# Molecular ecological methods to study fibrolytic ruminal bacteria: Phylogeny, competition, and persistence

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## ABSTRACT

Efficiency of fibre digestion in ruminants is critical for animal productivity. Bacteria most important in fibre digestion and utilization are *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Butyrivibrio fibrisolvens*. Of these four species, the *Fibrobacter* and *Ruminococcus* are the most cellulolytic and can potentially be manipulated genetically, or ecologically, for increased ruminal cellulolysis. However, transformation systems have only been developed for *B. fibrisolvens*. The taxonomy of *Ruminococcus*, and *B. fibrisolvens* is complex and 16S rDNA sequence indicates that both are polyphyletic. In addition to delineating phylogenetic relationships between bacteria, 16S rRNA based studies provide molecular targets to enable placement of ruminal manipulations in a phylogenetic context. These target sites can also be used to design tracking protocols to measure persistence of introduced bacteria into the rumen.

## Introduction

Fibre fermentation in the rumen is of critical importance to the productive efficiency of the animal, especially under extensive grazing conditions. Hungate described the cooperative host-microbial symbiosis in ruminants in which the microbes gain access to fibre with enzymes not produced by the host animal [13]. The degradation and fermentation of fibre contributes to microbial yield and volatile fatty acids which are an important source of protein and energy, respectively, to the animal [13]. Attempts to improve fibre breakdown and fermentation pre-gastrically would provide both additional energy and bacterial protein to the animal.

The diversity of microorganisms in the rumen is extensive and comprises bacteria, fungi, protozoa [11], and even phage [14, 15]. Estimates of the extent of ruminal bacterial diversity cultured is as little as 10% [18]. It has been suggested that representatives of all the major functional groups of ruminal microorganisms have been isolated and that the extent of diversity is irrelevant. This view is simplistic and is partly due to the traditionalist practice of studying pure cultures. In "real ecosystems" microorganisms exist and interact as members of consortia, and it is the phenotypes expressed as members of consortia that ultimately determine functionality [5]. In this manuscript we discuss attempts to increase

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fibre breakdown by manipulation of some of the most important fibre degrading bacteria. This data serves to highlight the complexity of the interactions involved.

## Major fibre degrading bacteria found in the rumen.

The major cellulolytic bacteria in the rumen are *Butyrivibrio fibrisolvens, Ruminococcus albus, R. flavefaciens,* and *Fibrobacter succinogenes*. The first three species are Gram-positive but *F. succinogenes* is Gram-negative and together with the ruminococci are the most fibrolytic. *F. succinogenes* will not be discussed in this manuscript but recent reviews can be consulted [8, 11, 30].

### *Butyrivibrio fibrisolvens*

Butyrivibrios were first isolated by Hungate [12] and fermented cellulose poorly. Bryant and Burkey [4] subsequently characterised similar isolates as gram-negative, butyrate producing rods. When a group of 22 *B. fibrisolvens* strains were assessed for their ability to digest spear grass (*Heteropogon contortus*) few could digest more than 10% of the dry matter (DM) [23]. Most strains digest hemicellulose and it is this feature, and not the ability to digest cellulose, that is the main contribution made by *B. fibrisolvens* to fibre digestion [11].

DNA-DNA hybridization studies have shown that butyrivibrios can be divided into at least five groups, suggestive of five different species [22]. More recent 16S rDNA analysis has confirmed the DNA-DNA hybridization results and show that *B. fibrisolvens* is polyphyletic [10] and all strains sequenced so far fall within cluster XIV of the colostridia [6]. Forster et al. [9] identified three sequences that were diagnostic of most sequenced strains and when hybridized with rRNA from sheep, deer and cattle consuming high forage diets, *B. fibrosolvens* made up less than 1% of the population. Recently these same probes showed that *B. fibrisolvens* was less than 2% of the ruminal population in sheep eating a tanniniferous diet [19]. Traditional microbiological enumeration estimates of *B. fibrisolvens* in rumen vary from 2% [21] on a high concentrate diet to as high as 38% on a high forage diet [20]. 16S rRNA based numbers are underestimates of *B. fibrisolvens* because the phenospecies is polyphyletic and current probes do not adequately cover diversity. However, culturable numbers have probably been overestimated and studies have used growth on glycerol tributyrate [20], or motility [28] to presumptively identify *B. fibrisolvens*.

### *Ruminococcus species*

Cellulolytic ruminococci are gram-positive and can be divided into two mains species; *R. albus* and *R. flavefaciens* [11]. It is difficult to divide these two species on the basis of morphology or phenotype but the ability of *R. flavefaciens* to produce succinate as a major end-product seems to be consistent with it's phylogenetic position [17]. In a survey of 22 strains of ruminococci it was found that the ability to digest DM or neutral detergent fibre (NDF) of rhodes grass (*Chloris gayana*), spear grass or lucerne (*Medicago sativa*) varied greatly [16]. Ability to digest filter paper cellulose discs was not highly correlated with the ability to digest NDF [16], a point previously noted by Stewart et al., [25].

*Ruminococcus* are phenotypically distinguishable from other *Peptococcaceae* by the presence of meso-diaminopimelic acid at position three in their peptidoglycan [7]. The "true" ruminococci of which the cellulolytic *R. flavefaciens* C94 is the type strain [3] are

placed within cluster IV of the colostridia [6] with *R. albus*, and *R. callidus* but several apparently non-cellulolytic ruminococci (eg. *R. torques, R. gnavus, R. obeum*, and *R. productus*) are in cluster XIVa. Total cellulolytic *Ruminococcus* have been estimated to be between 4% [20], and 11% [21] of the total ruminal population. We have been able to design a *Ruminococcus* genus specific 16S rDNA based probe to cluster IV ruminococci [17]. Hybridization to rRNA from sheep fed a predominantly tropical grass diet (rhodes grass) indicated that ruminococci were approximately 5% of the population.

## Improving plant cell wall degradation in the rumen

### *Is it possible to improve fibre degradation?*

A significant research effort has been invested in improving forage quality via classical breeding techniques, and more recently, molecular biological manipulation [31]. These breeding programs seek to improve various aspects of forage quality including plant cell wall digestibility [31]. Post-harvest modification of forage has also received considerable attention [2]. These include various treatments such as grinding, pelleting, steam treatment, and chemical modifications to improve the digestibility of fibre [2]. Modification of plant cell wall digestibility by microbial manipulation has traditionally received less attention partly because the ruminal ecosystem is so complex. Significant advances have however been made in our understanding during the last 10-15 years because of the application of molecular biology techniques.

There has been scepticism as to the value of microbial manipulation because of the recalcitrance of plant cell walls to digestibility but improvements in ruminal fibre digestibility may be possible. In the rumen there are two competing kinetic processes occurring simultaneously, the rate of digestion (Ks), and the rate of passage (Kp), which together make up the total disappearance of any feed fraction [29]. When $^{14}$C-labeled wheat plant cell wall was fed to sheep the rate of digestion was 9.2% per h, and the rate of passage was 2.1% per h. The portion escaping digestion was 18.5% (Kp/(Ks+Kp)) and could be considered potentially fermentable [29]. An obvious criticism would be that the 18.5% not digested in the rumen was made up of a fraction not available to rumen microorganisms. However, several experiments with sheep have demonstrated that 17 (high forage) to 60% (high concentrate) of dietary cellulose and hemicellulose enters the hindgut [1, 26, 27], and 19 (high forage) to 65% (high concentrate) of this material disappeared. If plant cell walls escaping digestion in the rumen can be digested in the hindgut, this suggests that ruminal fermentation may not be optimal and improvements in Ks would improve ruminal fibre digestion.

### *Recombinant ruminal bacteria*

One proposed mechanism for increasing fibre breakdown in the rumen is to construct bacteria by recombinant techniques so that they have enhanced fibrolytic activities [8, 30]. A major stumbling-block has been the inability to transform most of the important fibrolytic bacteria and only a limited amount of success has been obtained with *B. fibrisolvens* [30].

More recently success has been obtained with the introduction of domain II of the *xynA* gene from the ruminal fungus *Neocallimastix patriciarum* into *B. fibrisolvens* OB156 [32]. One of the constructs had α-amylase signal peptide that allowed only 15.6% of the xylanase activity to be detected extracellularly. However, when the signal peptide was mutated to

make it more hydrophobic 62% of the xylanase activity was detected extracelluarly. Recently, similar constructs of the same gene were transformed into *B. fibrisolvens* H17c (H17c pUBxynA [α-amylase signal peptide], H17c pUMSX [mutated α-amylase signal peptide]). The H17c pUBxynA construct appeared to by the fittest in competition studies with native strains, even though H17c pUMSX secreted more of the recombinant xylanase extracelluarly and digested slightly more fibre in monoculture. Because H17c pUBxynA appeared to be the fittest it was inoculated into the rumen of cattle and sheep eating a rhodes grass diet. This organism was detectable until 24 d post-inoculation and it's dilution rate (measured as the decrease in numbers) appeared to be slower than the dilution rate of particles (3% per h) [24] indicating that H17c pUBxynA was competing. Recombinant butyrivibrios are a valuable tool for manipulation of ruminal processes and remain the only fibrolytic ruminal bacterium that can be genetically manipulated.

### *Naturally occurring bacteria*

Surveys of cluster IV ruminococci have shown that the ability to digest fibre varies considerably among strains, but some strains can be considered superior fibre degraders [16]. Can we then not use the best fibre digesters as inoculants, and thereby circumvent all the problems associated with recombinant strains? Recently we repeatedly inoculated five strains of highly fibrolytic ruminococci into sheep eating a rhodes grass diet so that numbers increased approximately 10-fold over baseline. Measurements of cellulolytic activity in the rumen increased significantly over controls and introduced strains persisted (tracked with specific sequences) for at least 20 d post inoculation. The data strongly suggest that if ruminococci are at sufficiently high numbers in the rumen rates of fibre digestion may be improved. An interesting question still under investigation is why these highly cellulolytic bacteria decline in numbers in the rumen. The answer to this question may lie in specific ecological criteria that are required for establishment such as ability to attach to substrate or the utilization of metabolites from other bacteria.

An obvious criticism of this data would be that the numbers of introduced organisms were not persisting at all, but were slowly diluting out of the rumen. Passage rate of a rhodes grass diet in sheep has been determined to be ~3% per h [24], and the rate of decline of the *Ruminococcus* based on a genus specific 16S rRNA probe was ~0.3% per h, a dilution rate 10-fold less that that of rhodes grass. This indicates that the dosed organisms were not just diluting out, but were successfully competing for fibre in the rumen.

## Conclusions

There has been an ever increasing number of manuscripts that describe new techniques for studying microbial ecology in a variety of ecosystems. It should however be recognised that the study of microbial ecology is a study of ecology and not of techniques, and the techniques are a means to an end, and not the end itself. Fibre digestion is a complicated process but the true rate limiting steps have not been clearly identified. This manuscript gives some evidence supporting microbiological intervention but more work is required to address the problems related to establishment and persistence. There are still many questions to answer that can only be addressed by application of a suit of techniques that look at the microbial ecology of the rumen as a whole.

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