87\% \pm 2\%$ and the oxidation-reduction index of the products was 1.16 ± 0.05 (assuming a cell composition of CH_{1.8}O_{0.5}). The formation of the fermentation end-products was simultaneous (Fig. 3).

The temperature range for glucose fermentation by *Neocallimastix* sp. L2 was from 37 to 42 °C, with a sharp optimum around 38 °C. The pH range for glucose fermentation was from pH 6 to 7.5, showing a broad optimum between pH 6.5 and 7.5. *Neocallimastix* sp. L2 required CO₂ for growth. Glucose fermentation rates were 80 $\mu\text{mol l}^{-1} \text{h}^{-1}$ at 5% CO₂, gradually increasing to 140 $\mu\text{mol l}^{-1} \text{h}^{-1}$ at 100% CO₂ in the gas phase. In the absence of CO₂ in the gas phase no growth was observed.

Discussion

This study describes morphological and physiological characteristics of an anaerobic fungus isolated from llama faeces. Based on its growth characteristics, i.e. polyflagellated zoospores, extensively branched rhizoids and the production of monocentric colonies, the isolate was assigned to the genus *Neocallimastix*. The organism previously named *Neocallimastix joyonii* is a polycentric species (Breton *et al.*, 1989) and has therefore been reclassified as *Orpinomyces joyonii* (Li *et al.*, 1991). All *Neocallimastix* species described thus far were isolated from the rumen or faeces of ruminant foregut fermenters such as sheep (Orpin, 1975; Lowe *et al.*, 1985), cattle (Barr *et al.*, 1989; Kostyukovsky *et al.*, 1991), reindeer (Orpin *et al.*, 1985), llama and impala (this paper, Milne *et al.*, 1989). Anaerobic fungi isolated from hindgut fermenters were assigned to other genera, i.e. *Caecomyces* (Gold *et al.*, 1988) and *Piromyces* (Teunissen *et al.*,

1991). However, the isolation of a *Neocallimastix* strain from the tapir (*Tapirus terrestris*), a hindgut fermenter (F. D. Marvin-Sikkema, unpublished), suggests that the genus *Neocallimastix* is not restricted to foregut-fermenting herbivores.

When ultrastructural features of *Neocallimastix* sp. L2 were compared with those described for other strains of *Neocallimastix* and other genera of anaerobic fungi, the following can be concluded. So-called 'crystal bodies' and microbodies (hydrogenosomes according to Yarlett *et al.*, 1986) have been observed in the vegetative stage of all members of the *Neocallimasticaceae* studied so far (Gold *et al.*, 1988; Munn *et al.*, 1988). In zoospores of all genera they are located around the flagella pole (Gold *et al.*, 1988). The location of these structures close to the motility apparatus was also found in the rumen holotrichs *Dasytricha ruminantium* and *Isotricha* sp. (Yarlett *et al.*, 1981, 1983). In zoospores of *Neocallimastix* sp. L2 the hydrogenosomes appear to be very elongated towards the flagella pole, with a bulb at the opposite side. They also appear irregularly shaped and in many cases seem to contain regions of cytoplasm (Figs 1i and 2). It could not be decided with certainty whether these structures are indeed invaginations of the cytosol. Similar regions have been found in *Piromyces mae* and *Neocallimastix hurleyensis* (Li *et al.*, 1990; Webb & Theodorou, 1988, 1991). Hydrogenosomes with such structures have been termed megahydrogenosomes (Li *et al.*, 1990). The internal electron-transparent lines in some of the hydrogenosomes of *Neocallimastix* sp. L2 seem to be identical with the lines which were observed around hydrogenosomes in *Piromyces dubonica* (Li *et al.*, 1990). The presence of a single membrane around hydrogenosomes of *Neocallimastix* sp. L2 is in contrast to the double membranes around hydrogenosomes of anaerobic protozoa (Benchimol & De Souza, 1983) and supports the hypothesis of Cavalier-Smith (1987), that hydrogenosomes in anaerobic fungi were possibly derived from peroxisomes and did not, as in protozoa, evolve from mitochondria or from symbiotic bacteria. Ribosomal globules and arrays, as observed in all genera and species studied thus far (Heath *et al.*, 1983; Gold *et al.*, 1988; Munn *et al.*, 1988; Webb & Theodorou, 1988; Li *et al.*, 1990), are also present in *Neocallimastix* sp. L2. Interestingly, there are clear differences in the location of these structures in zoospores: they are located at the posterior in *Neocallimastix frontalis* (Heath *et al.*, 1983), at the anterior in *Neocallimastix hurleyensis*, *Piromyces mae* and *Piromyces dubonica* (Webb & Theodorou, 1988; Li *et al.*, 1990) and dispersed throughout the cell in all other genera and species (Gold *et al.*, 1988). The anterior location in *Neocallimastix* sp. L2 emphasizes the relationship of this strain with *Neocallimastix hurleyensis*. The flagellar apparatus of all anaerobic gut-fungi described so far is

strikingly similar. Only relatively small variations in some components, i.e. the presence or absence of skirts and struts, appear to exist. These variations are very subtle indeed, sometimes difficult to observe, and there are some contradictory reports on this subject (Munn *et al.*, 1988; Gold *et al.*, 1988). Careful examination of more isolates is needed before the structure of the kinetosome can be used as a trustworthy criterion for classification of anaerobic fungi.

The inability of *Neocallimastix* sp. L2 to grow in non-reduced medium, the low $V_{\max}$ value of O_2 utilization, the absence of cytochromes and the inability to use NO_3^- , SO_4^{2-} , $S_2O_3^{2-}$, Fe^{3+} or fumarate as electron acceptors clearly indicate that this fungus is a strictly fermentative organism. The observed apparent K_m value for O_2 ($1.0 \pm 0.5 \mu M$) is comparable with that found for *Dasytricha ruminantium* (Lloyd *et al.*, 1983) and *Neocallimastix patriciarum* (Yarlett *et al.*, 1987) ($1.7 \mu M$ and $4.0 \pm 0.3 \mu M$, respectively). It can be thus be concluded that *Neocallimastix* sp. L2 is well adapted to the rumen environment, in which dissolved O_2 levels can be up to $1.6 \mu M$ dissolved O_2 (Scott *et al.*, 1983).

Neocallimastix sp. L2 resembles other isolates within the genus *Neocallimastix* in the range of carbohydrates utilized, but differs in that it is unable to ferment inulin, which is fermented by other *Neocallimastix* isolates (Phillips & Gordon, 1988; Theodorou *et al.*, 1988; this paper). As found for *Neocallimastix patriciarum* (Orpin & Greenwood, 1986), amino acids can replace ammonium as nitrogen source for growth of *Neocallimastix* sp. L2. The failure of *Neocallimastix* sp. L2 to utilize peptides, bovine serum albumin or casein as nitrogen sources may be due to the absence of appropriate uptake mechanisms or an inability to liberate utilizable nitrogen containing compounds from these substrates. The sulphur requirements of *Neocallimastix* sp. L2 are identical to those found for *Neocallimastix patriciarum* (Orpin & Greenwood, 1986). The fermentation products, formed by *Neocallimastix* sp. L2 are very similar to those formed by other genera, although there are some differences in their relative proportions (Phillips & Gordon, 1988; Breton *et al.*, 1989, 1990). Since there are contradictory reports on whether formate is produced by *Neocallimastix patriciarum* (Orpin & Munn, 1986; Marvin-Sikkema *et al.*, 1990), formate production cannot be used to characterize *Neocallimastix* isolates.

It can be concluded from the morphological and physiological characteristics that isolate L2 belongs to the genus *Neocallimastix*. There are insufficient distinctive morphological and physiological features to decide whether *Neocallimastix* sp. L2 should be classified as a new species. However, it is clear that although *Neocallimastix* sp. L2 has many similarities to described isolates,

it also has a unique combination of characteristics in which it differs from all other isolates described so far: inability to ferment inulin, extremely sharp temperature optimum, the positioning of the ribosomal globules and arrays at the anterior of the zoospores, and the presence of extremely irregularly shaped hydrogenosomes and megahydrogenosomes in these zoospores. The similarities of this strain from llama faeces to *Neocallimastix* isolates obtained from other animals, especially *Neocallimastix hurleyensis*, suggests a lack of herbivore host-specificity in these fungi.

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