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Fibrolytic Rumen Bacteria: Their Ecology and Functions*

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ABSTRACT : Among rumen microbes, bacteria play important roles in the biological degradation of plant fiber due to their large biomass and high activity. To maximize the utilization of fiber components such as cellulose and hemicellulose by ruminant animals, the ecology and functions of rumen bacteria should be understood in detail. Recent genome sequencing analyses of representative fibrolytic bacterial species revealed that the number and variety of enzymes for plant fiber digestion clearly differ between *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*. Therefore, the mechanism of plant fiber digestion is also thought to differ between these two species. Ecology of individual fibrolytic bacterial species has been investigated using pure cultures and electron microscopy. Recent advances in molecular biology techniques complement the disadvantages of conventional techniques and allow accurate evaluation of the ecology of specific bacteria in mixed culture, even *in situ* and *in vivo*. Molecular monitoring of fibrolytic bacterial species in the rumen indicated the predominance of *F. succinogenes*. Nutritive interactions between fibrolytic and non-fibrolytic bacteria are important in maintaining and promoting fibrolytic activity, mainly in terms of crossfeeding of metabolites. Recent 16S rDNA-based analyses suggest that presently recognized fibrolytic species such as *F. succinogenes* and two *Ruminococcus* species with fibrolytic activity may represent only a small proportion of the total fibrolytic population and that uncultured bacteria may be responsible for fiber digestion in the rumen. Therefore, characterization of these unidentified bacteria is important to fully understand the physiology and ecology of fiber digestion. To achieve this, a combination of conventional and modern techniques could be useful. (**Key Words** : Fiber Digestion, Rumen Bacteria, Molecular Ecology, Uncultured Bacteria)

INTRODUCTION

Cellulose, a main component of the plant cell wall, is the most common carbohydrate on earth, and its production is estimated to be 100 billion tons per year (Leschine, 1995). Ruminant animals are able to utilize cellulose as an energy source because of a symbiotic relationship with microbes in the rumen. To maximize the utilization of cellulose by ruminant animals, the ecology and functions of rumen microbes should be understood in detail. Rumen microbes are comprised of bacteria (10^{10}-10^{11} per ml), fungi (10^{3}-10^{6} per ml) and protozoa (10^{4}-10^{6} per ml) (Hespell et al., 1997; Orpin and Joblin, 1997; Williams and Coleman, 1997). Bacteria and fungi produce a wide range of highly active plant fiber degrading enzymes, while the contribution of protozoa to plant fiber digestion is estimated to be less significant in terms of the proportion of total NDF degrading activity (Dijkstra and Tamminga, 1995). Although rumen fungi possess superior ability to penetrate the plant cell wall and solubilize lignin, their contribution to fiber digestion may be low due to their small biomass (8% of total microbial mass, Orpin and Joblin, 1997). Rumen bacteria play a particularly important role in the biological degradation of plant fiber because of their much larger biomass and higher activity. Here, we summarize the ecology and functions of rumen bacteria involved in plant fiber digestion.

REPRESENTATIVE FIBROLYTIC BACTERIA

Rumen bacteria have been the subject of intensive studies over the past 50 years, and numerous studies have described the isolation and characterization of a variety of bacterial strains from various ruminant animals (Bryant, 1959; Stewart et al., 1997). Among major rumen bacteria, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Butyrivibrio fibrisolvens*, *Prevotella ruminicola*, *Eubacterium cellulosolvens* and *Eubacterium ruminantium* are recognized as fibrolytic bacterial species

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(Stewart et al., 1997). Varel and Dehority (1989) reported that the proportions of *F. succinogenes*, *R. flavefaciens* and *R. albus* in the total cellulolytic bacteria in cattle rumen were 33.0%, 2.6% and 46.0%, respectively. In addition, the ability of these three species to digest cellulose is much higher than that of other cellulolytic ruminal species. Therefore, *F. succinogenes*, *R. flavefaciens* and *R. albus* have been considered representative cellulolytic bacterial species in the rumen.

Plant fiber is largely composed of cellulose and hemicellulose (Van Soest, 1982). Cellulose is composed of β-1,4-linked glucose residues, while hemicellulose mainly consists of xylan assembled from β-1,4-linked xylose residues. The xylan is substituted with acetyl, arabinosyl, and glucuronyl residues. To degrade this complex matrix of polymers, the synchronous action of a wide range of hydrolytic enzymes is necessary. Numerous genes encoding enzymes involved in plant fiber degradation have been isolated from *F. succinogenes*, *R. flavefaciens* and *R. albus* (Barros and Thomson, 1987; Kawai et al., 1987; Sipat et al., 1987; Ohmiya et al., 1988; White, 1988; Flint et al., 1989; Gong et al., 1989; McGavin et al., 1989).

Recent genome sequencing analyses revealed that *R. flavefaciens* FD1 possesses at least 65 genes encoding glycoside hydrolases (Flint et al., 2008). The components of cellulosome were found in *R. flavefaciens* 17, and this species probably has a unique cellulosome structure (Rincon et al., 2005). Cellulosomal protein from *R. flavefaciens* 17 is sorted to the cell surface by the mediation of sortase (Rincon et al., 2005), one of the enzymes involved in protein anchoring to the Gram-positive bacterial envelope (Ton-That et al., 2004). In other cellulosome-producing bacteria such as *Clostridium thermocellum*, *Acetivibrio cellulolyticus* and *Bacteroides cellulosolvens*, the anchoring of the cellulosome to the bacterial cell wall arises *via* attachment to cell-surface protein (Schwarz, 2001). Rincon et al. (2005) suggested that the cellulosome of *R. flavefaciens* seemed to be intricate and versatile both in its modular and subunit arrangement and in its capacity to incorporate numerous types of cellulosomal components. Although more investigation is needed, the cellulosome of *R. flavefaciens* surely allows this species to degrade plant fiber highly efficiently.

F. succinogenes may have another system for plant fiber degradation. The complete genome sequence of this species indicates that 104 open reading frames encode putative enzymes involved in plant fiber degradation including 83 glycosyl hydrolases, 7 pectate lyases and 14 carbohydrate esterases (Morrison et al., 2003). The number and variety of enzymes for plant fiber degradation are obviously greater than those of the other common fibrolytic bacteria including *R. flavefaciens* (Jun et al., 2007). However, no genes from *F. succinogenes* have similarity with genes encoding cellulosome-related proteins present in other cellulolytic bacteria and fungi. Therefore, the mechanism of plant fiber degradation by *F. succinogenes* may be very different from that of *R. flavefaciens*.

Several studies on the cellulosome of *R. albus* have been published. Wood et al. (1982) reported that the cellulase activity of *R. albus* SY3 is cell-associated and exists as an unstable high molecular mass complex (1.5 MDa). Although some genetic evidence for the cellulosome complex of *R. albus* was obtained (Morrison and Miron, 2000; Ohara et al., 2000), details such as its components and structure remain to be elucidated.

ECOLOGY OF *F. SUCCINOGENES*, *R. FLAVEFACIENS* AND *R. ALBUS*

Bacteria inhabiting the rumen have been classified into four groups depending on their environmental habitat: 1, free-living bacteria associated with the liquid phase in the rumen; 2, bacteria associated with feed particles; 3, bacteria associated with rumen epithelium; and 4, bacteria attached to the surface of protozoa (Czerkawski and Cheng, 1988; McAllister et al., 1994). Microbial populations associated with feed particles are estimated to be responsible for 88-91% of ruminal endoglucanase and xylanase activity (Williams and Strachan, 1984; Minato et al., 1993). Bacterial roles are particularly important because bacterial populations associated with feed particles are predominant numerically, accounting for up to 75% of the total microbial population (Minato et al., 1993). These data indicate that fiber-associated bacterial populations are pivotal for ruminal fiber digestion. Because attachment is an essential step for fibrolytic bacteria to initiate digestion of plant fiber in the rumen, numerous investigations have explored various aspects of bacterial attachment to feed particles.

The ability of *F. succinogenes*, *R. flavefaciens* and *R. albus* to attach to plant fibers, as well as the mechanism of this attachment, have been studied using pure cultures (Minato and Suto, 1978; Mosoni et al., 1997; Pegden et al., 1998) and the digestive activities of these cultures have been visualized by electron microscopy (Latham et al., 1978; Cheng et al., 1980, 1981). Minato and Suto (1978) evaluated the cellulose attachment ability of ruminal bacteria *in vitro* and reported that *F. succinogenes* showed the highest attachment ability among common ruminal isolates. Mosoni et al. (1997) reported that attachment of *R. flavefaciens* and *F. succinogenes* peaked after 45 min of contact with limited cellulose. Roger et al. (1990) reported that *R. flavefaciens* attached to Avicel within 1 min of first contact whereas *F. succinogenes* required 30 min, thus suggesting the superiority of *Ruminococcus* spp. to *F. succinogenes* with regard to initial fiber attachment. Other studies also demonstrated competition among the three

fibrolytic species *in vitro* using cellulose as a substrate (Odenyo et al., 1994; Shi et al., 1997). Sung et al. (2007) reported the effects of pH on bacterial attachment to rice straw and the attachment of *F. succinogenes*, *R. flavefaciens* and *R. albus* was clearly inhibited when the pH was lower than 6.0.

Previous microscopic observations demonstrated a different mode of fiber attachment between *F. succinogenes* and *R. flavefaciens*. *F. succinogenes* tightly attaches to the surface of plant fiber and releases small vesicles (Cheng et al., 1983/1984; Gaudet and Gaillard, 1987). No vesicle formation was observed in *R. flavefaciens* cells attached to plant fiber, although a very extensive extracellular glycocalyx had diffused from the cells (Latham et al., 1978a). Latham et al. (1978b) demonstrated that *F. succinogenes* and *R. flavefaciens* each had specific attachment sites on fresh perennial ryegrass *in vitro*; *F. succinogenes* was predominant on the cut edges of mesophyll cell walls and the intact faces of mesophyll, while *R. flavefaciens* was predominant on the cut edges of epidermal cell walls.

Recent advances in molecular biology techniques allow the specific and accurate evaluation of fiber attaching ability *in situ*. We carried out quantitative PCR-aided monitoring of the kinetics of fiber attachment of *F. succinogenes*, *R. flavefaciens* and *R. albus* in the rumen (Koike et al., 2003a). After *in situ* incubation for 5 min, the number of *F. succinogenes* and the two ruminococcal species attached to orchardgrass hay stems was 10^5 and 10^4/g dry matter (DM) of stem, respectively. At 10 min, the number of all three species attached to the stems increased 10-fold. Thereafter, the attached cell number of the three species gradually increased and peaked at 24 h (10^9/g DM for *F. succinogenes* and 10^7/g DM for *R. flavefaciens*) or 48 h (10^6/g DM for *R. albus*). Shinkai and Kobayashi (2007a) successfully established a fluorescence *in situ* hybridization (FISH) protocol to visualize and localize specific bacteria associated with plant materials and detected *F. succinogenes* and *R. flavefaciens* attached to orchardgrass hay. They reported that *F. succinogenes* was found firmly attached not only to the cut edges but also to the undamaged inner surface of the hay, while *R. flavefaciens* was frequently detected on the leaf sheath of the hay and was associated with the formation of many pits on the surface of the sheath. These observations indicate that *F. succinogenes* and *R. flavefaciens* have different ecological niches *in vivo* and that *F. succinogenes* is metabolically active on less digestible fiber tissues, while *R. flavefaciens* prefers easily digestible fibers. The flexibility in attachment and growth of *F. succinogenes* was confirmed by *in situ* experiments in which *F. succinogenes* grew even on cellulase-treated rice straw that was much less degradable (Shinkai et al., 2007b).

The distribution of *F. succinogenes*, *R. flavefaciens* and *R. albus* in the rumen as quantified by molecular-based techniques is shown in Table 1. Irrespective of sample source, target molecule and approach, *F. succinogenes* was present at higher proportions than the two ruminococci with the exception of the report by Weimer et al. (1999), in which *R. albus* was present at a higher proportion than the others. Although host animal and diet differed between the experiments and these factors can affect the distribution of rumen bacteria, *F. succinogenes* has been found to be a particularly important species for fiber digestion in the rumen.

INVOLVEMENT OF NON-FIBROLYTIC BACTERIA IN FIBER DIGESTION

Fiber digestion *in vitro* can be improved in co-cultures of fibrolytic and non-fibrolytic species. The combination of fibrolytic species *F. succinogenes*, *R. flavefaciens* or *R. albus* with non-fibrolytic *Treponema* or *Butyrivibrio* species accelerates the rate of cellulose digestion (Cheng et al., 1991). Nutritive interactions including hydrogen transfer and crossfeeding of fermentation products and of oligomers and monomers derived from polymer degradation are important to maintain fibrolytic activity (Flint, 1997).

Scheifinger and Wolin (1973) first demonstrated that growth of the non-fibrolytic *Selenomonas ruminantium* occurs on cellulose in co-culture with *F. succinogenes*. Russell (1985) subsequently showed that cellodextrins (primarily cellotetraose and cellopentaose) support growth of the non-fibrolytic *S. ruminantium* and *P. ruminicola*. Crossfeeding was also demonstrated in succinate-propionate metabolism. *F. succinogenes* and *R. flavefaciens* produce succinate during fiber digestion. However, succinate does not accumulate in the rumen, since it is rapidly converted into propionate. For this conversion, succinate-decarboxylating bacteria such as *S. ruminantium* are considered to play a central role in the rumen

Table 1. Distribution of *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* in the rumen quantified by molecular-based techniques

Sample	Animal	Method	Proportion (% total bacteria)			Literature
			F. succinogenes	*R. flavefaciens*	*R. albus*	
Rumen digesta	Lactating dairy cattle	16S rRNA-targeted oligonucleotide probe	0.09-0.40	0.06-0.32	0.59-1.59	Weimer et al. (1999)
Rumen digesta	Sheep	16S rRNA-targeted oligonucleotide probe	2.0	1.6	0.9	Michalet-Doreau et al. (2002)
Ruminally incubated rice straw	Sheep	16S rDNA-targeted real-time PCR	2.60	0.14	0.03	Koike et al. (2007)
Rumen digesta	Lactating dairy cattle	16S rDNA-targeted real-time PCR	0.61-1.00	0.34-0.80	0.001-0.008	Stevenson and Weimer (2007)
Rumen digesta	Dairy heifers	16S rRNA-targeted scissor probe	2.9-10.1	1.1-1.9	0.8-1.7	Uyeno et al. (2007)

Table 2. Interaction between fibrolytic and non-fibrolytic bacteria

Combination		Improved function	Increase[a]	Literature
Fibrolytics	Non-fibrolytics			
Fibrobacter succinogenes S85	*Selenomonas ruminantium* HD4	Propionate production[c]	0 mM →16 mM	Scheifinger and Wolin (1973)
Fibrobacter succinogenes BL2	*Treponema bryantii* $B_2$5	Dry matter digestibility[d]	34% → 38%	Kudo et al. (1987)
Fibrobacter succinogenes A3c	*Prevotella ruminicola* H2b	Hemicellulose digestibility[b]	60% → 70%	Osborne and Dehority (1989)
Fibrobacter succinogenes A3c	*Prevotella ruminicola* H2b	Cellulose digestibility[b]	56% → 59%	Fondevila and Dehority (1996)
Ruminococcus flavefaciens B34b	*Prevotella ruminicola* H2b	Cellulose digestibility[b]	33% → 42%	*Ibid.*
Ruminococcus flavefaciens C94	*Selenomonas ruminantium* S137	Dry matter digestibility[b]	22% → 25%	Sawanon and Kobayashi (2006)
Ruminococcus albus 6A41	*Treponema bryantii* $B_2$5	Dry matter digestibility[d]	24% → 26%	Kudo et al. (1987)

[a] Increase from the value obtained in mono-culture of fibrolytics to those in co-culture of fibrolytics and non-fibrolytics.
[b] Orchardgrass hay was used as substrate. [c] Ball-milled Whatman no.1 filter paper was used as substrate. [d] Barley straw was used as substrate.

(Scheifinger and Wolin, 1973; Wolin et al., 1997; Sawanon and Kobayashi, 2006). *S. ruminantium* has been detected as a member of the fiber-associated bacterial community in the rumen (Koike et al., 2003b), and its population size on plant fiber was estimated to be 9% (Koike et al., 2007). This ecological information suggests that *S. ruminantium* may be involved in fiber digestion. Sawanon *et al.* (2003) isolated *S. ruminantium* strains belonging to a phylogenetically novel group from sheep rumen. Subsequently, they reported that fiber digestion in a co-culture of *F. succinogenes* and the novel *S. ruminantium* strains exceeded the value achieved by *F. succinogenes* alone. Furthermore, propionate production and growth of *S. ruminantium* was notable in co-cultures, while succinate accumulated in monocultures of *F. succinogenes*. These results indicate that *F. succinogenes* provides fiber hydrolysis products to *S. ruminantium* as growth substrates, while *S. ruminantium* may activate *F. succinogenes* by rapidly consuming the products. The occurrence of similar events was confirmed in co-culture of *S. ruminantium* with *R. flavefaciens* (Sawanon and Kobayashi, 2006). Fondevila and Dehority (1996) reported that when a non-fibrolytic *P. ruminicola* strain was co-cultured with either *F. succinogenes* or *R. flavefaciens*, fiber digestion was improved compared to that of the fibrolytic species alone. Other published data on the interactions between fibrolytic and non-fibrolytic species are summarized in Table 2. Such crossfeeding between fibrolytic and non-fibrolytic bacteria could enhance fiber digestion in the rumen.

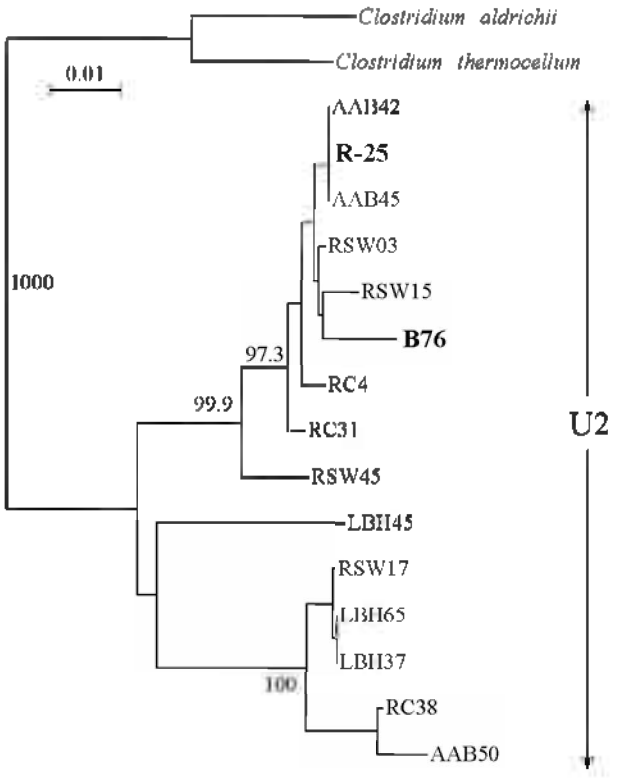

Figure 1. Phylogenetic placement of unknown bacterial group U2 belonging to low GC Gram-positive bacteria (Koike et al., 2003b). The tree was modified by adding two novel strains (shown in bold face).

POSSIBLE INVOLVEMENT OF UNCULTURED BACTERIA IN FIBER DIGESTION

Based on 16S rDNA-based analysis, it has been suggested that 300-400 different bacterial species are present in the rumen (Edwards et al., 2004; Yu et al., 2006). Among these, only 2-31% showed a close relationship (a 97% or higher sequence identity) with previously described species (Kobayashi, 2006). These data clearly suggest that the majority of the rumen bacterial community members are unidentified bacteria. In order to determine the members of a fibrolytic consortium, we carried out 16S rDNA library analysis on the bacteria attaching to ruminally incubated hay, most of which are thought to be involved in fiber degradation. The majority (77%) of the fiber-associated community members had less than 97% identity with 16S rDNA sequences of known bacteria, even though 17% were identified as ruminal fibrolytic species including *B. fibrisolvens*, *F. succinogenes*, *P. ruminicola* and *Pseudobutyrivibrio ruminis* (Koike et al., 2003b). This finding suggests that presently recognized fibrolytic species, such as *F. succinogenes* and the two ruminococci species known to have fibrolytic activity, may represent only a small proportion of the total fibrolytic population.

Among the unidentified group of bacteria in the fiber-associated community, we focused on unknown group 2 (U2), which was part of a group of low GC Gram-positive bacteria (Figure 1), since this group has branched

Table 3. Properties of new strains belonging to U2

	B76	R-25
Gram reaction	+	+
Shape	rod	coccoid
Size	0.5×0.8 μm	0.8 μm
Growth		
Glucose	+	+
Xylose	+	+
Arabinose	+	+
Cellobiose	+	+
Avicel	-	-
Enzyme activities		
CMCase	-	+
Xylanase	+	+
Arabinofuranosidase	+	+

phylogenetically from the fibrolytic species *C. thermocellum* and *Clostridium aldrichii*. In order to determine the ecology of U2, a specific real-time PCR assay and FISH assay were developed. Members belonging to U2 were distributed in the solid rather than the liquid phase of the rumen content and their time course of population size on ruminally incubated orchardgrass hay stems after feeding synchronized well with that of *F. succinogenes*, the dominant known fibrolytic bacterial species (Goto et al., 2006). Furthermore, FISH analysis revealed that the members of the U2 group attach themselves tightly to hay stems by coexisting with other bacteria rather than existing alone, strongly indicating that the bacteria placed in the U2 group participate in the development of a fiber-digesting bacterial consortium.

Recently, we succeeded in isolating strains belonging to U2 (Koike et al., Unpublished data, 2008). Throughout the isolation process, information from molecular analyses was combined with traditional culture techniques; *i.e.*, an anti-Gram-negative agent was employed to screen Gram-positive bacteria, and the proportion of U2 members in the medium was monitored by real-time PCR. Consequently, enrichment of U2 was successful and the enriched culture was used for isolation. The characteristics of U2 strains are summarized in Table 3. The 16S rDNA sequence of two strains designated B76 and R-25 showed 97% similarity with that of U2 clones deposited in the GenBank database. The strains were rod (B76) and coccoid (R-25) in shape and stained Gram-positive. Both strains grew in medium containing glucose, cellobiose, xylose or arabinose, whereas no growth was observed in Avicel medium. Therefore, B76 and R-25 are unlikely to degrade cellulose in the rumen. We confirmed that 30-40% of B76 and R-25 cells attached to Avicel and orchardgrass hay. This observation supports the ecological characteristics of U2 as a fiber-associated group. B76 possessed xylanase and arabinofuranosidase activity. In particular, xylanase activity of B76 was higher than that of xylanolytic *B. fibrisolvens* H17c under cellobiose-growing conditions. R-25 showed arabinofuranosidase activity that was higher than that of *B. fibrisolvens* H17c and B76. These results suggest that B76 and R-25 contribute to hemicellulose degradation in the rumen.

In addition to U2, we have focused our research on the ruminal *Bacteroides/Prevotella* group. This group contains diverse subgroups mainly comprised of previously uncultured bacteria (Ramšak et al., 2000). Recently, Stevenson and Weimer (2007) demonstrated the predominance of uncultured *Prevotella* in the rumen, the population size of which was estimated to be up to 60% of total bacteria. In contrast, classical ruminal *Prevotella* species such as *P. ruminicola*, *Prevotella bryantii* and *Prevotella brevis* made up only 2-4% of total bacteria in cattle rumen. Therefore, uncultured *Prevotella* could have a major role in ruminal fermentation. In order to isolate the previously uncultured *Prevotella*, we have been applying isolation procedures similar to those used for U2; *i.e.*, enrichment of *Prevotella* is being attempted by incubation of rumen digesta with antibiotics and monitored by real-time PCR assay with *Prevotella* genus-specific primers. Isolation efforts using this enrichment method are ongoing.

Considering the ecological significance of uncultured bacteria, it is surely important to cultivate and characterize them to fully understand the ecology of fiber digestion. To achieve this, a combination of conventional and modern techniques is proving useful.

CONCLUSION

In order to maximize the utilization of cellulose by ruminant animals, the ecology and functions of rumen bacteria should be understood in detail. Although the ecological and functional properties of representative cultured bacteria have been demonstrated, recent molecular approaches revealed that presently recognized fibrolytic species may represent only a small proportion of the total fibrolytic population. Therefore, previously uncultured bacteria need to be isolated and characterized to determine their contribution to plant fiber digestion in the rumen.

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