Role and Potential of Ruminal Fungi in Fiber Digestion
– Review –

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ABSTRACT : Anaerobic chytridiomycete fungi are now well recognized as one of the major components of rumen microflora. Since the discovery of anaerobic fungi, the knowledge upon their morphology and physiology has been accumulated. It is certain that they have roles in ruminal fiber digestion, although their quantitative contribution to rumen digestion is still unclear. Their role in fiber digestion is complicated by the dietary factors and the interaction with other microorganisms. We aim at reviewing such information in this article.

Considerable attention has been paid to the polysaccharidase of these fungi. Analysis on the fungal genes encoding these enzymes has been performed in several laboratories. This article also covers the genetical analysis of fungal polysaccharidases.

(Key Words: Rumen, Anaerobic Fungi, Chytridiomycetes, Fiber Degradation)

INTRODUCTION

Interests in the ruminal anaerobic fungi have been growing in recent decades especially on their capacity for fiber digestion. Several excellent reviews have been published so far (Orpin and Joblin, 1988; Joblin, 1990; Fonty and Joblin, 1991). We aim at discussing the roles of rumen chytridiomycete fungi in the ruminal fiber degradation. We have conducted a joint research program on this subject supported by Japan Society for Promoting Science and Korea Science and Engineering Foundation. The basic knowledge on classification and culture techniques of these particular microorganisms will also be presented.

MATERIALS AND METHODS

Taxonomy of anaerobic chytridiomycete fungi

The ruminal fungi are unique in that they are the only known strictly anaerobic fungi in the biosphere. However, monoflagellated zoospores of ruminal fungi were discovered long time ago by Liebetanz (1910) as monoflagellated protozoa and been given the names as *Piromonas communis* and *Sphaeromonas communis*. In 1913, Braune discovered a multiflagellated organism and named it *Callimastix frontalis*. From ultrastructural studies on *Callimastix* sp., Vavra and Joyon (1966) concluded that this organism belonged not to the protozoa, but to the fungi. They created a novel genus *Neocallimastix*. About ten years later, Orpin (1975) was the first to succeed in culturing these organisms and he defined their life cycle. Later he has demonstrated the presence of chitin in their cell-wall (Orpin, 1977a). He concluded that *N. frontalis*, *P. communis* and *S. communis* belong to the class *Chytridiomycetes* in subdivision *Mastigomycota*. This conclusion was confirmed by 18S rRNA sequence analyses on the rumen fungi (Doré and Stahl, 1991). Barr et al. (1989) involved the ruminal fungi into the order *Spizellomycetales* in sub-division *Mastigomycota* and separated ruminal fungi from *Chytridiomycetales* on the basis of the ultrastructural differences in zoospores. Heath et al. (1983) had proposed a new family *Neocallimastigaceas* in the order of *Spizellomycetales*. However, from recent rRNA analyses, Li and Heath (1992) and Li et al. (1993) have proposed to elevate the family *Neocallimastigaceae* to the new order *Neocallimasticales* in the *Chytridiomycota*. Five genera have been established so far; *Neocallimastix*, *Piromyces* (formerly *Piromonas*), *Caecomyces* (formerly *Sphaeromonas*), *Orpinomyces*, *Anaeromyces* (syn. *Ruminomyces*) and 17 species are known (table 1). The change in nomenclature for *Piromonas* and *Sphaeromonas* was proposed by Gold et al. (1988), because their former names were given as

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the protozoa. *Ruminomyces* and *Anaeromyces* have similar morphological features and considered to be synonymous (Ho et al., 1993a; Li et al., 1993). Species belonging to the former three genera develop monocentric thalli, and the latter two produce polycentric thalli. Among the rumen chytridiomycetes, species belonging to the genera *Neocallimastix* and *Orpinomyces* produce multiflagellated zoospores, and the remaining species produce monoflagellated zoospores.

Tabel 1. Classification of anaerobic chytridiomycete fungi

Genera and characteristics	Species	Source	Reference
Neocallimastix			
Monocentric, polyflagellate zoospore, extensive, filamentous rhizomycelium	*frontalis*	Sheep	Heath et al., 1983
	patriciarum	Sheep	Orpin and Munn, 1986
	hurleyensis	Sheep	Webb and Theodorou, 1991
	variabilis	Sheep	Ho et al., 1993b
Piromyces			
Monocentric, monoflagellate zoospore, filamentous rhizomycelium	*communis*	Sheep	Gold et al., 1988
	mae	Horse	Li and Heath, 1990
	*dumbonicus**	Elephant	Li and Heath, 1990
	*rhizinflatus**	Ass	Breton et al., 1991
	minutus	Deer	Ho et al., 1993c
	spiralis	Goat	Ho et al., 1993d
	citronii	Horse	Gaillard-Martinie et al., 1995
Orpinomyces			
Polycentric, polyflagellate zoospore, filamentous rhizomycelium	*joyonii*	Sheep	Li et al., 1991
	intercalaris	Cattle	Ho et al., 1994
Caecomyces			
Polycentric or monocentric, monoflagellate zoospore, spherical holdfasts	*communis*	Sheep	Gold et al., 1988
	equi	Horse	Gold et al., 1988
Anaeromyces			
Polycentric, monoflagellate zoospore, filamentous rhizomycelium	*mucronatus*	Cow	Breton et al., 1990
	elegans	Sheep	Ho et al., 1993a

* According to Ho and Barr, 1995.

The classification of fungi is based principally upon the morphological characteristics of the thallus. This is also true for the rumen chytridiomycetes fungi, but an analysis of the ultrastructural features of zoospores is often required for the species-level identification (Gold et al., 1988; Li et al., 1991). The traditional physiological tests, such as sugar utilization or end product formation for classification, do not seem to be efficient for identifying species, because little difference is usually observed in sugar utilization and end-product formation among genera (Phillips and Gordon, 1988; Gordon and Phillips, 1989). The G + C contents in fungal DNA has been measured in several strains (table 2). The G + C contents of these organisms are often as low as 13 to 20%, indicating the uniqueness of rumen fungi as true fungi. However the G + C contents of coding regions are usually as high as 40 to 45%, suggesting that the introns or non-coding sequences have very low G + C contents. Brownlee (1994) has proposed the use of DNA base sequence of the spacer non-coding region between 18 S rRNA gene and 28S rRNA gene. Further molecular level analyses on the rumen chytridiomycetes fungi are required for the efficient identification of the species.

Isolation of rumen chytridiomycetes

Most anaerobic chytridiomycetes fungi have been

isolated from the rumen, but some of them have been isolated from hind gut contents or feces of a range of herbivores belonging to orders Marsupialia, Rhodentia, Proboscidea, Perissodactyla, and Artiodactyla (Li and Heath, 1993). Usual techniques used for the isolation of rumen anaerobic bacteria are still useful for the isolation of rumen chytridiomycetes. The methods published by Joblin (1981) and Lowe et al. (1985) are routinely used in many laboratories. The selection of rumen chytridiomycetes is facilitated by the addition of antibacterial agents. Pure cultures are usually maintained by a frequent transfer (usually 2-3 days interval) to the fresh media. Cryopreservation of zoospores or mature thalli are also possible (Yarlett et al., 1986a), but it is not certain that all strains do persist during storge. However, the cultures can sometimes be maintained for long period at 39℃ under usual culture condition (Joblin, 1981). This suggests the presence of dormant spores or some kinds of resting cells.

Tabel 2. DNA Base composition of anaerobic fungi

Organisms	G + C mol %	Organisms	G + C mol %
Neocallimastix		*Caecomyces*	
N. sp. LM-2[a]	18	*C. communis*[a]	22.1
N. frontallis MCH3[c]	17.9	*Anaeromyces*	
N. sp. N1[c]	21.2	*A. mucronatus*[b]	15.7
N. sp. B10[c]	21.2	*Orpinomyces*	
N. variabilis Bm2[c]	23.7	*O. joyonii*[a]	14.6
		O. sp. 01[c]	24.5
Piromyces			
P. comunis[b]	15.5		
P. communis 93-3[c]	21.2		
P. sp. P1[c]	34.1		

[a] Brownlee, 1989.
[b] Billion-Grand et al., 1991.
[c] Kozawa et al., 1993.

Population sizes of chytridiomycetes in the rumen

The quantification of biomass of rumen chytridiomycetes is still difficult. Determination of only the relative population size is possible. The numbers of viable zoospores are usually estimated as colony forming units (CFU) by roll-tube techniques. Thallus forming units (TFU) based on the most probable number technique using filter strip broth has been proposed by Theodorou et al. (1990). Enumeration of zoosporangia developed on agar strips containing carbohydrates after incubation within the rumen has also been used (Ushida et al., 1989a). Recently, we have developed a novel *in vitro* culture of rumen fungi in the dual-phase medium, a solid medium containing carbohydrate overlaid by a liquid medium. Enumeration of zoosporangia under a binocular microscope was done after 6h incubation (Kojima et al. unpublished). Analysis of chitin or protein provides the good estimation of biomass of pure cultures. It does not work well *in vitro*, however. Recent development of 18S-rRNA targeted oligonucleotide probes may give more precise estimation for fungal biomass not only in the rumen, but also in duodenum or feces (Millet et al., 1996; G. J. Faichney, personal communication).

Chytridiomycetes fungi appear in the rumen of young ruminants about ten days after their birth (Fonty et al., 1987). The development of the subsequent population depends on the nature of diet. Forage-based diets promote the development of fungal populations, but starch-based diets lower the population from the young animals. In general, diets rich in fiber promote a larger population of chytridiomycetes fungi in the rumen of adult ruminants. Although their capacity for starch utilization has been stressed by certain researchers (McAllister et al., 1993), their dependence on fibrous feed is obvious (Grenet et al., 1989). Little information is available about generic composition of chytridiomycetes in the rumen. Monocentric species (mostly *Neocallimastix*) had seemed to be predominant in sheep rumen in earlier studies (Ushida et al., 1993), and polycentric species (*Orpiomyces* and *Ruminomyces*) have been isolated almost entirely from cattle. However recent DNA probe works suggested the predominance of polycentric species even in the sheep rumen (Millet et al., 1996). Since polycentric species often produce low numbers of zoospore in pure culture, it is often difficult to determine by the usual roll-tube technique whether polycentric species are the predominant chytridiomycetes in the rumen.

Carbohydrate fermentation and hydrolytic enzymes

The rumen chytridiomycetes fungi have an array of polysaccharidases (*endo*-glucanase, *exo*-glucanase, xylanase, cellodextrinase, amylase), glycosidases (α-and β-glyosidase, β-fructosidase, β-xylosidase, α-L-arabinofuranosidase etc.), and esterase (acetylxylan esterase, *p*-coumaroyl esterase, feruroyl esterase) (Pearce and Bauchop, 1985; Williams and Orpin, 1987ab; Williams and Withers, 1981, 1982; Joblin et al., 1990; Borneman et al., 1990, 1991; Matsui et al., 1992). Therefore rumen chytridiomycetes can ferment a wide range of carbohydrates in plant materials. Most species ferment plant polymers, such as cellulose, xylan and starch, but cannot

ferment pectin and inulin. Certain species cannot grow on cellulose without coexistence of soluble sugar such as cellobiose (Ushida and Matsui, unpublished observation).

Rumen chytridiomycetes also have protease activities (Wallace and Joblin, 1986; Asao et al., 1993). Proteases may have role in cell-wall degradation, because the plant structural protein, such as extensin, increases the integrity of plant cell-wall (Fry, 1986). To test this hypothesis, the effect of protease inhibitors on plant cell-wall digestion by fungus was estimated. Either L-cystein, a metalloprotease inhibitor, or phenylmethylsuphonyl fluoride (PMSF), a serine protease inhibitor, was added to the fungal culture. These inhibitors were effective against protease produced by ruminal fungi (Asao et al., 1993). PMSF did not affect fungal *endo*-glucanase and xylanase activities. However it may inhibit *exo*-glucanase activity as it reduced crystalline cellulose (Avicel) degradation. Thus the effect of PMSF on timothy hay degradation was not further determined. Metalloprotease inhibitor (L-cystein) reduced timothy hay degradation (dry matter, neutral detergent fiber and acid detergent fiber) by fungus though it did not affect *endo*-glucanase and xylanase activitites as well as Avicel degradation (table 3). Possession of protease is a unique characteristic of rumen chytridiomycetes as rumen cellulolytic organisms, because the major ruminal celluloytic bacteria are not proteolytic.

Tabel 3. Effect of Protease inhibitors on fiber digesting capacity of ruminal polycentric fungus

Inhibitor	L-cystein		PMSF	
	0 mM	2 mM	0 mM	1.5 mM
Extrcellular CMC ase[a)]	100	96.0	100	103.6
Extracellular xylanase[a)]	100	107.2	100	105.0
Avicel degradability[b)]	29.2 ± 1.4	30.7 ± 1.1	37.5 ± 2.1	23.1 ± 1.8**
Extracellular proteinase[bc)]	11.1 ± 1.1	6.6 ± 1.0*	13.8 ± 1.4	10.4 ± 0.7*
Timothy hay degradation[ad)]				
Dry matter	42.2 ± 0.5	39.4 ± 0.6**	ND	ND
NDF	50.2 ± 0.3	46.7 ± 0.4**	ND	ND
ADF	47.9 ± 0.4	46.3 ± 0.3**	ND	ND

[a)] Relative activity to control (100) on the day 7 of culture on timothy hay medium. n = 3.
Sugars in Lowe's B medium was replaced by ground timothy hay (1% w/v).
[b)] % digested during 7 days. n = 3. Means ± Standard deviation.
Sugars in Lowe's B medium was replaced by crystalline cellulose (3% w/v).
[c)] unit/ml culture spernatant (1 unit = 1 mg azocasein hydrolyzed/h).
Assayed on the same samples as avicel degradability. n = 3. Means ± Standard deviation.
[d)] % digested during 7 days. n = 3. Means ± Standard deviation.
NDF, neutral detergent fibr; ADF, acid detergent fiber. Medium, see (a).
* $p < 0.05$, ** $p < 0.01$, ND, not determined. PMSF, phenylmethylsulfonyl fuloride.

Most of the strains produce hydrogen, carbon dioxide, formate, lactate, succinate, acetate and ethanol. Certain species do not produce succinate and ethanol. Unlike bacteria, fermentation pathway may not vary between genera since the fermentation end products are quite similar. Metabolic pathway has been defined in three strains of *Neocallimastix* (Yarlett et al., 1986b; O' Fallon et al., 1991; Marvin-Sikkema et al., 1994). *N. patriciarum* metabolizes glucose through EMP pathway to phosphoenol pyruvate that is further metabolized to pyruvate with two intermediate products, oxaloacetate and malate. Reoxidation of NADH generated during glycolysis is coupled with hydrogen production, the reduction of pyruvate and acetoaldehyde. Since this strain produces only trace amount of ethanol and formate, hydrogen and lactate become the major electron sink. *N. frontalis* possess a similar carbon and electron flow system.

Role of chytridiomycetes fungi in ruminal fiber degradation

Rumen chytridiomycetes appear to release zoospores within 30 minutes after feeding (Orpin, 1977b; Orpin and Bountiff, 1978). Free zoospores move in the liquid by chemotactic response to soluble carbohydrates towards damaged area or stomate of the feed particles newly ingested by the host. After attachment of the zoospores to

the feed particles, flagella are detached from the zoospores, then encystement and germination occur, followed by penetration of the plant tissues by the rhizoids and form the sporangia. This type of life cycle implies that chytridiomycetes may have a unique mode of action in rumen fiber digestion. Their colonization certainly weakens the integrity of plant tissues and fragmentation of feed particles would proceed (Carderon-Cortes et al., 1989; Akin et al., 1990). The rumen chytridiomycetes appear to preferentially colonize lignified tissues such as sclerenchyme and xyleme that remain within the reticulo-rumen longer than other tissues (Bauchop, 1979; Grenet et al., 1989).

This observation may be explained by the life cycle of the rumen chytridiomycetes and the maturation time of 24 to 32 h after encystement in the rumen (Lowe et al., 1987).

Most strains tested so far degrade hay particles such as timothy or ryegrass by 40 to 70% during 6 to 8 days under batch culture conditions. In a continuous culture system(*i.e.* Rusitech), an addition of one strain of *Neocallimastix* to the mixed rumen bacteria increased degradation rate of wheat straw by 15% (Hillaire and Jouany, 1989). Fonty and Gouet (1989) inoculated either *N. frontalis, P. communis* or mixed rumen chytridiomycetes to the rumen of new born lambs which had been individually introduced into a sterile isolator before the establishment of cellulolytic flora in the rumen. They also prepared the lambs which harbored *Fibrobacter succinogenes* or *Ruminococcus flavefaciens* as the sole cellulolytic organisms. Then they deterimined the *in sacco* digestibility of wheat straw or ryegrass hay in the rumen of these lambs.

The results indicated that lambs harbouring chytridiomycetes digested fiber less efficiently than those harboring cellulolytic bacteria. However, in the case of the mature rumen, elimination of chytridiomycetes fungi from the rumen by the chemical means often decreases the plant fiber digestion (Windham and Akin, 1984; Ford et al., 1987, Carderon-Cortes et al., 1989; Ushida et al., 1989b; Gordon and Phillips, 1993). Introduction of fungal isolates into the sheep rumen enhanced feed degradation in the rumen (Ha et al., 1994). The overall role of rumen chytridiomycetes in fiber digestion is still debatable.

Interaction between chytridiomycetes and other microorganisms in the rumen

The fiber degrading activity of rumen chytridiomycetes is complicated by the interaction with other microorganisms. Coculture studies have been done in several laboratories in order to define interactions between bacteria and chytridiomycetes. Coculture with methanogenic bacteria enhanced fiber digestion by chytridiomycetes with few exceptions (Ushida, 1993). This kind of interaction is defined as inter-species hydrogen transfer which improves metabolic activity of hydrogen producing organisms (Wolin and Miller, 1983).

The presence of methanogens also improves the specific activity of fungal cellulases (Joblin and Williams, 1991). However the presence of sulfate reducing bacteria, same hydrogentroph as methanogens, did not enhance metabolic activity of rumen chytridiomycetes (Ushida et al., 1995; Morvan, 1996).

Coculture with saccharolytic bacteria including several celluloytics often decreased fiber digestion by rumen chytridiomycetes (Ushida, 1993). The negative effect of some strains of *Ruminococcus*, *Butyrivibrio*, and *Megasphaera* were evident. The mechanisms involved in this negative interaction have not been well defined except for *R. flavefaciens* 007. This bacterium produces the protein that inhibits *endo*-glucanase of chytridiomycetes (Bernalier et al., 1993). These authors identified two inhibitory proteins of 24 kDa and 100 kDa in the bacterial supernatant. These proteins were suggested to be enzymes that act on the fungal cellulase complex. These inhibitory proteins may have chytinolytic activity that can release a cellulase complex from fungal cell wall to reduce cellulolytic activity (Kopecny et al., 1996). The significance of this inhibition under *in vivo* situation is not known. The effects of cellulolytic bacteria on the ability in fiber digestions of chytridiomycetes may depend on species and strain under investigation. Non significant or increased fiber digestion has been observed when different strains of cellulolytic bacteria were cocultured with rumen chytridiomycetes (Kim, 1997).

Rumen ciliate protozoa predate chytridiomycetes (Williams et al., 1994) and digest them (Morgavi et al., 1993), therefore defaunation (elimination of protozoa) often increase the population size of rumen chytridiomycetes (Ushida et al., 1989a, 1991).

Cloning and expression of polysaccharidase gene of rumen chytridiomycetes

Several *endo*-glucanases, xylanases, β-glucosidases and β-xylosidases have been purified from strains belonging to genera *Neocallimastix, Piromyces* and *Orpinomyces* (Hebraud and Fevre 1988, 1990; Li and Calza, 1991; Teunissen et al., 1992; Wilson and Wood, 1992; Garcia-Campayo and Wood, 1993; Gomez de Serga and Fevre, 1993; Chen et al., 1994; Zhou et al.,

1994).

These enzymes are all extra-cellular, and have acidic pH (5 to 6) and mesophillic temperature (50 to 55℃) optima. Rumen chytridiomycetes secrete potent polysaccharidases into the medium (Wood et al., 1986; Barichievich and Calza, 1990; Matsui et al., 1992; Wilson and Wood, 1992), but the protein production is usually small.

More than ten genes encoding polysaccharidases, such as *endo*-glucanases and xylanase have been cloned and expressed in *E. coli* so far (table 4). The *endo*-glucanases of rumen chytridiomycetes that have been sequenced so far were classified into *family* 5 – *subfamily* 4 – endoglucanase. Family 5 cellulases are also produced by aerobic true fungi such as *Trichoderma* spp., but these enzymes are classified into subfamily 5. Interestingly most of the *family* 5 *sub* 4 cellulases of anaerobic microbes are ruminal origin. High homology between rumen fungal endoglucanase genes and those of *Clostridia* and of *Ruminococci* suggested the horizontal transfer of cellulase gene. The *cel* B gene of *N. patriciarum* and *cel* A gene of *N. frontalis* having no intron (Zhou et al., 1994; Fujino et al., 1995) may support this hypothesis.

Cellulase genes of *Neocallimastix* have a reiterated sequence at the C-terminal region which is considered to code a binding structure with the scaffolding protein (Xue et al., 1992; Fanutti et al., 1995; Fujino et al., 1995).

These reiterated sequences strongly suggest the presence of cellulosomes in this group of fungi. Indeed, high molecular weight protein complexes have been detected in the culture supernatant of *Neocallimastix* (Wood et al., 1988) and *Piromyces* (Teunissen et al., 1992).

Tabel 4. List of sequence data on fungal polysaccharidases

Organism	Enzyme	gene	family	Genbank accession number	authors
N. patriciarum	Endoglucanase	*celB*	5	Z31364 X 77186	Zhou et al., 1994
N. patriciarum	Xylanase	*xynA*	11	X65526	Gilbert et al., 1992
N. frontalis	Endoglucanase	*celA*	5	U38843	Fujino et al., 1995
N. frontalis	Endoxylanase	*xyn1*	11	X82266	Durand et al., 1994
N. frontalis	Endoxylanase	*xyn2*	11	X82439	Durand and Févre, 1994
N. patriciarum	Xylanase	*xynB*	10	S71569 X 76919	Black et al., 1994
N. patriciarum	Cellobiohydrolase	*celA*	6	U29872	Denman et al., 1995
Piromyces sp.	Mannanase	*manA*	26	X91857	Fanutti et al., 1996
Piromyces sp.	Endomannanase	*manB*	26	X97408	Millward-Sadler et al., 1996
Piromyces sp.	Endomannanase	*manC*	26	X97520	Gilbert, 1996
Piromyces sp.	Xylanase	*xynA*	11	X91858	Fanutti et al., 1995
Orpinpmyces sp.	Xylanase	*xynA*	11	U57819	Li et al., 1996
Orpinpmyces sp.	Endoglucanase	*celB*	5	U57818	Li et al., 1996
Orpinpmyces	Cellobiohydrolase	*celA*	6	U63837	Li et al., 1996
Orpinpmyces	Cellobiohydrolase	*celC*	6	U63838	Li et al., 1996

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