

The diversity and taxonomy of anaerobic gut fungi

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Anaerobic gut fungi have been isolated from the gut of a wide range of herbivores and their distribution is world-wide. Many characteristics such as life cycle, reproductive structures, zoospore ultrastructures, thallus morphology and development, molecular affinities and isozyme profiles of the anaerobic gut fungi show that they are more closely related to the Chytridiomycetes than to any other group of fungi. Thus, they are assigned to the class Chytridiomycetes, but a new order, Neocallimastigales, and a new family, *Neocallimastigaceae*, were erected to accommodate them. This review describes the distribution, life cycle, morphological diversity, taxonomy and phylogeny of the anaerobic gut fungi.

Key words: anaerobic fungi, diversity, gut fungi, phylogeny, taxonomy.

Introduction

For a long time, the rumen microbial population was believed to be composed of a diverse population consisting of anaerobic bacteria and protozoa. It was only in 1975 that the anaerobic rumen fungi were identified and recognised as an integral part of the rumen microbiota (Orpin, 1975). The presence of chitin in their cell walls (Orpin, 1977a), their morphology and life cycle confirmed that they are true fungi. The significance and possible role of the rumen fungi in fibre digestion was recognised when extensive colonisation of fibrous plant materials by the fungi was observed in the rumen of sheep and cattle (Bauchop, 1979a,b). The fungi preferentially colonise the thick-walled sclerenchyma and vascular tissues (Akin *et al.*, 1983; Ho *et al.*, 1988a,b, 1991, 1996) (Fig. 1) and are able to contribute to the overall digestion of various forages and wheat straw (Akin *et al.*, 1983; Gordon and Ashes, 1984). Anaerobic fungi have now been found to occur in the guts of a wide range of herbivores and are now commonly known as anaerobic gut fungi. Given their common occurrence and abundance, particularly in animals fed fibrous diets, it seems amazing that the anaerobic fungi in the rumen remained unrecognised for so long whilst the other anaerobic rumen microorganisms have been

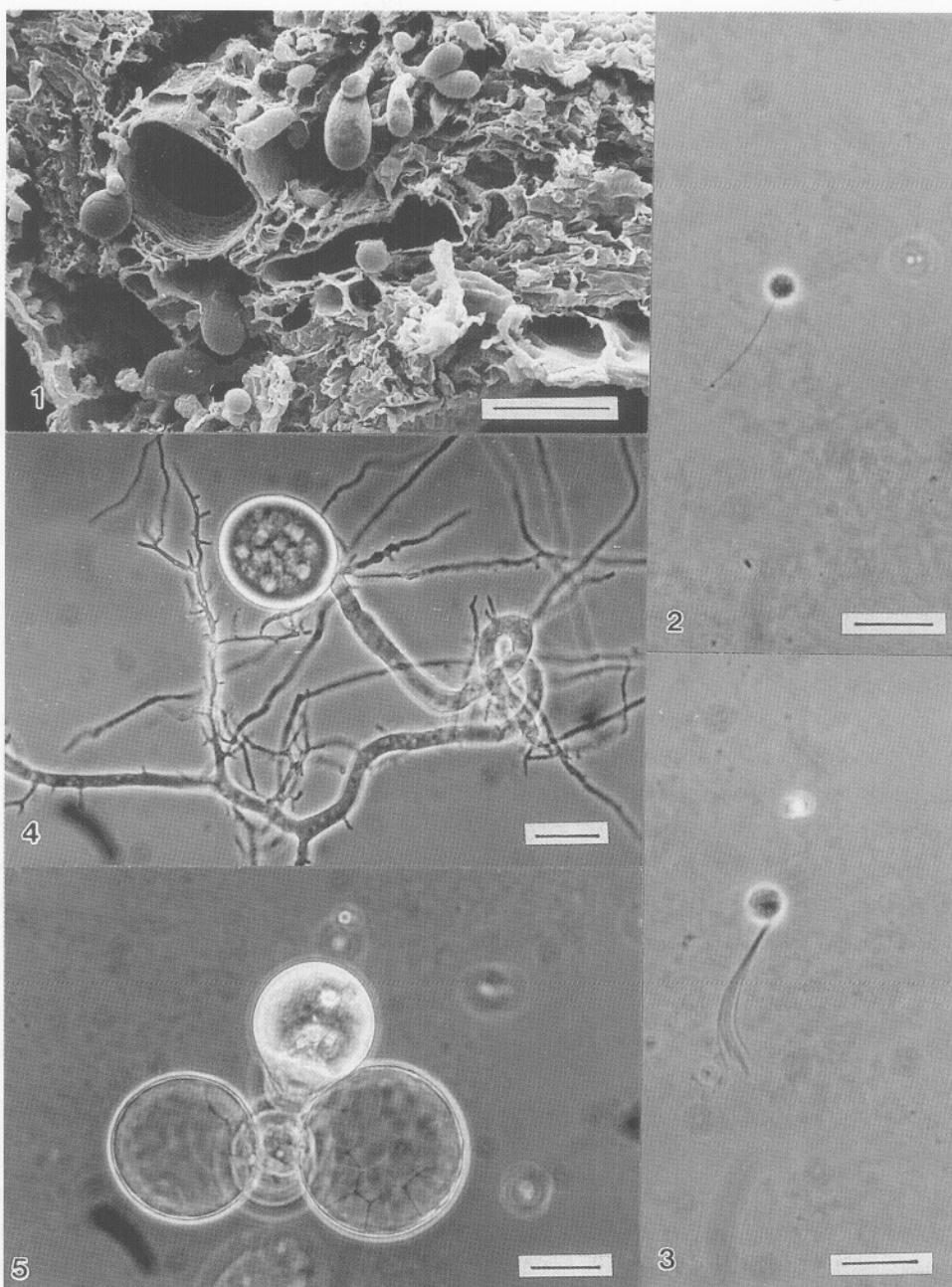
discovered and studied for about a hundred years. One of the reasons for this is probably because most studies on rumen microorganisms are by using rumen fluid or filtered rumen contents instead of the solid rumen digesta that is usually discarded. It is in these rumen digesta that the fungal thalli are attached. Hence, the fungal zoospores swimming freely in the rumen fluid are often the structures observed by early rumen microbiologists who thought that they were flagellated protozoa (Liebetanz, 1910; Braune, 1913).

Distribution

The distribution of anaerobic gut fungi is worldwide. They are commonly found in many herbivores of tropical as well as temperate regions. Anaerobic gut fungi seem to be confined to the alimentary tract of herbivores and have not been isolated in other anaerobic terrestrial or aquatic habitats (Orpin and Joblin, 1988; Bauchop, 1989). Table 1 shows the range of ruminants and non-ruminants from which anaerobic gut fungi have been isolated.

Herbivores where anaerobic fungi have not been found are the giant panda (*Ailuropoda melanoleuca*: Milne *et al.*, 1989), rabbit (*Oryctolagus cuniculus*: Bauchop, 1989), possum (*Trichosurus vulpecula*: Bauchop, 1989), giraffe (*Giraffa camelopardalis*: Milne *et al.*, 1989), blackbuck (*Antilope cervicapra*: Milne *et al.*, 1989), okapi (*Okapia johnstonii*: Milne *et al.*, 1989), Brazilian tapir (*Tapirus terrestris*: Milne *et al.*, 1989), lesser mouse deer (*Tragulus javanicus*: Y.W. Ho, unpublished) and larger mouse deer (*Tragulus napu*: Y.W. Ho, unpublished).

The distribution of anaerobic fungi in the alimentary tract of a cattle had been studied by Davies *et al.* (1993). They found fungi throughout the whole digestive tract, including the faeces. The highest number of fungi was in the digesta contents from the reticulo-rumen and omasum (Davies *et al.*, 1993). The fungi in the reticulo-rumen accounted for about 89% of the total fungi in the whole digestive tract. Fungal populations were considerably less in the abomasum, small intestine and hindgut. Fungal populations in the hindgut ranged from 0.5-2.3% of the total fungal population of the digestive tract (Davies *et al.*, 1993). It is rather surprising that anaerobic fungi are present in the abomasum where gastric digestion takes place and the digesta contents are acidic with pH values ranging from 3.3 to 4.9. Besides the digestive tract and faeces, anaerobic fungi have also been isolated from saliva of a sheep (Lowe *et al.*, 1987a). Although anaerobic fungi have been isolated from all parts of the alimentary tract of the cattle, there is little evidence to indicate that growth takes place in any organ except the rumen. Fungi found in other parts of the alimentary tract probably originated from the rumen (Davis *et al.*, 1993; Wong *et al.*, 1995).



Figs. 1-5. Scanning electron micrograph and light micrographs. 1. Fungi colonising the thick-walled sclerenchyma and vascular tissues of a rice straw fragment in the rumen of a goat. 2. A uniflagellated zoospore (*P. minutus*). 3. A polyflagellated zoospore (*N. frontalis*). 4. An anaerobic rumen fungus (*N. frontalis*) with filamentous myceloid rhizoids. 5. An anaerobic rumen fungus (*C. communis*) with bulbous rhizoids. Bars: 1 = 25 μm ; 2-5 = 20 μm .

Morphology

Zoospores of anaerobic gut fungi are either uniflagellated (Fig. 2) or polyflagellated (Fig. 3). In the uniflagellate species, the zoospores are predominantly uniflagellated, but occasionally they can be bi- or quadriflagellated. Zoospores of polyflagellate species always have more than four flagella. However, during encystment the flagella are not shed simultaneously and very often a single flagellum is left attached to the zoospore. Occasionally, the flagella of polyflagellated zoospores may cluster together appearing as one, especially when they are in a stationery stage. Thus, it is important that a number of zoospores are examined before making a conclusion on whether they are uniflagellated or polyflagellated. The size of zoospores varies, but generally, uniflagellated zoospores are smaller (4-11 µm) than polyflagellated zoospores (7-22 µm).

The fungal thallus development of anaerobic gut fungi is either monocentric or polycentric. In the monocentric development, the fungal thallus predominantly develops a single sporangium and the rhizoidal system is anucleate. During germination of the zoospore, the nucleus does not enter the germ tube. The anucleate germ tube branches to become the rhizoidal system. There are two types of rhizoids; filamentous myceloid and bulbous.

There are two further developmental stages, namely, endogenous and exogenous, among monocentric species. In endogenous development, the nucleus remains in the zoospore cyst that eventually enlarges and develops into a sporangium. In exogenous development there is bipolar germination. Rhizoids develop from one side of the zoospore cyst and a wider outgrowth develops from the other side. The nucleus moves into the broader outgrowth that develops into a sporangiophore, and a sporangium forms at the end. The length of the sporangiophore varies from very short to over 100 µm long. The sporangiophore is easily distinguishable from the main rhizoid by the absence of lateral outgrowths, and the point where the zoospore cyst germinated is often swollen.

The monocentric forms include *Neocallimastix*, *Piromyces* and *Caecomyces*. The zoospores of *Neocallimastix* are polyflagellated, while those of *Piromyces* and *Caecomyces* are uniflagellated. *Piromyces* is distinguished from *Caecomyces* by its rhizoidal system. The rhizoids of *Piromyces*, like *Neocallimastix*, are filamentous myceloid (Fig. 4), but those of *Caecomyces* are bulbous (Fig. 5). Most species of *Neocallimastix*, *Piromyces* and *Caecomyces* exhibit both endogenous and exogenous developmental forms.

In some monocentric species (*Piromyces* spp. and *Caecomyces communis*), two or three sporangia may be formed. These monocentric-multisporangiate

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Table 1. Occurrence of anaerobic gut fungi in ruminant and non-ruminant herbivores.

	Species of animal	Reference
Ruminant	Antelope (<i>Hippotragus equinus</i>)	Milne <i>et al.</i> , 1989
	Arabian oryx (<i>Oryx leucoryx</i>)	Milne <i>et al.</i> , 1989
	Camel (<i>Camelus bactrianus</i>)	Milne <i>et al.</i> , 1989
	Cattle (<i>Bos indicus</i>)	Ho <i>et al.</i> , 1988a,b, 1990, 1993a, 1994a,b
	Cattle (<i>Bos taurus</i>)	Bauchop, 1979a; Akin and Rigsby, 1987; Barr <i>et al.</i> , 1989; Ho and Bauchop, 1991
	Gaur (<i>Bos gaurus</i>)	Milne <i>et al.</i> , 1989
	Goat (<i>Capra hircus</i>)	Orpin and Joblin, 1988; Jalaludin <i>et al.</i> , 1992; Ho <i>et al.</i> , 1993b, 1996
	Grey kangaroo (<i>Macropus giganicus</i>)	Bauchop, 1989
	Impala (<i>Aepyceros melampus</i>)	Bauchop, 1980; Milne <i>et al.</i> , 1989
	Llama (<i>Lama glama</i> , <i>L. pacos</i> , <i>L.</i> <i>guanicoe</i>)	Milne <i>et al.</i> , 1989; Bauchop, 1989
	Musk ox (<i>Ovibos moschatus</i>)	Orpin and Joblin, 1988
	Red deer (<i>Cervus elaphus</i>)	Bauchop, 1980
	Redneck wallaby (<i>M. rufogriseus</i>)	Bauchop, 1989
	Reindeer (<i>Rangifer tarandus</i>)	Orpin and Joblin, 1988; Bauchop, 1980
	Sheep (<i>Ovis aries</i>)	Orpin, 1975, 1976, 1977b; Bauchop, 1979a; Lowe <i>et al.</i> , 1987b; Phillips and Gordon, 1989; Wong <i>et al.</i> , 1995
	Sika deer (<i>Cervus nippon</i>)	Ho <i>et al.</i> , 1993c
	Swamp wallaby (<i>Wallabia bicolor</i>)	Bauchop, 1989
	Wallaroo (<i>M. robustus</i>)	Bauchop, 1989
	Water buffalo (<i>Bubalus bubalis</i>)	Ho <i>et al.</i> , 1988a,b, 1991, 1994b
Non-ruminant	Asian elephant (<i>Elephas maximus</i>)	Milne <i>et al.</i> , 1989; Li <i>et al.</i> , 1990; Teunissen <i>et al.</i> , 1991
	African elephant (<i>Loxodonta africana</i>)	Bauchop, 1980; Teunissen <i>et al.</i> , 1991
	Horse (<i>Equus caballus</i>)	Bauchop, 1980; Orpin, 1981; Gold <i>et</i> <i>al.</i> , 1988; Li <i>et al.</i> , 1990
	Donkey (<i>Equus asinus</i>)	Bauchop, 1983
	Zebra (<i>Equus burchelli</i>)	Bauchop, 1983; Milne <i>et al.</i> , 1989
	Black rhinoceros (<i>Diceros bicornis</i>)	Teunissen <i>et al.</i> , 1991
	Indian rhinoceros (<i>Rhinoceros unicornis</i>)	Teunissen <i>et al.</i> , 1991
	Mara (<i>Diplochotis patagonum</i>)	Teunissen <i>et al.</i> , 1991
	Guinea pig (<i>Cavia aperea</i>)	Orpin, 1976

forms are uncommon, and in most cases, only one or none of the sporangia develops zoospores.

In the polycentric development, the thallus develops numerous sporangia, the number being limited by available substrate (Barr *et al.*, 1989). During germination of the zoospore cyst, the nucleus migrates into the germ tube and divides repeatedly as the germ tube grows and develops into a network of

nucleated mycelium (rhizomycelium) which forms many sporangia. Sporangia are either borne on sporangiophores which are lateral outgrowths of the hyphae or they develop in the hyphae as intercalary sporangia. Viable sporangia that differentiate and release zoospores are abundantly formed in freshly isolated cultures. However, prolonged culturing usually results in the production of undifferentiated sporangia or a complete cessation of sporangial production, and identification of the species becomes problematic.

The polycentric forms are *Orpinomyces* and *Anaeromyces*. *Orpinomyces* produces polyflagellated zoospores while *Anaeromyces* produces uniflagellated zoospores. *Orpinomyces* can be further distinguished from *Anaeromyces* by the presence of many sporangiophore initials (swollen outgrowths from the hyphae) and globose sporangia formed either intercalarily in the hyphae or terminally on mature sporangiophores which can be single or branched complexes. *Anaeromyces* does not form sporangiophore complexes and the sporangia, which are predominantly ellipsoidal and fusiform with an elongated or pointed tip, are borne terminally on single sporangiophores.

Taxonomy

The generic names *Sphaeromonas* and *Piromonas* were established by Liebetanz in 1910 for a group of uniflagellate rumen organisms presumed at that time to be flagellated protozoa. A few years later, Braune (1913) named a polyflagellate rumen organism *Callimastix frontalis* because of its similarity to *Callimastix cyclopsis*, a polyflagellate parasite of copepod. Subsequently, in 1966 Vavra and Joyon studied the ultrastructures of *C. cyclopsis* and suggested that it was most probably a chytridiomycete fungus. They established a new zooflagellate genus, *Neocallimastix*, and *C. frontalis* was assigned to it as the type species. It was Orpin (1975) who showed that the rumen zooflagellate, *N. frontalis*, was actually the zoosporic stage of a fungus. However, its taxonomic status was not established until Heath *et al.* (1983) studied its ultrastructures and formally assigned it to the class Chytridiomycete, order Spizellomycetales and a new family, *Neocallimasticaceae*, was erected to accommodate it. *Neocallimasticaceae* was later corrected to *Neocallimastigaceae* (Index of Fungi, 1989).

Many characters such as life cycle, reproductive structures and vegetative thalli of the anaerobic gut fungi show that they are more closely related to the Chytridiomycetes than to any other group of fungi. Molecular data (18S rRNA sequences) of the fungi also show that they are indeed chytridiomycete fungi (Dore and Stahl, 1991; Bowman *et al.*, 1992; Li and Heath, 1992). However, studies by Heath and Bauchop (1985) and Munn *et al.* (1987) showed distinct differences in some of the mitotic and ultrastructural characteristics of the anaerobic fungi from the other fungi in the Spizellomycetales and they

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suggested the establishment of a new order for the anaerobic fungi. Later, Li *et al.* (1993) using cladistic analysis of structural data found the anaerobic gut fungi to be distinct from both the Spizellomycetales and the Chytridales and a new order, Neocallimastigales (= Neocallimasticales), was established for them.

At present, anaerobic gut fungi are classified and placed in:

Class	Chytridiomycetes
Order	Neocallimastigales
Family	Neocallimastigaceae

So far, five genera have been established for the anaerobic gut fungi. They are *Neocallimastix* (Vavra and Joyon, 1966), *Piromyces* (Gold *et al.*, 1988), *Caecomyces* (Gold *et al.*, 1988), *Orpinomyces* (Barr *et al.*, 1989) and *Anaeromyces* (Breton *et al.*, 1990). The classification of the genera is based mainly on the number of flagella present in zoospores, fungal thallus morphology and development, and ultrastructures of the zoospores. A total of seventeen species have been formally named and described. *Neocallimastix joyonii* (Breton *et al.*, 1989) was later transferred to *Orpinomyces* and *O. bovis* was placed in synonymy with *O. joyoni* (Li *et al.*, 1991). Later, Ho and Barr (1995) revised the classification of anaerobic gut fungi and placed *N. patriciarum* (Orpin and Munn, 1986) and *N. variabilis* (Ho *et al.*, 1993a) in synonymy with *N. frontalis*, thus reducing the number of species to fourteen. They also provided a detailed description and a key for all the genera and species in their revised classification.

The identification of anaerobic gut fungal species is complicated and difficult as natural morphological variation occurs within a species. At times there is sufficient morphological variation that the fungal thallus of one species may look like another. Artificial culture media probably cause the most variations. In rich medium such as glucose medium, sporangia may become abnormally large and they usually abort before zoospores are differentiated. Thus, it is important that conclusions on the identification of species are made only after examination of sufficient materials.

Although ultrastructures of zoospores provide valuable and essential information on the classification of anaerobic gut fungi, it is totally impractical and unnecessary to use them for their identification. Species of anaerobic gut fungi can be identified solely on characters seen in the light microscope.

Phylogeny

Dore and Stahl (1991), Li and Heath (1992) and Li *et al.* (1993) assessed the phylogenetic relationships among the anaerobic gut fungi and their relationships with other eukaryotes using 18S rRNA sequences and cladistic

analysis of structural data and concluded that the anaerobic gut fungi are monophyletic and form a closely related group in the Chytridiomycetes. The isozymatic characteristics (Ho *et al.*, 1994b) and the G+C contents (Brownlee, 1989) of the anaerobic gut fungi also indicate that they are a monophyletic group. Anaerobic gut fungi are thought to have derived from free-living chytridiomycetes which have evolved the ability to survive the anaerobic conditions in the digestive tract of their herbivorous hosts (Heath, 1988). The absence of mitochondria and lipid droplets and presence of hydrogenosomes are undoubtedly adaptations to anaerobiosis; the unique surface layer of the zoospores is probably an adaptation to the harsh chemical environment of the rumen (Heath *et al.*, 1983).

Based on zoospore ultrastructures, thallus morphology and development, molecular affinities and isozymatic characteristics, the anaerobic gut fungi are more closely related to the chytridiomycete fungi in the order Spizellomycetales than those in the other orders (Barr, 1988, Bowman *et al.*, 1992; Ho *et al.*, 1994b). Despite this close relationship, the anaerobic gut fungi, nevertheless, show distinct differences and form a separate cluster (on dendograms showing phylogenetic relationships) from the Spizellomycetales (Li *et al.*, 1993; Ho *et al.*, 1994b). As mentioned earlier, a new order, the Neocallimastigales has been established for the anaerobic gut fungi.

At the generic level, the phylogenetic relationships among *Neocallimastix*, *Piromyces*, *Caecomyces* and *Orpinomyces* are still unclear. Ho *et al.* (1994b) using Nei's genetic identity calculated from the isozymatic characteristics of the anaerobic fungi found that *Piromyces* was more closely related to *Caecomyces* than to *Neocallimastix*. In contrast, Dore and Stahl (1991) using 18S rRNA sequences found *Piromyces* to be more closely related to *Neocallimastix* than to *Caecomyces* (*C. communis* = *Sphaeromonas comminis*, Gold *et al.*, 1988). Li *et al.* (1993), on the other hand, using cladistic analysis of structural data found that *P. dumponica* and *C. equi* were more closely related, forming a cluster but *C. communis* was consistently isolated and was closer to *Neocallimastix*. Matrix of genetic similarity and distance coefficients showed that *Orpinomyces* is more related to *Piromyces* and *Caecomyces* than to *Neocallimastix* (Ho *et al.*, 1994b). 18S rRNA sequences also showed *Orpinomyces* (*N. joyonii* = *O. joyonii*; Li *et al.*, 1991) and *Piromyces* to be closely related but they are closer to *Neocallimastix* than to *Caecomyces*. Cladistic analysis of rRNA sequences and cladistic analysis of structural data showed that the relationships between *Orpinomyces*, *Piromyces* and *Neocallimastix* are not clear and depending on the algorithms or set of data used or outgroup chosen, *Orpinomyces* can be close to *Piromyces* and remote from *Neocallimastix* or close to *Neocallimastix* and remote from *Piromyces* (Li

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and Heath, 1992; Li *et al.*, 1993). To resolve the relationships among these four genera, further studies and analyses need to be done. In the case of *Anaeromyces*, it is found to be more distantly related to *Orpinomyces*, *Piromyces* and *Neocallimastix* than these three genera are to one another (Li and Heath, 1992).

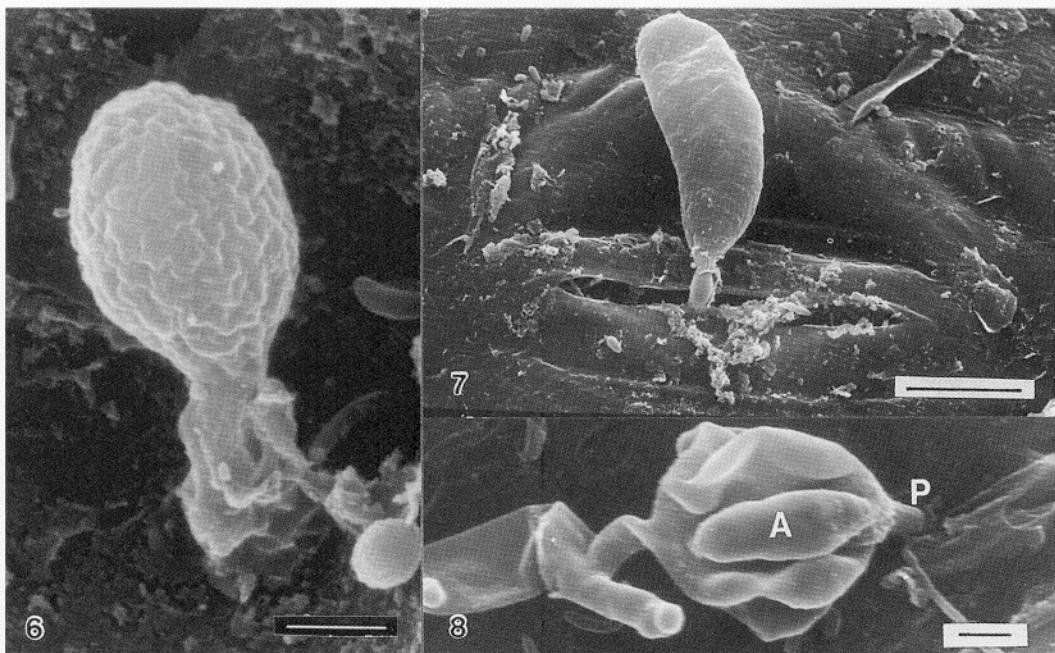
Life cycles

All anaerobic gut fungi have very similar life cycles. Basically, it consists of a motile flagellated zoospore stage swimming freely in the fluid contents and a non-motile, vegetative and reproductive stage attached to the digesta materials. The life cycle of monocentric anaerobic fungi is about 24-32 h (Bauchop, 1981; Lowe *et al.*, 1987b). The polycentric anaerobic fungi, besides producing zoospores for reproduction, are also capable of vegetative reproduction by fragmentation of their mycelium.

Zoospores of *Neocallimastix* show very sensitive chemotactic response to soluble carbohydrates and they probably locate freshly ingested plant fragments by migrating up a soluble-carbohydrate gradient to stomata and damaged areas (Orpin and Bountiff, 1978). Four chemoreceptors, sensing fructose, glucose, mannitol and mannose, have been identified and mixtures of low concentrations of glucose, sucrose and fructose caused synergistic response which could be responsible for location of specific plant tissues by the zoospores (Orpin and Bountiff, 1978). Zoospores of *N. frontalis*, *P. communis*, *O. joyonii* (= *O. bovis*) and *Anaeromyces* sp. (= *Ruminomyces*) also show chemotactic response to phenolic acids such as p-coumaric acid, ferulic acids and syringic acid which are often found in the lignified tissues of plants (Wubah and Kim, 1996).

Once the zoospores have located a suitable site on the plant fragments, they shed their flagella and attach themselves on their flagellar pole with material of unknown nature. The material for attachment can be fibrillar and radiates out as fine fibrils from the point of attachment (Munn *et al.*, 1981) or not distinctly fibrillar and spread out irregularly at the point of attachment (Ho *et al.*, 1988b) (Fig. 6). The flagellum is shed with its perikinetosomal apparatus (Heath *et al.*, 1986).

The zoospore cell encysts and then germinates with a single germ tube. The sites of encystment and germination on the plant fragments are damaged surfaces, cut-ends and stomata (Orpin, 1977b; Bauchop, 1979b; Akin *et al.*, 1983; Ho *et al.*, 1988b, 1991, 1996a) (Fig. 7). The germ tube very rapidly elongates, and branches into a network of rhizoids or hyphae in the plant tissues. Some fungi produce appressoria for penetration of cell walls (Ho *et al.*, 1988 a,b, 1991) (Fig. 8).



Figs. 6-8. Scanning electron micrographs. 6. An attached zoospore. 7. Germinated zoospores in a stoma of a grass fragment in the rumen of buffalo. 8. An appressorium (A) with a penetration peg (P) for penetration of the plant cell wall. Bars: 6, 8 = 1 µm; 7 = 5 µm.

Zoosporogenesis has been reported to be induced by a water-soluble component in the diet of the host animal (Orpin, 1977c). One of the components is haem (Orpin and Greenwood, 1986). For *N. frontalis*, maximum production of zoospores occurs 15-30 min after the onset of feeding by the host animal (Orpin, 1975) but for *P. communis* and *C. communis*, maximum production occurs about 1 h after feeding (Orpin, 1976, 1977b). Several haem-containing compounds have also been found to induce zoosporogenesis and it has been proposed that haem in synergism with other compounds in the diets induced abundant zoosporogenesis in the rumen (Orpin and Greenwood, 1986).

Zoospores are discharged from the sporangia in several ways. It can be through an apical pore in the sporangium (*N. hurleyensis*, Webb and Theodorou, 1991; *P. rhizinflatus*, Breton *et al.*, 1991), through the dissolution of a wide apical portion of the sporangium wall (*P. minutus*, Ho *et al.*, 1993c; *C. communis*, Ho and Barr, 1995), through rupture and dissolution of the apical part of the sporangium followed by collapse and dissolution of the sporangial wall (*N. frontalis*, Heath *et al.*, 1983; *O. joyonii*, *P. comminis*, Ho and Barr, 1995; *O. intercalaris*, Ho *et al.*, 1994a), through rapid dissolution of the whole

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sporangial wall (*P. spiralis*, Ho *et al.*, 1993b) or through dissolution of papillae (*P. mae*, Li *et al.*, 1990).

The ability of the anaerobic fungi in faeces to survive long periods of desiccation, reduced temperature and exposure to air (Davies, *et al.*, 1993) has led to the suggestion that an additional survival or resistant stage be included in the life cycle (Davies *et al.*, 1993). This stage occurs when conditions become unfavourable for vegetative growth, and survival structures are formed to withstand the adverse conditions. However, the survival structures have not yet been positively identified. Wubah *et al.* (1991) have reported that a *Neocallimastix* sp. produced dark brown resistant sporangia as the culture grew older. These resistant sporangia had melanised cell walls and nuclear DNA levels of two to four times that of normal sporangia. However, none of these resistant sporangia could germinate.

Conclusions

Increased interest and research activities in the anaerobic gut fungi in the last decade or so have provided much information on their biology, taxonomy, physiology and biochemistry. However, at present, there is still very little information on the range and diversity of fungal species inhabiting different host species, different substrates and different parts of the alimentary tract. It is not known whether the gut fungi are host- or substrate-specific. Further work is needed to elucidate this.

The taxonomic position of some of the established species and the phylogenetic relatedness among species also need to be resolved. More exacting technique will be required for this and it is likely that it lies with molecular technology.

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