Molecular Analysis of *Archaea, Bacteria* and *Eucarya Communities* in the Rumen* - Review -

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ABSTRACT : If rumen bacteria can be manipulated to utilize nutrients (i.e., ammonia and plant cell wall carbohydrates) more completely and efficiently, the need for protein supplementation can be reduced or eliminated and the digestion of fiber in forage or agricultural residue-based diets could be enhanced. However, these approaches require a complete and accurate description of the rumen community, as well as methods for the rapid and accurate detection of microbial density, diversity, phylogeny, and gene expression. Molecular ecology techniques based on small subunit (SSU) rRNA sequences, nucleic acid probes and the polymerase chain reaction (PCR) can potentially provide a complete description of the microbial ecology of the rumen of ruminant animals. The development of these molecular tools will result in greater insights into community structure and activity of gut microbial ecosystems in relation to functional interactions between different bacteria, spatial and temporal relationships between different microorganisms and between microorganisms and feed particles. Molecular approaches based on SSU rRNA serve to evaluate the presence of specific sequences in the community and provide a link between knowledge obtained from pure cultures and the microbial populations they represent in the rumen. The successful development and application of these methods promises to provide opportunities to link distribution and identity of gastrointestinal microbes in their natural environment with their genetic potential and in situ activities. The use of approaches for assessing population dynamics as well as for assessing community functionality will result in an increased understanding and a complete description of the gastrointestinal communities of production animals fed under different dietary regimes, and lead to new strategies for improving animal growth.

(**Key Words** : Rumen Microbes, DNA, RNA, Ecology, Molecular Analysis, DGGE, Review)

INTRODUCTION

The microbial community inhabiting the rumen is characterized by its high population density, wide diversity and complexity of interactions. All major groups of microbes are represented in the rumen. Bacteria are predominant but a variety of protozoa occur widely (Clarke, 1977; Clark and Bauchop, 1997). The anaerobic fungi are widely distributed in the gut of herbivores (Orpin and Joblin, 1988; Ushida et al., 1997). Yeasts are frequently present (Hespell et al., 1997) and the occurrence of bacteriophage is well documented (Klieve and Bauchop, 1988). Importantly, the rumen contains representatives of all three domains of life (*Archaea, Bacteria and Eucarya*) described by Woese and co-workers (Olsen and Woese, 1993; Woese et al., 1990). Many animals of a wide range of orders have a portion of the digestive system adapted to accommodate a fermentation which aids them in digestion and provides them with a variety of nutritional and health benefits. Microbial populations have been described in herbivores, *omnivores and carnivores and* in all zoological classes (Clarke, 1977; Clark and Bauchop, 1997).

The rumen, the most extensively studied gut community, contains large numbers of bacteria (up to 10^{11} viable cells per ml comprising 200 species), ciliate

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protozoa (10^4-10^6 per ml spread over 25 genera), anaerobic rumen fungi (zoospore population densities of 10^3-10^5 per ml divided into 4 genera) and bacteriophage (10^7-10^9 particles per ml) (Hespell et al., 1997; Klieve and Swain, 1993; Orpin and Joblin, 1988; Stewart and Bryant, 1988; Williams and Coleman, 1988). Despite this vast amount of knowledge, the basic prerequisites for ecological studies, namely enumeration and identification of community members, have tremendous limitations. The two major problems faced by microbial ecologists studying the gastrointestinal community are the inevitable bias introduced by culture based enumeration and characterization techniques and the lack of a phylogenetically based classification scheme (Amann et al., 1990; Amann et al., 1994; Pace et al., 1985; Raskin et al., 1997; Stahl, 1993a; Ward, 1989). Indeed, most of the information in the literature related to rumen microbiology is based on organisms found in dated culture collections which were isolated by conventional culture techniques. This paradigm is clearly evident in studies of *Fibrobacter succinogenes*. Comparative 16S rRNA sequence analysis revealed that *Fibrobacter* isolates (formerly one species, *Bacteroides succinogenes*) from culture collections actually represent two distinct species in a genetically diverse yet phylogenetically coherent genus (Amann et al., 1992; Lin and Stahl, 1995; Montgomery et al., 1988; Stahl et al., 1988). Surprisingly, it is apparent that the diversity within the *Fibrobacters* (92% 16S rRNA sequence similarity and 20% DNA/DNA homology) is as great as that seen between *Escherichia coli* and *Proteus vulgaris*, representatives of two different genera (Montgomery et al., 1988). This great genetic diversity was also revealed in studies using genome mapping and restriction fragment length polymorphism (RFLP) analysis (Ogata et al., 1997), and genus and species level 16S rRNA probes (Stahl et al., 1988). Two observations from the use of 16S rRNA probes illustrate the power of these molecular tools and the paradigm that rumen microbiology must address. First, these studies revealed a large portion of *F. succinogenes*-like organisms that were unaccounted for by the genus specific probes, suggesting that there is another assemblage of *F. succinogenes*-like organisms that have not been characterized (Stahl et al., 1988). These yet to be characterized microorganisms may be important contributors to plant cell wall hydrolysis, and their presence would not be known without the application of molecular based techniques. Second, these studies estimated *F. succinogenes* population levels to be approximately 1% of the total bacterial population of the rumen (Stahl et al., 1988). This estimate is at least 10-fold higher than studies using habitat-simulating culture techniques (Dehority and Orpin, 1988).

It is evident that there is a gap between the information derived from the classical culture based enumeration and characterization techniques and the information being derived from emerging molecular ecology techniques. The classic studies of Marvin P. Bryant and others have provided a wealth of information related to the rumen and a rich appreciation for its complexity. However, as we continue to isolate new and novel organisms from the rumen, it is clear that the system is not fully characterized. Thus, it is clearly time to use novel techniques that are now available to critically examine this well characterized microbial community. The application of an array of molecular ecology techniques based on SSU rRNA sequences to the microbial ecology of the rumen will lead to rapid and reproducible methodologies for assessing rumen microbial population density, diversity, and functionality. Moreover, these approaches will give tremendous insight into not only the microbial community structure of the rumen, but the functional status of the community.

APPLICATION OF NUCLEIC ACID BASED ANALYSIS OF GASTROINTESTINAL COMMUNITIES

In molecular ecology it is important to distinguish between identification, quantification and monitoring of function and activity. The information derived from each approach is highly dependent on the type of target nucleic acid employed and the conceptual and technical basis used for nucleic acid probe design. Generally these can be divided into DNA-based methods employing empirically characterized probes and rRNA-based methods based on comparative sequence analysis for design and interpretation of "rational" probes (Olsen and Woese, 1993; Pace et al., 1985; Raskin et al., 1997; Stahl, 1993b; Stahl and Amann, 1991; Ward et al., 1992). Molecular approaches for the characterization of microbial communities have been reviewed extensively. Two recent reviews (Hugenholtz and Pace, 1996; Stahl, 1993b), as well as numerous books (for an example see Stackebrandt and Goodfellow, 1991) describe these approaches in detail.

DNA-BASED STUDIES OF RUMEN ECOLOGY

Survival of bacteria reintroduced into the rumen is of considerable interest to rumen microbiologists. Attwood et al. (1988) reported the use of a 1.9 kb randomly cloned

and labeled fragment of genomic DNA from the laboratory strain *Prevotella* (*Bacteroides*) ruminicola subsp. brevis to determine survival time of this organism when introduced into the rumen. Results showed that strain B14 had a half life of 9 h in *in vitro* mixed cultures but only 30 min following inoculation into the rumen. In fact, the organism dropped below the detection limit (2×10^7 cells per ml rumen fluid) within 3 hours, most likely due to bacteriocin-like activity in fresh ruminal fluid. A number of studies have used randomly cloned DNA fragments targeting either plasmid or chromosomal DNA as hybridization probes for bacterial identification after isolation from fecal or gut samples. *Streptococcus bovis*, a normal inhabitant of the rumen, has been implicated in human colonic carcinoma. Whitehead and Cotta (1993) tested a cloned amylase gene as a DNA probe for rapid and accurate identification of *S. bovis* derived from human and bovine sources.

RFLP analysis of genes coding for rRNA has been used for taxonomic studies of many different bacteria. This method has been called ribotyping and has proven useful for differentiating between species as well as sub-species. Ribotyping use in epidemiology has also been proposed because of great intra-species variations in the ribopatterns. Diversity of ribopatterns among closely related isolates varies depending on restriction endonuclease digestion pattern and probe sequence. In general, these DNA-based methods for studying gastrointestinal molecular ecology can have high specificity and sensitivity. However, it is necessary to identify those methods that require pure culture isolates (RFLP and ribotyping) and have the disadvantage of cultural bias. In addition, DNA probes have also been used to investigate species diversity in the gastrointestinal tract including studies on *Fibrobacter* (Flint et al., 1990), *Selenomonas ruminantium* (Ning et al., 1991), and *Butyrivibrio fibrisolvens* (Mannarelli et al., 1990). However, these techniques are less suitable for detailed characterization of community structure of gastrointestinal communities compared to rRNA techniques which employ rational probes designed within the framework of comparative sequence analysis and are therefore highly specific and span a greater taxonomic diversity.

RNA-BASED STUDIES OF RUMEN ECOLOGY

The principles and practice involved in 16S rRNA based methods have been extensively reviewed (Hugenholtz and Pace, 1996; Olsen and Woese, 1993; Pace et al., 1985; Raskin et al., 1997; Sayler and Layton, 1990; Stahl, 1986; Stahl, 1988; Stahl, 1993a; Stahl, 1993b; Stahl, 1997; Stahl and Amann, 1991; Ward, 1989; Ward et al., 1992). These methods are also applicable to 23S rRNA which will likely be used more extensively in the future since it contains more information (3,000 bp compared to 1,500 bp for 16S rRNA). Extensive comparative sequence analysis of 16S rRNA molecules representing a wide diversity of organisms shows that different regions of the molecule vary in sequence conservation. Oligonucleotides complementing regions of universally conserved 16S rRNA sequence are used as universal probes while those complementing more variable regions of sequence are useful as selective probes targeting species, genus, or phylogenetic groups.

The use of 16S rRNA-based methods for ecological studies in the rumen was first demonstrated by Stahl et al. (1988). Species specific 16S rRNA targeted oligonucleotide probes were developed to enumerate strains of *Fibrobacter* (*Bacteroides*) *succinogenes* and *Lachnospira multiparus* in the bovine rumen to monitor shifts in population abundance or changes in population activity in complex microbial communities. Culture based enumeration of *F. succinogenes* was largely unsuccessful in the same study. These techniques formed the basis for further studies of bacterial populations in gut environments. One such study recently published by this same research group, addresses the use of group specific rRNA targeted probes (Lin et al., 1997). Group specific 16S rRNA targeted oligonucleotide probes specific for bacterial, eukaryotic, and archaeal rRNA were used to enumerate these three groups in the gastrointestinal tract of various domestic animals (bovine, ovine, caprine and porcine). The results indicated that the bacterial, eukaryotic and archaeal populations varied from approximately 60-90%, 3-30% and 0.5-3%, respectively in the gastrointestinal tract of most of the animals examined (Lin et al., 1997). By using different probes for classes of methanogenic bacteria, it was also shown that the predominant methanogens differed in different animal hosts (Lin et al., 1997). Moreover, these studies revealed a dominant archaeal group that were unaccounted for by the current methanogen specific probes, suggesting that there is another assemblage of rumen methanogens that have not been characterized (Lin et al., 1997). Other in vivo studies include the work of Krause and Russell (1996), who developed probes for obligate amino acid-fermenting ruminal bacteria and then used these probes to examine the effect of monesin addition on the levels of these bacteria and their role in amino acid deamination, and the examination of molecular beacons as an alternative to radioisotopic labeling of 16S rRNA-targeted probes (Schofield et al., 1997).

We have developed probes for the other major ruminal cellulolytic bacteria *Ruminococcus albus* and *R. flavefaciens* and these were used in *in vitro* studies of the dynamics of bacterial interactions during fermentation of cellobiose, cellulose and alkaline hydrogen peroxide treated wheat straw (Odenyo et al., 1994a; Odenyo et al., 1994b). The results showed that 16S rRNA targeted oligonucleotide hybridization probes were effective in quantifying specific bacteria in defined mixed cultures and provided useful information on bacterial competition during growth on insoluble substrates. Furthermore, this study revealed for the first time the production of a bacteriocin-like substance by a ruminal bacterium, a mechanism which may be used in the competition for nutrients. These probes have also been used by other research groups to study the competition for cellobiose or cellulose by *R. albus, R. flavefaciens* and *F. succinogenes* in chemostats under substrate-excess and substrate-limited conditions (Shi and Weimer, 1997; Shi et al., 1997). rRNA targeted probes have also been used to study the ruminal bacterium *Synergistes jonesii*, which has attracted considerable interest due to its limited geographical distribution and its ability to degrade 3,4-DHP, the toxic principle of the tropical leguminous shrub *Leucaena leucocephala* (Allison et al., 1990; Allison et al., 1992). The 16S rRNA sequence of *S. jonesii* was not closely related to any of the bacteria so far characterized and was an ideal candidate to evaluate the use of oligonucleotide probes for tracking bacteria in the ruminal community. In this laboratory, radiolabeled and fluorescent-dye-conjugated probes were developed for quantitation of *S. jonesii* in a mixed-culture chemostat (McSweeney et al., 1993). Further ecological studies on transmission, colonization, persistence and population studies *in vivo* are under investigation.

Recently, two groups, one from Canada and one from Japan, have investigated the microbial diversity of the rumen by use of comparative sequence analysis of 16S rDNA libraries generated by PCR (Forster et al., 1997; Tajma et al., In Press). In both cases, comparative sequence analysis placed approximately 50% of the clones into the low G+C gram-positive bacteria and 30-40% of the clones were assigned to the *Cytophaga-Flexibacter-Bacteroides* group (which includes Prevotella). The remaining clones were placed in either the *Spirochaetes or Proteobacteria* (Tajma et al., In Press). Additional studies using SSU rDNA libraries of *Archaeal and Eukarya* populations are underway. These studies are critical to the success of other molecular based approaches. Ultimately, these approaches will result in greater insights into community structure and activity of gut microbial ecosystems in relation to functional interactions between different bacteria, spatial and temporal relationships between different microorganisms and between microorganisms and feed particles.

Surprisingly, a limited number of studies have used rRNA based methods to study ruminal protozoal and fungal populations. Initially, sequence analysis of the 18S rRNA genes from *Polyplastron multivesculatum* (originally deposited as *Entodinium simplex*) and *Dasytricha ruminantium* was used to phylogenetically position these protozoa within the hydrogenosome containing protozoa (Embley et al., 1995). More recently, seven other rumen ciliate protozoa, as well as two additional isolates of *P. multivesculatum* and *D. ruminantium*, have been analyzed and these studies indicate that the rumen ciliates are monophyletic and fall into three distinct groups within the Class Litostomatea (Wright and Lynn, 1997a; Wright and Lynn, 1997b; Wright et al., 1997). Signature probes for *Entodinium caudatum, Epidinium caudatum*, and *P. multivesculatum* have been developed and are currently being analyzed for use in the study of rumen microbial ecology (Wright et al., 1997; R. J. Forster, personal communication). Fluorescently labeled oligonucleotide probes targeting the 16S rRNA of the Archaea were used by Finlay et al. (28) to demonstrate that *Entodinium* species and *D. ruminantium* contained methanogenic endosymbionts outside digestive vacuoles. Exosymbiotic methanogens had been well documented previously based on characteristic auto- fluorescence of these bacteria. Also, comparative sequence analysis of the 18S rRNA genes was used to phylogenetically position *Neocallimastix* in the *Chytridiomycete* class of fungi (Dore and Stahl, 1991). We have recently determined the 18S rRNA sequences for four additional rumen fungi and these data support the previous comparative analysis and suggest that the anaerobic rumen fungi are monophyletic (Thill et al., 1997).

So, it is evident that gastrointestinal molecular microecology is only just becoming an established approach in rumen microbiology. At present the use of some of these procedures is limited because of the less extensive sequence collection available. Within the ruminal microbial world, the SSU rRNA sequences for numerous predominant bacteria have been determined. These representatives include (not an exhaustive list, derived from GeneBank and the Ribosomal Database Project, Madik et al., 1996): *Acetitomaculum ruminis, Butyrivibrio fibrisolvens, Clostridium aminophilum, Clostridium sticklandii, Fibrobacter intestinalis, Fibrobacter succinogenes, Methanobrevibacter ruminantium, Methanobacterium formicicum, Methanomicrobium mobile,*

Methanosarcina barkerii, Peptostreptococcus productus, Prevotella ruminicola, Ruminobacter amylophilus, Ruminococcus albus, Ruminococcus flavefaciens, Selenomonas ruminantium, and Wolinella succinogenes. However, genus and species specific 16S rRNA targeted oligonucleotide probes that have been validated for use in the rumen have only been developed for members of the genus Fibrobacter (Lin and Stahl, 1995; Stahl et al., 1988). 16S rRNA targeted oligonucleotide probes for other genus-species members of the rumen have been only used to track population dynamics in *in vitro* studies.

CURRENT AND FUTURE RESEARCH APPROACHES

The long term goals of our research group are to advance our understanding of rumen microbial ecology, community structure and functionality by the application of molecular approaches for assessing microbial density, diversity, phylogeny, and gene expression. The successful development and application of these methods, promises to provide the first opportunity to link distribution and identity of gastrointestinal microbes in their natural environment with their genetic potential and in situ activities. We are currently using numerous approaches, as it is clear that all of the techniques used to describe community structure have limitations. Therefore, it is critical that one use a variety of these methodologies.

DENATURING GRADIENT GEL ELECTROPHORESIS

One approach for directly determining the genetic diversity of complex microbial populations is based on electrophoresis of PCR-amplified 16S rRNA fragments in polyacrylamide gels containing a linear gradient of denaturants. In denaturing gradient gel electrophoresis (DGGE, Muyzer et al., 1993), DNA fragments of the same length but different base-pair sequences can be separated. This procedure has been applied to the analysis of PCR fragments derived from the V3 region of the 16S rRNA (Muyzer et al., 1993). These fragments were obtained after amplification of 16 S rRNA genes from genomic DNA from uncharacterized mixtures of microorganisms. Subsequent hybridization with group-specific oligonucleotide probes was used to identify particular constituents of the population (Muyzer et al., 1993). This procedure allows one to directly identify the presence and relative abundance of different species and to profile microbial populations in both a qualitative and semiquantitative way. Thus, the DGGE profiling method can be used for assessing the relative abundance of microorganisms such as *Archaea* and *Bacteria* in samples obtained from different communities.

We have applied this procedure to pure cultures of ruminal fibrolytic bacteria *F. succinogenes*, *R. albus*, and *R. flavefaciens* as well as other members of the genus Ruminococcus which may not be true members of this genus (*R. bromii, R. gnavus, R. lactaris, R. obeum, and R. torques*). Nine strains of *R. albus*, nine strains of *R. flavefaciens*, two strains of *R. bromii* and single representatives of the other microorganisms were analyzed (Cann et al., 1996). DGGE profiling of the nine *R. flavefaciens* strains showed that the PCR amplified V3 region of the 16S rRNA gene from these strains all migrated to the same location in the DGGE gel. These results suggest a lack of sequence diversity in this region of the 16S rRNA gene from *R. flavefaciens*. In contrast, DGGE profiling of the nine R. albus strains suggested a high degree of sequence diversity in the PCR amplified V3 region of the 16S rRNA gene from these strains. The PCR amplified V3 region of the 16S rRNA genes from the *R. albus* strains migrated to different locations in the DGGE gels and these locations differed from that of the *R. flavefaciens* strains. Six of the *R. albus* strains, including the type strain, yielded PCR amplified V3 regions of the 16S rRNA gene which all migrated to the same location in the DGGE gel. The PCR amplified V3 regions of the 16S rRNA gene from *R. albus* strains 8 and RO13 migrated slightly faster than the V3 region PCR product from the type strain of *R. albus* (strain 7). Furthermore, the V3 region PCR product from *R. albus* strain SY3 migrated much slower in the DGGE gel than the V3 region PCR products from the other eight *R. albus* strains. These results suggest that there is significant sequence diversity and phylogenetic heterogeneity within the V3 region of the 16S rRNA gene from *R. albus*, and that the V3 region of the 16S rRNA gene from *R. albus* SY3 has a much lower G+C content than other representatives of this species. The PCR amplified V3 regions of the 16S rRNA gene from *R. gnavus, R. lactaris, R. obeum*, and R. torques all migrated to slightly different locations in the DGGE gel, and these locations differed from those of the other *Ruminococcus* strains. The PCR amplified V3 regions of the 16S rRNA gene from the two *R. bromii* strains migrated to a similar location as that for the V3 region of *R. albus* SY3. All of the *Ruminococcus* DGGE profiles differed from the profile for *F. succinogenes*, and mixtures of PCR amplified V3 regions from the strains of *F. succinogenes, R. albus*, and *R. flavefaciens* were easily resolved in DGGE gel. Further studies with different dilutions of input DNAs from these

strains showed that the DGGE technique is quantitative. These results show that DGGE can be used to differentiate between closely related bacterial strains. Thus this sensitive techniques is highly suitable to the analysis of microbial diversity and population dynamics for the major fibrolytic bacteria from the rumen.

We have also applied this procedure to rumen samples from steers fed different diets in a preliminary study to determine the utility of these techniques for the analysis of a complex microbial community (Kocherginskaya et al., 1997). Rumen samples were collected from four steers fed a medium-quality grass-legume hay at maintenance intake, and four steers were fed a diet of 20% hay, 52% corn, 5% corn steep liquor, 3% minerals, and 20% of corn byproducts. Rumen samples were harvested approximately one hour prior to feeding, passed through cheesecloth, and centrifuged. Total genomic DNA was isolated from cell pellets and used for amplification of either the V3 or V9 region of the 16S rDNA gene. PCR products were then analyzed by DGGE (Muyzer et al., 1993). When the different PCR profiles obtained from amplification of the V3 region of the 16S rDNA were compared for samples from those animals fed the medium-quality grass-legume hay diet, the patterns were remarkably similar. Nonetheless, the DGGE profiles demonstrated at least 16 distinguishable bands, with five of them being more predominant than the others. PCR profiles obtained from amplification of the V3 region of the 16S rDNA from samples from those animals fed the different corn based diets, showed profiles which were different from samples collected from animals fed the medium-quality grass-legume hay diet. Profiles from each of the four corn-based diet-fed animals also differed from each other. In general, these profiles were just as complex, with four to five predominant bands and, depending on the animal, at least eight distinguishable bands of lesser intensity. The profile obtained from one grain fed animal was distinctly different from profiles from the other three animals, with three different predominant species being present. These results demonstrate that this technique will contribute to our understanding of the genetic diversity and population structure of the rumen community, both *in vitro* and *in vivo*.

EUCARYA POPULATIONS

Other current or future projects also relate to a complete description of the microbial community structure of the rumen. These are centered on the ruminal members of the *Eucarya*, who must also be taken into consideration. Therefore, we have initiated a program to phylogenetically describe the remaining anaerobic rumen protozoa by comparative sequence analysis of 18S rDNA genes from representative genera in these two rumen *Eucarya*. Two approaches are being taken for the analysis of 18S rRNA genes from anaerobic rumen protozoa. First, we are using single cell-PCR to individually isolate single protozoal species based on morphology, as determined by microscopic morphological examination, from which single cells are harvested by micromanipulation and used for amplification of the 18S rDNA genes by procedures for single cell-PCR described by Lewin and co-workers (Lewin and Stewart-Haynes, 1992; Mirsky et al., 1993). 18S rDNA genes from *Entodinium caudatum* and *Polyplastron* species have been isolated and are currently being sequenced. This approach will be complemented by the shotgun PCR cloning approach for Eucaryal specific SSU rDNA PCR products. Our goal is to generate comparative sequence data for as many of the 22 genera and 66 species of anaerobic rumen protozoa (Hespell et al., 1997) as possible. This will provide a phylogenetic description of these group of microbes as well as providing a database for the development of oligonucleotide probes. These analyses will allow us to develop molecular ecology techniques based on 18S rRNA targeted oligonucleotide probes and DGGE for the study of population dynamics of these phylogenetic groups in the rumen gastrointestinal community.

FUNCTIONALITY OF RUMEN POPULATIONS

The approaches described above have not yet addressed how measured changes in microbial diversity and community structure of the rumen can be interpreted in relation to functionality. We are addressing these questions by using techniques to access cellulose degradation rates in the rumen samples and then correlating these results with the community structure and cellulase gene expression data. Specifically, we are applying PCR profiling of cellulase gene diversity and density, and in situ RT-PCR as a measure of cellulase gene expression in the samples from the rumen which have been used for community structure determination.

We have begun to use conserved cellulase family-specific sequences to generate cellulase gene sequence diversity profiles of samples from the rumen (Kocherginskaya and White, 1997). We are testing the cellulase-specific PCR primer pairs for family B, C, F and G cellulases (Sheppard et al., 1994) for generation of profiles of cellulase specific PCR products. First, we have used these primer sets with various cellulolytic isolates from the *Ruminococci*. It is clear that different strains

contain different genes and that some of these genes differ between strains (PCR products of different sizes). When these primer sets were used with DNA from *Streptococcus bovis* (a predominant starch degrader), no PCR products were detected, suggesting that these primer sets are specific for cellulolytic microorganisms. Also of interest is the complexity of PCR products generated with these cellulase family primers which will necessitate the design of more rumen specific primer sets and optimization of PCR conditions. Nevertheless, we have used these cellulase specific primers and PCR conditions with the samples from a hay fed steer and grain fed steer. Again, it is clear that different samples give different patterns of genes and that all of the genes tested show a PCR product. In addition, there are two very surprising observations from this preliminary screening. First, the overall complexity of the PCR product profiles appears to be much simpler than those derived from pure cultures. Second, the PCR patterns derived from the microbial populations from the hay fed steer are much simpler than the patterns derived from the grain fed steer. This is a paradigm shift from what one might expect, and suggests that the hay diet selects for a highly specialized and active cellulolytic population, whereas the grain diet seems to select for a more diverse non-specific cellulolytic population. This is an exciting preliminary finding and illustrates the potential power of this approach. Thus, we believe that this method can be refined to give us a measure of cellulase gene diversity and density which can then be correlated with community structure data to provide an accurate picture of both community diversity and functionality.

While the PCR profiles of cellulase genes will give us a measure of density and diversity in relation to the overall numbers of cellulolytics (determined from the community structure studies), these techniques will not reveal the levels of cellulase gene expression. We are developing *in situ* PCR techniques, based on the work of Amman and colleagues (Amann, 1995; Amann et al., 1990; Amann et al., 1992 Amann et al., 1994 Amann et al., 1995; Amann et al., 1996), Hodson et al. (1995), Seidman et al. (1994) and Colombi et al. (1993), that can be used to characterize the microscale genetic (gene expression) and taxonomic (community structure) properties present within the samples from the rumen used for community structure studies. This procedure allows amplification and detection of specific target nucleic acid sequences within bacterial cells rather than on bulk extracted nucleic acid (Nuovo, 1992). Individual genes, mRNA and rRNA are all candidates for detection and multiple targets can be detected in single cells by use of different fluorescent tags. Thus these techniques will allow one to determine the number of cells which are actively expressing cellulase gene homologues as well as correlation with the proportion of those cells that are members of the *Fibrobacter* or *Ruminococcus* taxons. These data, when analyzed with the community structure data, will give tremendous insight into not only the community structure of the rumen, but the functional structure of the microbial community.

CONCLUSIONS

The use of molecular ecology techniques based on nucleic acid probes is likely to revolutionize our approach to microbial ecology in the gastrointestinal tract and will provide, not simply a refinement or increased understanding but, a complete description of gastrointestinal community for the first time. Modern molecular ecology techniques based on sequence comparisons of nucleic acids (DNA or RNA) can be used to provide molecular characterization while at the same time providing a classification scheme which predicts natural evolutionary relationships. In principle, nucleic acid probes can be designed to hybridize with a complementary target sequence and thus provide a complete description independent of the growth conditions and media used. An example of the power of these modern molecular ecology techniques is provided by the analysis of SSU rRNA sequences. The highly conserved regions of the SSU rRNA molecule can serve as primer binding sites for *in vitro* amplification by PCR. The more conserved regions are also useful, serving as targets for universal probes that react with all living organisms or for discriminating between broad phylogenetic groups such as the domains *Archaea, Bacteria* and *Eucarya*. The more variable sequence regions are more appropriate for genus, species and sometimes even strain specific hybridization probes. Thus nucleic acid probes serve to evaluate the presence of specific sequences in the environment and provide a link between knowledge obtained from pure cultures and the microbial populations they represent in the gastrointestinal tract. Furthermore, whole-cell hybridization using *in situ* PCR is a powerful technique which can be used to describe an organisms expression of key enzymes. Thus development of these procedures and techniques will result in greater insights into community structure and activity of gut microbial communities in relation to functional interactions between different bacteria, spatial and temporal relationships between different microorganisms and between microorganisms and feed particles. The successful development and application of these

methods promises to provide the first opportunity to link distribution and identity of gastrointestinal microbes in their natural environment with their genetic potential and *in situ* activities.

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