RUMEN FUNGI - THEIR ROLES AND POTENTIAL FOR MANIPULATION

T. BAUCHOP

Summary

Large populations of obligately anaerobic fungi colonise plant fragments in the rumen of cattle and sheep on fibrous diets. Highest numbers are present on stem fragments and the fungi are found associated mainly with the lignified tissues that remain in the rumen for prolonged periods. Fungal populations are highest in animals receiving high-fibre diets and are absent from the rumen contents of animal receiving soft leafy forages, presumably due to the short retention-times of the latter feedstuffs. The major route of fungal invasion is via areas of damaged epidermis that permit access to internal plant tissues. The anaerobic fungi possess the range of glycosidases required for digestion of cellulose and hemicellulose to simple sugars and the products of the resultant fermentations include acetate, lactate, ethanol, formate, carbon dioxide and hydrogen. The properties of the fungi together with the extent of colonisation and growth on fibrous plant fragments suggests a significant role in cellulose digestion in the rumen. However the magnitude of the fungal contribution to rumen fermentation remains to be quantified and awaits development of a reliable method for determination of the fungal mass in the rumen. Despite this gap in our knowlege of the fungi, their properties, including their positive association with high-fibre diets, bring them to attention as possible candidates that might be manipulated to produce improved utilisation of high-fibre forages.

I. INTRODUCTION

Microbial fermentation of ingested plant materials in the rumen is central to the digestion and nutrition of ruminants. As a result of extensive studies over several decades the rumen microbiota had been shown to comprise a dense complex population of microbes consisting predominantly of anaerobic bacteria and protozoa. In the past few years however, large populations of fungi, possessing the distinctive property of being obligate anaerobes, have been found to form an integral part of the rumen microbiota. These fungi colonise and grow on the plant fragments in the rumen. The failure of earlier workers to detect these fungi may be explained by the fact that microorganisms associated with rumen 'solids' had been little studied, and by the practice, standard in studies of rumen microbiology, of using strained rumen contents and discarding the rumen 'solids'. The commonly discarded 'solids' fraction is now known to contain the vegetative stages of the rumen anaerobic fungi.

The fundamentally aerobic nature of virtually all fungi had precluded serious consideration being given to a role for fungi in the anaerobic environment of the rumen. Although in the past common saprophytic fungi had been isolated from rumen contents they were not regarded as indigenous rumen microbes. These fungi were considered to be merely transients as they continually enter the rumen on feed, are present there in relatively low numbers, and are unable to grow under the anaerobic conditions of the rumen environment.

The recently discovered rumen anaerobic fungi belong to an entirely different physiological category - unlike all other known fungi they are obligate anaerobes. High populations are present in the rumen of cattle and sheep and similar anaerobic fungi have been found so far only in the rumen or

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Department of Biochemistry, Microbiology and Nutrition, University of New England, Armidale, New South Wales 2351

hindgut of large herbivorous animals (Bauchop 1979b, 1983).

The obligately anaerobic fungi were first discovered in the rumen of sheep by Orpin (1975, 1977). However the importance of this discovery was not immediately obvious because of uncertainties about the classification of the microorganisms and because they appeared to be present in the rumen only in low numbers (Clarke, 1977).

The importance of this new group of microbes was brought to attention by the demonstration by means of scanning electron microscopy (SEM) that the fungi were present in large populations colonising fibrous plant fragments in the rumen of cattle and sheep (Bauchop, 1979a). Their close association with the more refractory plant fragments in the rumen digesta indicated a possible role in fibre digestion. Confirmation of cellulose digestion was obtained with pure cultures of fungi (Bauchop, 1979b; Orpin and Letcher, 1979), and later they were shown to carry out an active fermentation of cellulose (Bauchop and Mountfort, 1981). Recent studies on the glycosidase activities of one of these fungi, Neocallimastix frontalis, have demonstrated production of the range of extracellular enzymes required to degrade cellulose and hemicellulose to simple sugars (Pearce and Bauchop, 1985).

Fungi similar to the rumen organisms have also been shown to be present colonising plant fragments in the foregut or hindgut of herbivorous animals from different mammalian families from diverse geographical locations (Bauchop, 1981, 1983). Thus it appears that anaerobic fungi may be ubiquitous in at least the larger forms of herbivorous animals. Although as yet these non-ruminant organisms have been little studied their general properties are similar to those of the rumen fungi with regard to substrates utilized and fermentation products formed (T. Bauchop, unpublished).

The discovery of large populations of cellulolytic anaerobic fungi in the rumen has extended the known range of cellulose-fermenting microbes there. Present evidence suggests that the anaerobic fungi fill an important role in the rumen ecosystem with their preferential colonisation and growth on fibrous plant fragments contributing to degradation of fibrous feedstuffs.

Finding means to increase utilisation of high-fibre forages presents a major challenge to ruminant nutritionists and the potential for manipulation of the rumen anaerobic fungi would appear to present an important avenue for exploration.

II. EXPERIMENTAL AND RESULTS

(a) Life-cycle of rumen anaerobic fungi

The life cycle of the anaerobic fungi consists of a stage involving a non-motile, vegetative, reproductive form and a stage where a motile flagellated cell is produced. The developmental cycle of a common anaerobic fungus, N. frontalis, is shown in Fig. 1.

The mature vegetative organism consists of a sporangium and a much-branched rhizoid of thread-like hyphae that grow within the tissues of the invaded plant fragment. The sporangium develops on the exterior of the plant fragment and at maturity is commonly about 100μm long. Zoospores are formed within the sporangium and the eventual dissolution of part of the sporangium wail results in the release of flagellated zoospores, each of which can attach to a suitable plant fragment and develop into a new vegetative organism, thus repeating the life-cycle.

The development of these fungi was first determined during growth in culture medium with glucose as substrate (Orpin, 1975). However the key to understanding the life-cycle in the rumen was the appreciation of the central role of rumen digesta plant fragments as the substrata for the colonisation, growth and development of the fungi (Bauchop, 1979a).

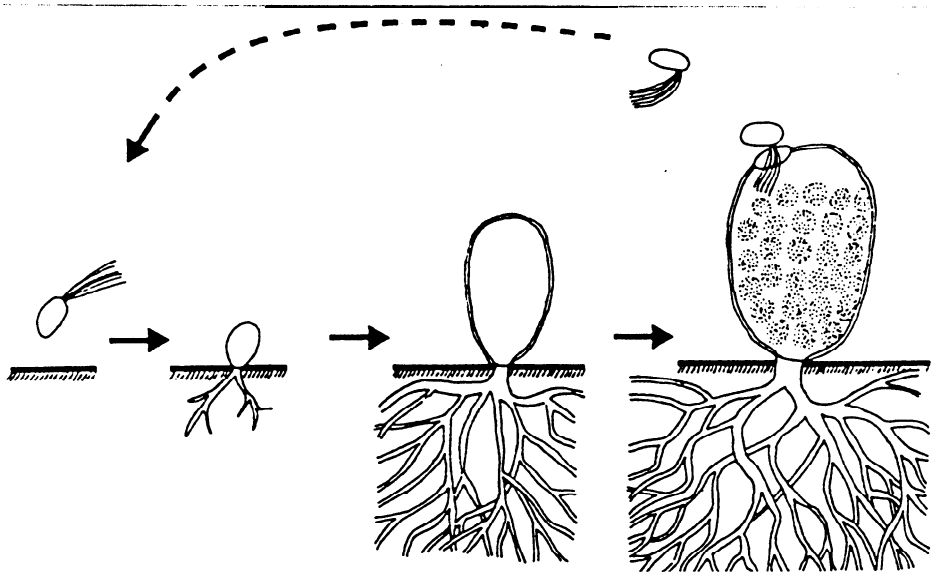

Fig. 1. Developmental cycle of the rumen anaerobic fungus Neocallimastix frontalis

In experiments with plant fragments suspended in nylon bags in the rumen of sheep, zoospores were found to invade plant fragments within 15 min and by 2 h large numbers had colonised exposed vascular cylinder of stem fragments. By 3 h, development of rhizoid was demonstrated by means of trypan-blue stain using samples of wheat straw leaf as substrate. The thinness of the leaf tissue together with the absence of masking effects of chlorophyll facilitated observation of rhizoid within the tissues of these stained preparations. Subsequent growth of fungi over a period up to 24 h resulted in development of large rhizoids with extensive invasion of leaf tissues.

The sporangium was found to develop from the original attached spore but did not begin to increase in size until about 12 h, although by this time rhizoid development was already extensive. By approximately 24 h the sporangium reached maximum size, Ruptured sporangia found attached to plant fragments after 24-30 h in the rumen indicated the time span of the life-cycle under conditions of once-daily feeding.

The existence of a number of different fungi in the rumen of cattle and sheep was indicated by morphological differences of zoospores and thalli, as well as by differences in their growth characteristics in pure culture. However detailed descriptions and classification of these fungi have not yet been completed. The first important step in setting the classification of these fungi on a firm basis was the assignment of the organism, N. frontalis, to a new family, the Neocallimasticaceae, in the Spizellomycetales of the Chytridiomycetes (Heath et al., 1982). Although some doubts remain about the phylogenetic affinities of Neocallimastix due to the many unusual properties of this organism (Heath and Bauchop, 1985) it is clear that the rumen anaerobic fungi are classed with the more primitive aquatic fungi possessing relatively simple life-cycles.

(b) Colonisation of plant fragments in the rumen

Large populations of rumen anaerobic fungi attached to plant fragments were demonstrated first in the rumen of sheep and cattle by means of SEM (Bauchop, 1979a). Following the discovery that fibrous plant fragments were the major colonisation sites it was shown that the rumen fungal populations could be observed readily by means of light microscopy using either stained or unstained preparations of plant fragments from the rumen (Bauchop, 1980). Examination of plant fragments by either SEM or light microscopy revealed that highest populations of fungi were present in animals receiving stalky high-fibre feeds such as chaffed lucerne hay' (Medicago sativa L.) in sheep (Fig. 2), and meadow hay in both cattle and sheep (Fig. 3). Under grazing conditions also, many fungi were present colonising rumen plant fragments, particularly when pasture plants were mature andstalky, Cattle grazing pastures of predominantly perennial ryegrass (Lolium perenne L.) and sheep grazing a pure stand of perennial ryegrass also provided examples of high fungal populations. In sheep grazing a pure stand of the legume lotus (Lotus pendunculatus L.) somewhat lower populations of fungi were present, although these plants likewise had developed to a stalky stage. By comparison, with sheep fed diets relatively low in fibre, anaerobic fungi could not be detected in rumen contents, either by direct microscopic examinations of plant fragments or by culture techniques for isolation of anaerobic fungi. The diets that gave negative results included: continuously grazed pasture that did not have the opportunity to develop stalks or seedheads; young pure stands of lucerne, red clover (Trifolium pratense L.) or white clover (Trifolium repens L.); barley grain (85%) diet.

These results, summarised in Table 1, demonstrated that high-fibre diets supported the greatest populations of anaerobic fungi. With once-daily feeding the fungi appeared to require approximately 24 h to complete their life cycle (Bauchop, 1979a) and thus require substrates of long turnover-time (>24h) in the rumen. Conversely the absence of fungi from the rumen of sheep ingesting soft leafy plants was explained by the short turnover-time (5-8h) of these feeds in the rumen resulting in failure of the fungi to maintain themselves.

Table 1 Populations of anaerobic fungi in the rumen of sheep and cattle on different diets

Animal	Diet	Fungal population[a]
Cattle/sheep	Meadow hay	4
Sheep	Chaffed lucerne hay	4
Sheep	Pelleted lucerne	2
Cattle/sheep	Perennial ryegrass	2
Sheep	Lotus pedunculatus	1
Sheep	Barley grain (85%) diet	N.D.[b]
Sheep	Continuously grazed pasture	N.D.
Sheep	Young lucerne, red clover or white clover	N.D.

[a]Fungal populations scored on a sale of 1 to 4 (high)
[b]N.D. = Not detected

Additional information on the size of the fungal populations has been obtained from in vivo experiments. Extensive fungal colonisation has been demonstrated with many different plant materials suspended in nylon bags in the rumen of cattle and sheep fed meadow hay or chaffed lucerne hay (Bauchop, 1979a). In nylon-bag experiments both fresh and dried materials were used and included leaf and stem fragments of the grasses and legumes that comprise common

Fig. 2. SEM of sporangia of rumen anaerobic fungi attached to lucerne stem fragment from natural digesta of a sheep, 24 h after feeding with chaffed lucerne hay. Bar marker = 50 µm.

Fig. 3. SEM of sporangia of rumen anaerobic fungi attached to a stem fragment of red clover from natural digesta of a steer, 24 h after feeding with meadow hay. Bar marker = 50 µm.

feedstuffs, Samples of the hard fibre, sisal (from Agave sisalona L.), commonly used as garden twine, was likewise heavily colonised. Normally several pieces of individual samples were suspended together in the nylon bags, and in all experiments every piece of plant material was found to be colonised by dense populations of fungi,

(c) Sites of colonisation

The rumen anaerobic fungi were found associated mianly with vascular tissues of stems and leaves, the plant tissues retained for the longest periods in the rumen. Principally stems were colonised with fungi attached to the vascular cylinders, both in legumes and grasses. However it was clear from microscopy studies that thin-walled tissues were colonised also (Bauchop, 1979a). Following fungal invasion, rhizoids eventually extended from the invasion sites to the vascular tissues where penetration and anchorage to this refractory material ensured extended retention in the rumen. Leaves were found to be colonised by fungi also but the relatively soft leaves of lucerne were invaded to only a limited extent, and fungi were found attached mainly to vascular tissues. Leaves of grasses were found to be more heavily colonised than lucerne leaves. Leaves of the warm-season forage Digitaria pentzii (Stent) were found likewise to be readily invaded by anaerobic fungi in the rumen of sheep (Akin et al., 1983). More refractory leaf material, such as wheat straw leaf, when suspended in nylon bags in the rumen of cattle and sheep for periods up to 24h, was heavily colonised by anaerobic fungi, with areas around the leaf ribs being densely populated by fungi (Bauchop, 1979a).

Like other rumen microbes the fungi appear unable to invade intact plant epidermal surfaces (Bauchop, 1980). The major route of invasion by fungi, with all of the plant fragments studied, was via areas of epidermal damage. The hollow stems of graminaceous plants were colonised predominantly on the interior surfaces. Colonisation via stomata was observed also, but appeared comparatively unimportant with stem fragments of both legumes and grasses. However stomata1 invasion was found more frequently with leaves of grasses and was found most extensively with wheat straw leaves. However as with stem fragments, damaged areas of leaves were most heavily colonised, Hyphal penetration through stomata has also been reported to occur frequently with leaves of D. pentzii (Akin et al., 1983).

(d) Fermentative activities of rumen anaerobic fungi

Rumen anaerobic fungi were isolated readily using anaerobic roll-tube techniques with addition of antibiotics to the culture media. Pure cultures of the fungi were found to colonise and grow readily on fragments of any of the common fibrous feedstuffs incorporated into anaerobic culture media. The discovery of the extent of attachment, colonisation, and growth of the fungi on fibrous plant fragments in the rumen had indicated a role in fibre digestion (Bauchop, 1979a). Information on the enzymic and fermentative activities of the fungi has come from both in vitro and in vivo studies. Digestion of plant cell walls was demonstrated most clearly with wheat straw leaf as substrate and the extensive degradation involved digestion of cell walls of epidermal long cells (Bauchop, 1979a, 1981). By comparison adjacent epidermal short cells resisted digestion, presumably because they are highly silicified. In experiments with sheep fed the forage D. pentzii, nylon bag digestibility studies showed that by 6h fungi preferentially colonised the lignified cells of leaf blade sclerenchyma and by 24h caused extensive degradation (Akin et al,, 1983).

Direct evidence for production of cellulase was provided by culturing fungi on strips of filter paper (Bauchop, 1979b, 1981; Orpin and Letcher, 1979). Initially following zoospore attachment the fungi developed as distinct colonies and the paper was digested only in areas of fungal growth. Successive sporulations

resulted in extension of the area of colonisation and eventually, after approximately 5 days, the paper strip was almost completely digested with the paper fibres replaced by a semi-transparent mat of fungal tissues.

In similar experiments carried out with the rumen fungus, N. frontalis, the products of cellulose fermentation were found to be acetate, lactate, ethanol, formate, CO_2 and H_2 (Bauchop and Mountfort, 1981)(Table 2). Hydrogen formation has not been reported previously in fungi, a further indication of the unusual nature of these microorganisms.

The discovery that the fungi form stable cocultures with rumen methanogenic bacterium with resultant conversion of the fungal products H_2 and CO_2 to methane (Bauchop and Mountford, 1981; Mountfort et al,, 1982)(Table 2) provides an example of one form of microbial interaction that is probably common in the rumen ecosystem. In the fungus-methanogen coculture, cellulose degradation commenced earlier, the rate was faster and the quantity digested was greater than in the monoculture (82 versus 53%). Recently the yield of cellulase also was shown to be increased in the coculture compared with the monoculture (Mountford and Asher, 1985).

Table 2 Fermentation of cellulose by Neocallimastix frontalis in the absence and presence of rumen H_2-formate-ulitising methanogenic bacteria

Product	mol/100 mol of hexose units	
	Fungus alone	Fungus plus rumen methanogenic bacteria
Acetate	72.7	134.7
Lactate	67.0	2.9
Ethanol	37.4	19.0
Formate	83.1	1.0
Carbon dioxide	37.6	88.7
Hydrogen	35.3	<0.5
Methane	0.0	58.7

Anaerobic fungi have also been cultured on Avicel, carboxymethylcellulose, cellophane, xylans and starch as well as on a range of simple sugars (Orpin and Letcher, 1979; Joblin, 1981; Pearce and Bauchop, 1985). Recently the cellular distribution and nature of the glycosidases produced by N. frontalis have been investigated (Pearce and Bauchop, 1985).

Cellulose-grown fungal cultures were fractionated into extracellular, membrane and intracellular fractions and assayed for glycosidase activity using as substrates, Avicel, carboxymethylcellulose, xylan, starch, polygalucturonic acid, and p-nitrophenyl-derivatives of galactose, glucose and xylose. Enzymic activity was highest in the extracellular fraction although the membrane fraction also displayed appreciable activity. The intracellular fraction was inactive towards all substrates. Polygalacturonic acid was the only substrate not hydrolysed by the active fractions indicating that pectinase was absent. Inability to ferment pectin or polygalacturonic acid has been found consistently in several as yet unclassified anaerobic fungi (T. Bauchop, unpublished). Of the major plant fibre components in forage, pectin is the most rapidly fermented by the rumen microbiota, The lack of pectinase in anaerobic fungi may thus relate to the relatively long time (c. 24 h) required for the fungal life cycle in the rumen.

The results of the glycosidase studies demonstrated that the common rumen fungus, N. frontalis, produced the range of enzymes necessary to degrade cellulose and hemicellulose to simple sugars,

(e) Potential for manipulation of rumen fungi

The discovery that highest fungal populations were present in the rumen of animals receiving the most fibrous diets indicates an area with potential for exploitation by manipulation of the fungi,

Increases in fungal populations or in digestive activities would present the obvious goals. However the great complexities of the rumen ecosystem and of the interrelationships within the microbiota make the outcome of manipulative efforts difficult to predict. Nevertheless the fungi, like the ciliate protozoa, have the considerable advantage over most rumen bacteria in that changes in levels of populations can be observed relatively easily by direct microscopic examination.

At present two treatments have been discovered that give rise to increased fungal populations in the rumen. In both cases feed intake and digestibility were also increased but casual relationships could not be established as other changes in the microbiota could not be excluded. However the results are of considerable interest.

Anaerobic fungi were not detected in sheep fed sulphur-deficient D. pentzii hay whereas hay collected following sulphur fertilisation supported an active population of rumen fungi (Akin et al, 1983). Marked increases in the numbers of rumen fungi were also obtained by dietary supplementation of low-sulphur hay with DL-methionine (Cordon, 1985). These results indicate a need for fundamental information on the nutritional requirements of the anaerobic fungi.

In the second treatment, using sheep offered a straw-based diet, defaunation of the ciliate protozoa with the anionic detergent Alkanate 3SL3 was found to be accompanied by increased rumen numbers of fungal zoospores and of fungi on feed fragments (Soetanto, 1985). Changes in rumen bacteria were not monitored.

These two findings show at least that it is possible to effect changes in fungal populations of the rumen and that increased populations were associated with improved intake and digestibility of roughage diets.

Investigations to increase digestive activity of rumen fungi offer an additional avenue for exploration. In this case biochemical information on the enzymes involved and on related regulatory controls are required. Preliminary work in this area has been initiated already (Pearce and Bauchop, 1985; Mountfort and Asher, 1985). Mutagenesis and selective screening for high yielding mutants is a simple approach to this line of work which has achieved successes with the production of high yielding cellulase mutants of the fungus Trichoderma reesei from soil.

III. CONCLUSION

The rumen anaerobic fungi have been shown to be active cellulolytic organisms that specifically colonise and grow on fibrous plant fragments, These properties taken together with the apparent extent of the rumen populations on plant fragments in animals receiving high-fibre diets indicates a significant role for the fungi in fibre digestion. In addition fungi may bring special properties to the degradation of plant materials in the rumen. As a result of their mode of growth involving hyphal extension, once attached to plant fragments, fungi can exert a pressure enabling them to penetrate deeply into tissues inaccessible to rumen bacteria or protozoa,

Although it now seems clear that the anaerobic fungi are well equipped enzymically to contribute to rumen fermentation, the major question of the extent of this contribution remains to be answered, Unlike. the rumen bacteria or protozoa the magnitude of the fungal populations cannot be measured by enumeration of any of the stages of their life cycle, Instead, it would seem necessary to determine the quantity of fungal tissues in the rumen in order to assess their importance, and this requires the development of a reliable method for determination of the fungal mass.

Despite this query about the precise quantitation of the role played by the rumen anaerobic fungi, their high populations and their special properties suggest them strongly as one group of microbes with potential for manipulation to produce improved rumen fermentation. Production of high-yielding cellulase mutants would seem to be one obvious starting point in the attack on the complex problem of improved high-fibre forage utilisation.

IV REFERENCES

AKIN, D.E., GORDON, G.L.R. and HOGAN, J.P. (1983). Appl, Environ. Microbiol. 46: 738.

BAUCHOP, T. (1979a). Appl. Environ. Microbiol. 38: 148.

BAUCHOP, T. (1979b). Annal. Rech. Vet. 10: 246.

BAUCHOP, T. (1980). In 'Contemporary Microbial Ecology', p. 305, eds. D.C. Ellwood, J.N. Hedger, M.J. Latham, J.M. Lynch and J.H. Slater. (Academic Press: London).

BAUCHOP, T. (1981). Agriculture and Environment 6: 339.

BAUCHOP, T. (1983). In 'Fibre in Human and Animal-Nutrition', p. 143, eds. G. Wallace and L. Bell. (Royal Society of New Zealand: Wellington).

BAUCHOP, T. and MOUNTFORT, D.O. (1981). Appl. Environ. Microbiol. 42: 1103.

CLARKE, R.T. J. (1977). In 'Microbial Ecology of the Gut', p. 251, eds. R.T.J. Clarke and T. Bauchop. (Academic Press: London).

GORDON, G.L.R. (1985). In 'Biotechnology and Recombinant DNA Technology in the Animal Production Industries*, p. 124, eds. R.A. Leng, J.S.F. Barker D.B. Adams and K.J. Hutchinson. (University of New England).

HEATH, I.B. and BAUCHOP, T. (1985). Can. J. Bot. 63: 1595.

HEATH; I.B., BAUCHOP, T. and SKIPP, R.A. (1982). Can. J. Bot. 60: 295.

JOBLIN, K.N. (1981). Appl. Environ. Microbiol. 42: 1119.

MOUNTFORT, D.O. and ASHER, R.A. (1985). Appl. Environ. Microbiol. 49: 1314.

MOUNTFORT, D.O., ASHER, R.A. and BAUCHOP, T. (1982). Appl. Environ. Microbiol. 44: 128.

ORPIN, C.G. (1975). J. Gen. Microbiol. 91: 249.

ORPIN, C.G. (1977). J. Gen. Microbiol. 99: 215.

ORPIN, C.G. and LETCHER, A.J. (1979). Curr. Microbiol. 3: 121.

PEARCE, P.D. and BAUCHOP, T. (1985). Appl. Environ. Microbiol. 49: 1265.

SOETANTO, H. (1985). M.Sc. Thesis, University of New England.-