Exploring rumen microbe-derived fibre-degrading activities for improving feed digestibility

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ABSTRACT

Ruminal fibre degradation is mediated by a complex community of rumen microbes, and its efficiency is crucial for optimal dairy productivity. Enzymes produced by rumen microbes are primarily responsible for degrading the complex structural polysaccharides that comprise fibre in the plant cell walls of feed materials. Because rumen microbes have evolved with their ruminant hosts over millions of years to perform this task, their enzymes are hypothesised to be optimally suited for activity at the temperature, pH range, and anaerobic environment of the rumen. However, fibre-rich diets are not fully digested, which represents a loss in potential animal productivity. Thus, there is opportunity to improve fibre utilisation through treating feeds with rumen microbe-derived fibrolytic enzymes and associated activities that enhance fibre degradation. This research aims to gain a better understanding of the key rumen microbes involved in fibre degradation and the mechanisms they employ to degrade fibre, by applying cultivation-based and culture-independent genomics approaches to rumen microbial communities of New Zealand dairy cattle. Using this knowledge, we aim to identify new opportunities for improving fibre degradation to enhance dairy productivity.

Rumen content samples were taken over the course of a year from a Waikato dairy production herd. Over 1,000 rumen bacterial cultures were obtained from the plant-adherent fraction of the rumen contents. Among these cultures, two, 59 and 103 potentially new families, genera and species of rumen bacteria were identified, respectively. Many of the novel strains are being genome sequenced within the Hungate 1000 rumen microbial reference genome programme, which is providing deeper insights into the range of mechanisms used by the individual strains for fibre degradation. This information has been used to guide the selection of rumen bacterial strains with considerable potential as fibrolytic enzyme producers *in vitro*, with the intent of developing the strains so that their enzymes may be used as feed pre-treatments for use on farm. Culture-independent metagenomic approaches were also used to explore the activities involved in fibre degradation from the rumen microbial communities. Functional screening has revealed a range of novel enzymes and a novel fibre disrupting activity. Enrichment for the cell-secreted proteins from the community revealed evidence of a diverse range of cellulosomes, which are cell-surface associated multi-enzyme complexes that efficiently degrade plant cell wall polysaccharides. Biochemical and structural characterisation of these proteins has been conducted.

In conclusion, cultivation and culture-independent genomic approaches have been applied to New Zealand bovine rumen microbial communities, and have provided considerable new insights into ruminal fibre degradation processes. Novel activities and bacterial species that display desirable activities on fibrous substrates *in vitro* are now being explored for their potential to improve ruminal fibre degradation, to allow the development of new technologies that will enhance dairy productivity.

Keywords: Digestibility; enzymes; fibre; nutrition; plant cell walls; rumen microbes; supplemental feeds.

INTRODUCTION

Inefficiencies in fibre digestion in the rumen limit dairy cow productivity, which is an issue of particular importance in New Zealand as our pasture and supplemental feed-based diets are fibre-rich. Rumen microbes are essential to digest fibrous feeds. To maximise ruminant productivity, it is necessary to better understand the role of the rumen microbiome and its bioactivities in fibre degradation. However, the rumen microbiome is highly complex, and contains thousands of species of microbes, with only an estimated 7-10% of these available in culture (Edwards *et al.* 2004). Cultures are desirable as they allow the organism to be studied directly, and they facilitate genome sequencing. To gain a more comprehensive understanding of the rumen microbiome, it is necessary to improve the representation of the community in culture, as well as use culture-independent approaches. New cultivation approaches have been highly successful in isolating previously uncultured and novel bacterial genera from the sheep rumen (Kenters *et al.* 2011), and hold great promise for application in dairy cattle. The microbiome may also be accessed by analysing the collective genomes

of the microbial community, an approach commonly known as "metagenomics". Metagenomics can reveal the phylogenetic structure of the entire community, as well as allowing access to their functions by sequencing metagenomic DNA directly, or cloning genomic DNA fragments to express bioactivities and capture the genes encoding them.

Feed enzymes are widely used in the swine and poultry industries to improve the digestion of dietary components, resulting in improved animal performance. Such enzymes are typically derived from soil fungi. Fibre-degrading enzymes produced by rumen microbes are highly attractive for developing similar products for ruminants since they are likely to be optimally suited for activity in rumen conditions. Through gaining a better understanding of the key rumen microbes involved in fibre degradation, the mechanisms they employ to degrade fibre, and rate-limiting steps in fibre degradation, we aim to identify new opportunities for improving ruminal fibre degradation to enhance dairy productivity.

MATERIALS AND METHODS

Seasonal samples of rumen contents were obtained from *via* the rumen fistulae of five dairy cows in a Waikato production herd over a year. Fibre-adherent rumen microbes were cultivated from rumen contents using a dilution to extinction approach, in combination with a liquid medium that mimics the physico-chemical composition of the rumen (Kenters *et al.* 2011). Cultures were identified *via* their 16S rRNA gene sequences. The total rumen community composition was determined using pyrotag sequencing of bacterial 16S rRNA amplicons (Noel 2013). *In vitro* fibrolytic activities of cultures were determined by culturing bacteria on a range of fibrous substrates, and assaying the culture supernatants and cell pellets for fibre-degrading enzyme activities (Yoshida *et al.* 2011).

To functionally identify bioactivities from the rumen microbiome, the bovine rumen content samples were fractionated to obtain the feed-adherent, feed-associated, and liquid microbiota (Ciric *et al.* 2014). Metagenomic DNA was extracted from each fraction and libraries were constructed in a large-insert fosmid vector. Library clones were assayed in agar plate based assays with substrates to detect endoglucanase, cellobiohydrolase, beta-glucosidase, endoxylanase, xylosidase, arabinofuranosidase, phenolic acid esterase and amylase activities. Positive clones were DNA sequenced and putative genes involved in fibre degradation were identified *in silico via* the manual curation of GAMOLA annotated sequences (Altermann and Klaenhammer 2003). Secreted proteins encoded by the microbiome (the metasecretome) were identified using a conditional phage-display system (Jankovic *et al.* 2007). Secretome protein-containing phagemid particles were DNA pyrosequenced (Ciric *et al.* 2014), and data were screened for carbohydrate active enzyme (CAZyme) protein domains that are associated with carbohydrate utilisation (Ciric *et al.* 2014). Genes encoding putative fibre-degrading enzymes were expressed in *Escherichia coli* (Goldstone *et al.* 2010). Purified enzymes were biochemically characterised to determine the range of activities they possess, and to determine their biochemical kinetics. Enzymes were also structurally characterised using established methods (Goldstone *et al.* 2010).

RESULTS

Over 1,000 anaerobic rumen bacterial isolate cultures were obtained from the plant-adherent microbiota, of which 626 were unique *via* 16S rRNA gene sequencing, and these included rarely-cultured bacterial groups (Noel 2013). Putative novel taxa were identified at several taxonomic levels: family (2), genus (59) and species (103). At the species-equivalent level, cultivated members represented 7.7% of the overall diversity, which represented almost a third of the plant-adherent bacteria by sequence abundance. The genomes of many of the novel bacteria within this collection are being sequenced through the Hungate 1000: A catalogue of reference genomes from the rumen microbiome project (Kelly *et al.* 2014). Genome analysis has enabled us to identify novel strains with industrial potential for fibre degradation. The fibrolytic activities of strains were tested *in vitro* (Table 1) and greater activities were observed than when previously well characterised model rumen fibrolytic bacterial strains were used.

Table 1: Relative *in vitro* fibrolytic activity of rumen bacterial culture enzymes

Strain	Substrate	Relative activity[a]
Bacteroides strain A	cellulose	2.56
Ruminococcus strain A	cellulose	1.23
Clostridiales strain A	cellulose	0.83
Ruminococcus strain A	xylan	1.62
Bacteroides strain A	xylan	1.08
Ruminococcus strain B	xylan	1.02

[a] Activities on cellulose (filter paper) and xylan, relative to those of model cellulolytic strain *R. flavefaciens* FD1, and model xylanolytic strain *Butyrivibrio proteoclasticus* B316, respectively.

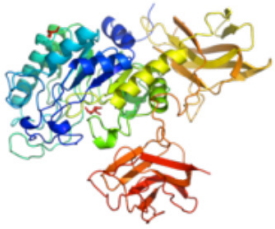

Figure 1: Structure of novel glycoside hydrolase family 29 protein identified from metagenomic library sequence.

Over 27,000 bovine rumen metagenomic fosmid library clones were assayed for activities involved in cellulose and arabinoxylan degradation. Approximately 3.4% of clones were positive, with xylosidase and arabinofuranosidase activities being the most prevalent. Over 300 positive clones that had high or multiple activities were sequenced. Twenty nine candidate genes predicted to be responsible for the observed activities were expressed, and a selection was biochemically and structurally characterised. Among these, multifunctional enzymes displaying activities on a range of bonds found within lignocellulose were identified. The protein structures of novel glycoside hydrolase family 5 and family 29 (Figure 1) enzymes were elucidated, where the domain structure of the latter was found to be novel.

Selection for the rumen microbial metasecretome resulted in a 29-fold enrichment of sequences encoding a diverse range of putative fibrolytic enzymes. An unexpected finding was the enrichment of sequences associated with cellulosomes, which are cell-surface "nanomachines" that are specialised for the efficient degradation of cellulose.

DISCUSSION AND CONCLUSION

Cultivation and culture-independent approaches have yielded an abundance of new cultures, fibrolytic enzymes and genetic information from New Zealand rumen microbiomes, and have revealed new insights into ruminal fibre degradation processes. These resources are being explored for their potential to improve ruminal fibre degradation, and provide a valuable foundation for the development of new technologies to enhance dairy productivity from fibrous feeds.

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