Molecular techniques for monitoring bacterial and bacteriophage populations in the rumen

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ABSTRACT

Molecular techniques are increasingly being used in microbial ecology to identify microorganisms, infer phylogenetic relationships, and describe community structure. We have used rRNA sequence information to develop a competitive PCR technique which enumerates proteolytic bacterial populations from the forestomach of pasture-grazed ruminants. PCR primer pairs, which circumscribe several proteolytic rumen bacterial populations, were used in co-amplifications of target and internal control DNAs, to quantify individual microbial populations. The results from enumerations in pasture-fed animals will be discussed in relation to the sensitivity, specificity and quantitative aspects of the technique. Rumen bacteriophage also impact significantly on rumen metabolism and are being quantified by DNA-based techniques. Studies using pulsed-field gel electrophoresis and laser densitometry of purified phage DNA have shown large variations in ruminal bacteriophage numbers and bacteriophage types. These molecular techniques provide better descriptions of individual ruminal populations and eventually will allow for a complete description of the rumen ecosystem.

Introduction

All animals harbor microbes within their gastrointestinal tracts and in mammals both foregut and hindgut microbial fermentations occur. Ruminants are the most successful foregut fermentors where high densities of rumen microbes carry out extensive degradation of low quality forage. Because microbes are essential to this process a full appreciation of rumen digestion and nutrition can only be obtained from a detailed knowledge of rumen microbial ecology. The aim of microbial ecology is "to understand the relationship of all organisms to their environment" [6] and this requires qualitative and quantitative answers to the following questions:

- What microorganisms are present and how abundant are they?
- What activities do these microorganisms carry out?
- To what extent are these activities performed?

In the past, culture-based studies have been used to address these questions but have not been able to exactly mimic the conditions found in the natural habitat. This has resulted in a large proportion of rumen microbes being "unculturable". Also, the descriptions of rumen microorganisms were phenotypically-based and have proved unsuitable for accurate differentiation of organisms. Because the evolutionary history of microoganisms is recorded in their nucleic acid sequences, the difference in sequences can be related to

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evolutionary distance between microorganisms. Molecular techniques based on the comparison of nucleic acid sequences are now being used in microbial ecology to identify organisms and describe community structure. Small subunit ribosomal RNAs (ssrRNAs) have proved useful as indicators of microbial phylogeny [24,15] and have the special utility of retrieving sequences without culturing organisms, allowing analysis of viable but unculturable populations [2]. Analysis of ssRNA sequences also places descriptions of organisms on a phylogenetic base which has resolved some of the confusion over the already described ruminal species [12,3]. Competitive PCR (cPCR) is a technique which uses an internal control DNA within a conventional PCR amplification reaction to allow quantitative determination of products [17,18,9-11]. We have developed a rDNA-targeted cPCR technique for enumerating specific bacterial populations in pasture-grazed ruminants, and here we use the assay developed for *Clostridium proteoclasticum* as an example.

The rumen is also home to a large and diverse population of bacteriophage which have significant impacts on rumen bacterial populations and may be responsible for considerable nitrogen recycling in the rumen [4,8,13,14]. Because bacteriophage lack rRNA genes they cannot be detected using the rRNA techniques described above. The second part of this paper describes a pulsed field gel electrophoreses (PFGE) method for quantifying ruminal phage DNA and its application to identifying phage diversity and abundance in rumen samples.

The use of competitive PCR to enumerate rumen bacterial populations

Theoretical considerations

In competitive PCR an internal control DNA acts as a competitive template and competes with the target DNA for reagents in the PCR reactions. This allows quantitation of the PCR products by comparing the amount of amplified target DNA with the amount of amplified internal control DNA using the following equation:

$$\text{Log}\,(N_{n1}/N_{n2}) = \text{Log}\,(N_{01}/N_{02}) + n\,\text{log}\,(eff_1/eff_2)$$

where eff_1 and eff_2 are the amplification efficiencies of target and internal control respectively, n is the number of cycles, and N_{n1}/N_{n2} and N_{01}/N_{02} are the ratios of products and initial templates respectively [25]. The log ratio of intensities of PCR products can be related to standard curves derived from serial dilutions of DNA extracted from known numbers of bacterial cells allowing the PCR product ratios to be converted to a bacterial cell equivalent.

Sequence alignments and primer design

Alignments of rDNA sequences from target and closely related organisms are used to identify unique regions for designing primers. Primers are designed within these regions guided both by the principles for developing oligonucleotide probes [22] and the criteria for designing PCR primers [7]. The primers are checked for specificity using the PROBE CHECK program of the Ribosomal Database Project [16] and the BLAST (Basic Local Alignment Search Tool) facility at the National Center for Biotechnology. In our experiments we "anchor" the PCR reaction at the 5' end of the ssrRNA gene using the fd1* primer and place the specific primer at the 3' end of the amplicon. This means that the specific primer should be as similar as possible to fd1* in respect to its length, %G+C and T_m.

PCR conditions and specificity testing

Thermocycling was carried out in sealed capillary tips using a Corbett FTS-1 Capillary Thermal Sequencer (Corbett Research, Sydney, Australia) using the conditions described previously [19]. Determining the conditions under which the primer pair specifically amplifies target DNA is largely an empirical process, involving testing a range of annealing temperatures. Initially, DNA from a small number of closely related organisms is tested as this is the most stringent test of primer specificity. Once specific conditions have been determined, they are applied to DNA from a wider range of organisms. The extent of specificity testing is determined by the anticipated complexity of the environment to be assayed and the availability of purified DNAs from that environment. The primers used in this study were tested for specificity against DNA from 85 bacterial strains mostly of rumen origin (Table 1).

Table 1. Specificity testing of cPCR primers

		Primers used*				
Bacterial species tested	# strains	fd1*/rd1*	B316/fd1*	B315/fd1*	C211/fd1*	C12b/fd1*
C. proteoclasticum	1	+	+	-	-	-
B. fibrisolvens D1 group	3	+	-	-	+	-
B. fibrisolvens group	6	+	-	-	-	-
Clostridium spp.	4	+	-	-	-	-
Ruminococcus spp.	3	+	-	-	-	-
Succinomonas amylolytica	1	+	-	-	-	-
Succinivibrio dextrinosolvens	1	+	-	-	-	-
Streptococcus bovis	38	+	-	+	-	-
Eubacterium spp C12b group	10	+	-	-	-	+
Eubacterium spp.	3	+	-	-	-	-
Selenomonas ruminantium	3	+	-	-	-	-
Prevotella spp.	4	+	-	-	-	-
Fibrobacter succinogenes	1	+	-	-	-	-
Lachnospira multiparus	1	+	-	-	-	-
Megasphaera elsdenii	1	+	-	-	-	-
Enterococcus faecalis	2	+	-	-	-	-

* PCR conditions for each of the specific primer pairs were 3 min at 95^{o}C, 6 cycles of 95 ^{o}C for 30 s, 62 ^{o}C for 15 s and 72 ^{o}C for 30 s, and 25 cycles of 95 ^{o}C for 15 s, 62 ^{o}C for 5 s and 72 ^{o}C for 30s with a final cycle of 72 ^{o}C for 3 min. Amplification with fd1*/rd1* differed only in the annealing temperature which was 55 ^{o}C.

Internal control DNAs

The aim of internal control DNA construction is to produce a template with the same priming sites as the target molecule, but whose PCR products can be differentiated from those of the target DNA. A number of techniques are available for constructing internal controls [5,21,23]. In this study internal controls were generated by deleting internal restriction fragments from specifically-amplified rRNA genes, religating the terminal fragments, and reamplifying. DNA bands representing the predicted deletion product for each specific target DNA were excised from the gel and purified.

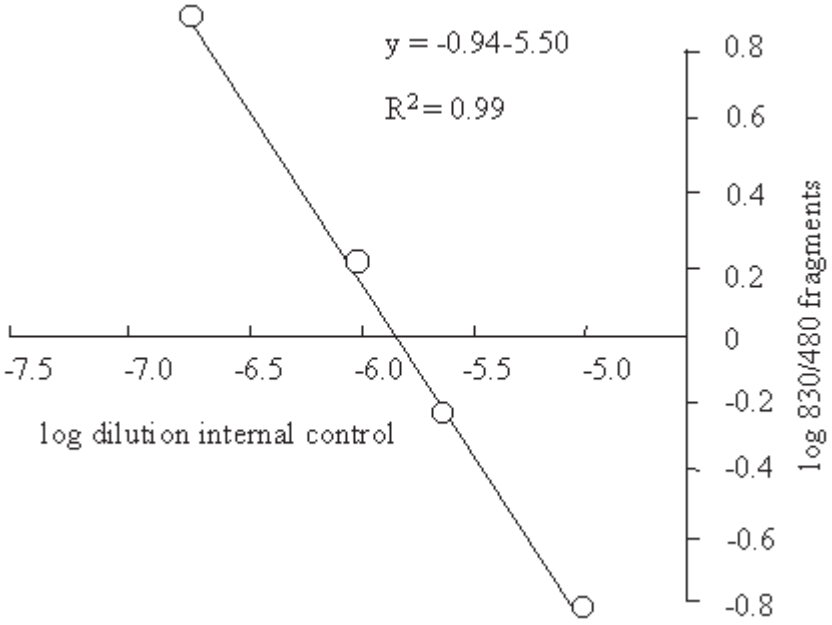
Internal control amplification efficiency

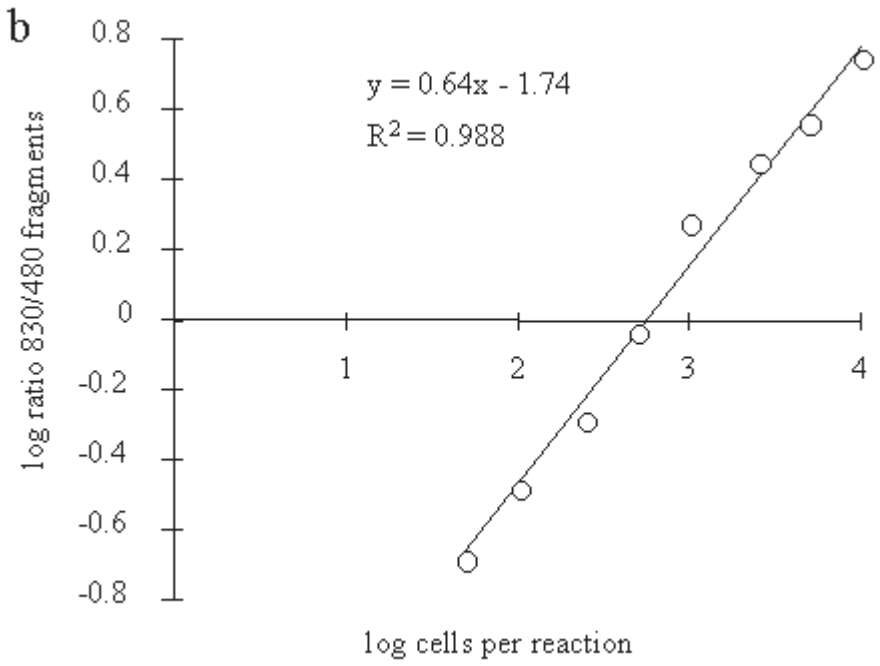
Ideally the target and internal control should amplify with equal efficiency (see theoretical considerations) and this can be verified by amplifying a range of internal control DNA concentrations with a constant amount of target DNA. The plot of log target/internal control versus log internal control dilution should produce a line with a slope of -1.0. Co-amplification of DNA from 1 x 10^3 *C. proteoclasticum* cells with dilutions of the internal control (Fig. 1a) shows a line with a slope of -0.94 with a regression coefficient of 0.99 indicating essentially equal amplification efficiency between the target and control DNAs.

Standard curves and sensitivity testing

cPCR is most accurate when target and internal control are co-amplified in equimolar proportions; therefore, it is necessary to determine the optimal internal control concentrations to use with DNA extracted from rumen samples. The optimal concentration of internal control is then used to construct a standard curve by co-amplification with target DNA extracted from a known number of cells (Fig. 1b). DNA from 5 x 10^1 to 1 x 10^4 *C. proteoclasticum* cells gave a linear response and could be used for quantitation of samples within this range. One can also determine the absolute sensitivity of the cPCR assay by diluting target and internal control DNAs to the point where they no longer coamplify. The lower limit of detection was 50 fg of *C. proteoclasticum* DNA (the equivalent of 25 cells) when co-amplified with a 2 x 10^6 dilution of the internal control DNA.

a





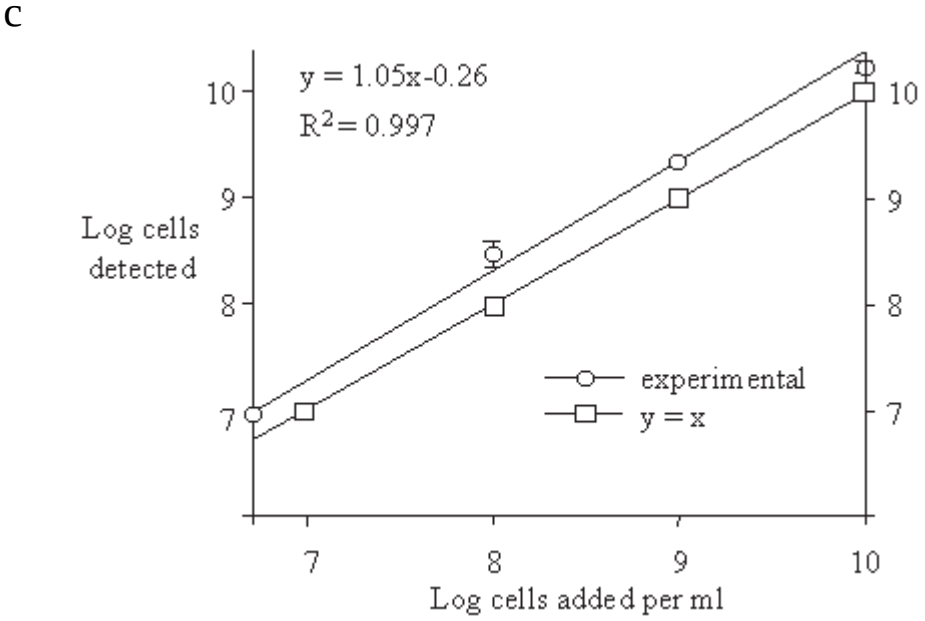


Fig. 1. Internal control amplification efficiency and standard curve construction. a) Dilutions of the internal control were co-amplified with DNA from 1 x 10^3 *C. proteoclasticum* cells and the log ratio of the intensities of internal control and target DNAs calculated and plotted against log dilution of internal control. b) DNA extracted from 1 x 10^{10} *C. proteoclasticum* cells ml^{-1} was serially diluted and co-amplified with a 1 x 10^6 dilution of the internal control. The results are expressed as *C. proteoclasticum* cell equivalents based on the amount of DNA extracted per cell. c) Ratios of the intensities of internal control to target DNA were quantitated by scanning densitometry and plotted on a log scale.

Detection of C. proteoclasticum added to rumen fluid

Rumen contents were spiked with known numbers of *C. proteoclasticum* cells to test the assay in a background of non-specific DNA (Fig. 1c). A linear relationship between the number of cells added and the cells detected was observed. The assay slightly overestimated the number of *C. proteoclasticum* cells added and this was particularly evident at the higher concentrations of cells. A background population of 6.25×10^6 ml^{-1} of *C. proteoclasticum* cell equivalents was detected in the unspiked rumen samples.

Detection of proteolytic rumen bacteria in vivo

The utility of the cPCR assay was demonstrated *in vivo* by enumerating proteolytic rumen bacterial populations directly from rumen samples. Samples were collected from eight lactating dairy cows fed four different diets in rotation and the populations of four proteolytic bacterial populations were determined (Fig. 2). The *C. proteoclasticum* probe detected populations of around 1×10^7 ml^- in samples from each of the diets but there were no significant differences in populations due to diet. The *Streptococcus* spp. probe detected higher levels of organisms (~ 5.5 to 7×10^7 cells ml^{-1}) but these also did not differ significantly. However, significant differences were observed in populations detected using the *Butyrivibrio fibrisolvens* and *Eubacterium* spp. probes. *Butyrivibrio fibrisolvens* numbers were significantly higher on high N+CHO than on high N alone, and the same trend was apparent on low N+CHO compared with low N, although this was not significant. This suggests that these *B. fibrisolvens* populations are stimulated by the addition of CHO and that this is more pronounced when more nitrogen is in the diet. Interestingly, the *Eubacterium* spp. probe detected the opposite trend, suggesting that CHO addition results in fewer of these particular *Eubacterium* spp. in the rumen.

Rumen bacteriophage quantitation

Theoretical considerations

From electron microscopy studies it has been observed that approximately 95% of all ruminal bacteriophage are tailed. Free bacteriophage can be separated from rumen fluid by differential centrifugation and filtration and their DNAs isolated [8]. As a general rule, tailed phage contain linear double stranded DNA genomes with a single copy in each phage particle [1]. Therefore, it is possible to determine phage genome size by comparing the relative migration of rumen phage DNA and concatomerised λ phage DNA in pulsed field agarose gels [8]. Laser densitometry of gel photographs enables calculation of the relative amounts of DNA present in each region of the gel (Fig. 3). Ruminal phage DNA concentration can also be determined by comparison with a known amount of plasmid DNA in conventional agarose gels. Therefore, by knowing the phage DNA concentration of each band and its size, one can calculate the number of linear double-stranded DNA molecules present in each region. As the ratio of double stranded DNA molecules to phage particles (tailed phages) is at unity, this also gives the number of phage represented by each region. Addition of phage numbers represented in each region gives the total phage present in that volume of sample. Hence, the concentration of phage present in the original rumen fluid sample can be calculated.

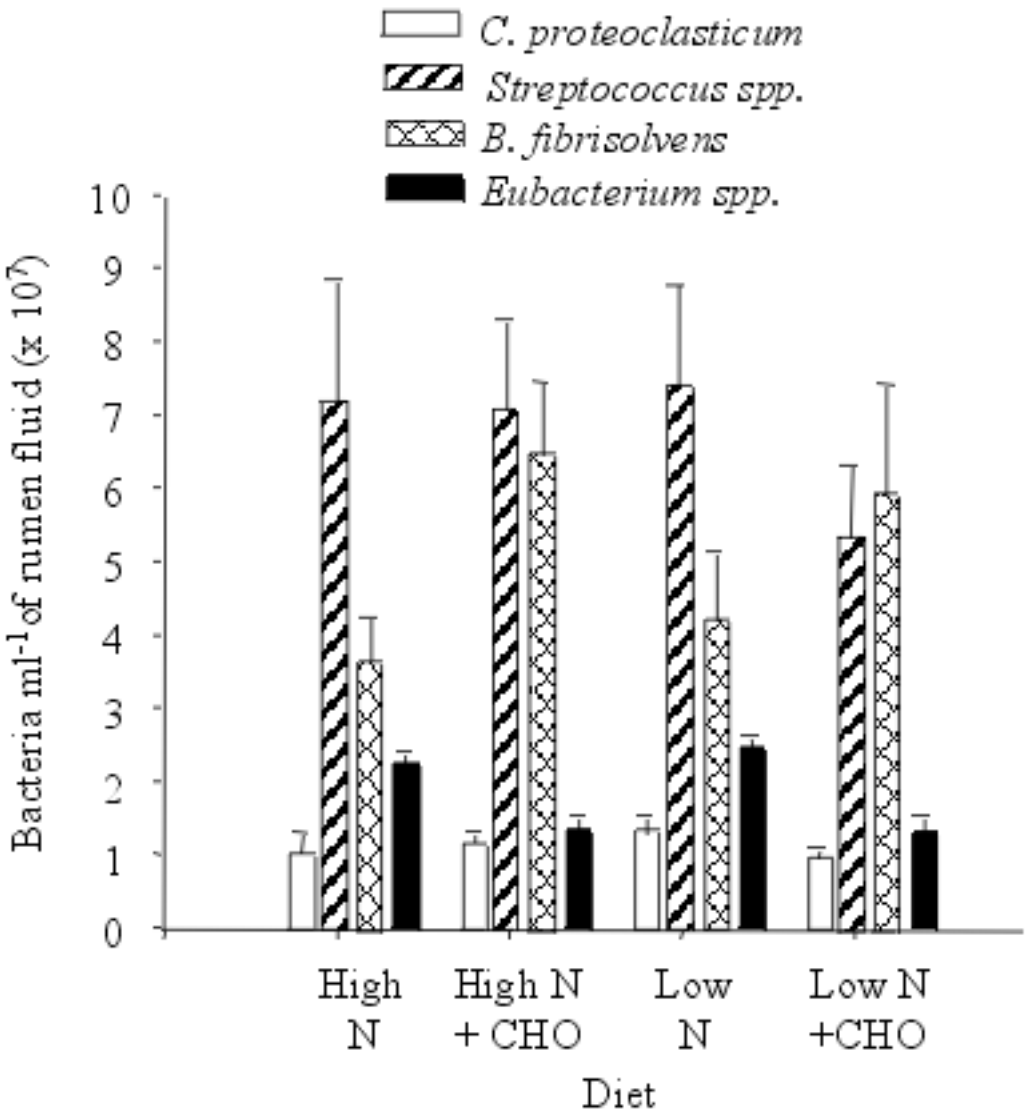


Fig. 2. cPCR quantification of proteolytic bacterial populations *in vivo.* Eight fistulated, lactating, Friesian dairy cows were fed 4 different diets in rotation: high nitrogen (High N; ryegrass-clover pasture, N 2.82% of dry matter); high nitrogen with carbohydrate (High N+CHO; 50:50 mixture of dextrose/cornflour, supplying 10% of minimum energy intake); low nitrogen (Low N;N 2.11% of dry matter) and low nitrogen with carbohydrate (low N+CHO). Rumen samples were collected and DNA extracted and probed for bacterial populations as previously described [19]. Results are the means of triplicate determinations and error bars represent standard error of the mean.

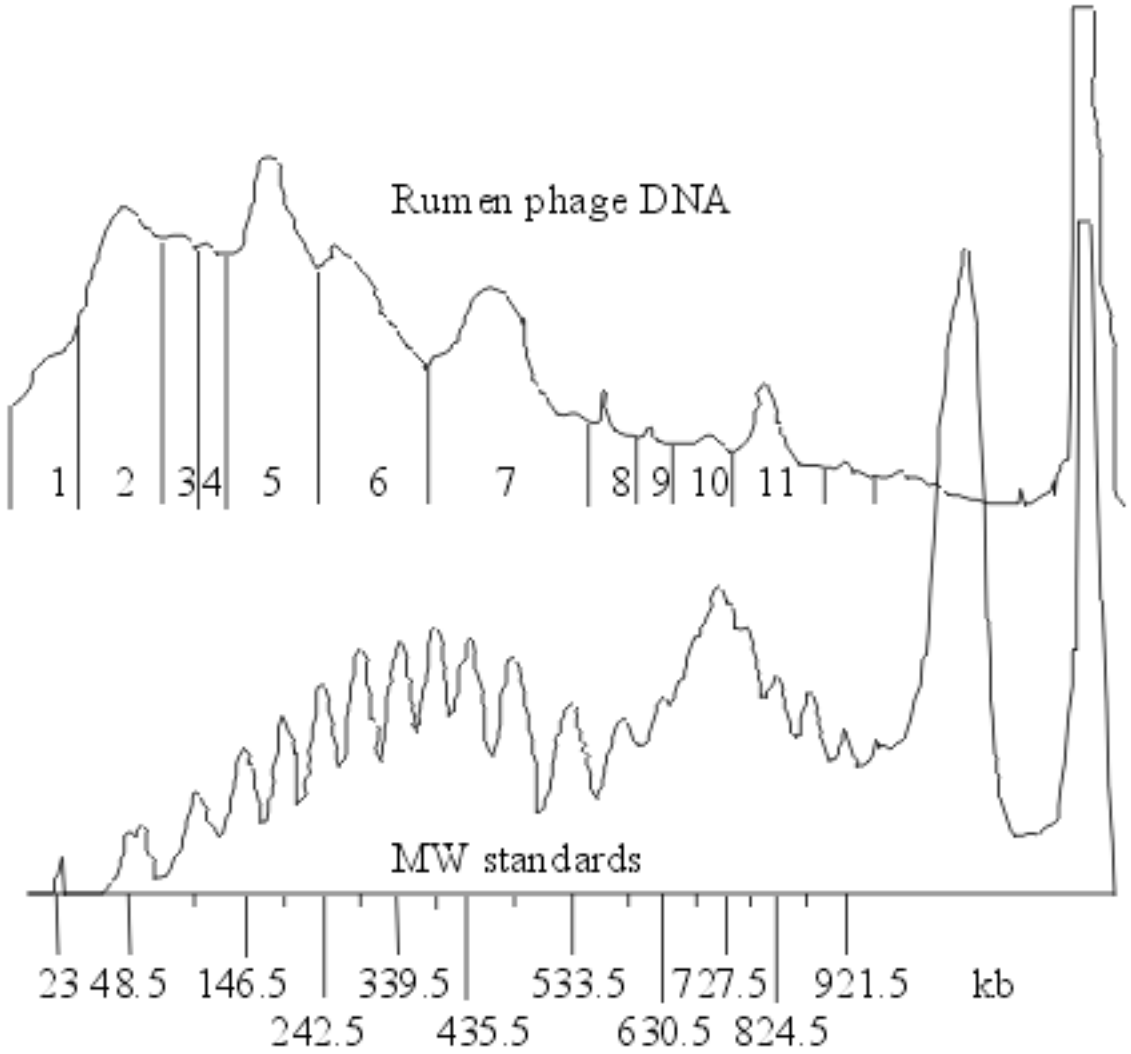


Fig. 3. Laser densitometric scans of ruminal phage DNA and lambda ladder DNA size marker separated by pulsed field gel electrophoresis.

Phage diversity and abundance

Using the PFGE and laser densitometry techniques outlined above, rumen samples have been found to contain a wide range of phage DNAs. Phage genomes vary widely in size from 10 to 800kb, although not all genome sizes are represented in every rumen sample. Analyses of many different rumen samples have shown that a broad region of phage DNA of size 30 to 200kb is always present. Distinct phage DNA bands of various sizes also occur but these appear less commonly and probably represent blooms of particular phage types. Phage densities determined using the technique are high, ranging from 10^9 to 10^{10} particles ml^{-1} of rumen fluid, and these agree well with the estimates from direct counts using electron microscopy.

Diurnal fluctuation

Diurnal fluctuations in ruminal phage were also followed by sampling 3 fistulated sheep every two hours over a period of 24 hours [20]. Another 8 sheep were also examined,

these samples were pooled and processed as a single sample. The results (Fig. 4) show a marked diurnal fluctuation in extracted phage DNA with a large drop approximately two hours after feeding. Phage DNA concentrations then increased and peaked 8 to 10 hours post-feeding, then slowly declined to pre-feeding levels over the remaining 14 to 16 hours. This fluctuation in phage DNA may be related to the availability of phage receptor sites on the outer membrane of rumen bacteria. Prior to feeding, the rumen bacterial population is likely to be substrate-limited and metabolically not fully active. In this situation one would expect the cells not to express substrate receptor proteins which are often the sites for bacteriophage absorption and therefore the free phage population in the rumen would be high. After ingestion of fresh feed, substrates for bacterial metabolism would become

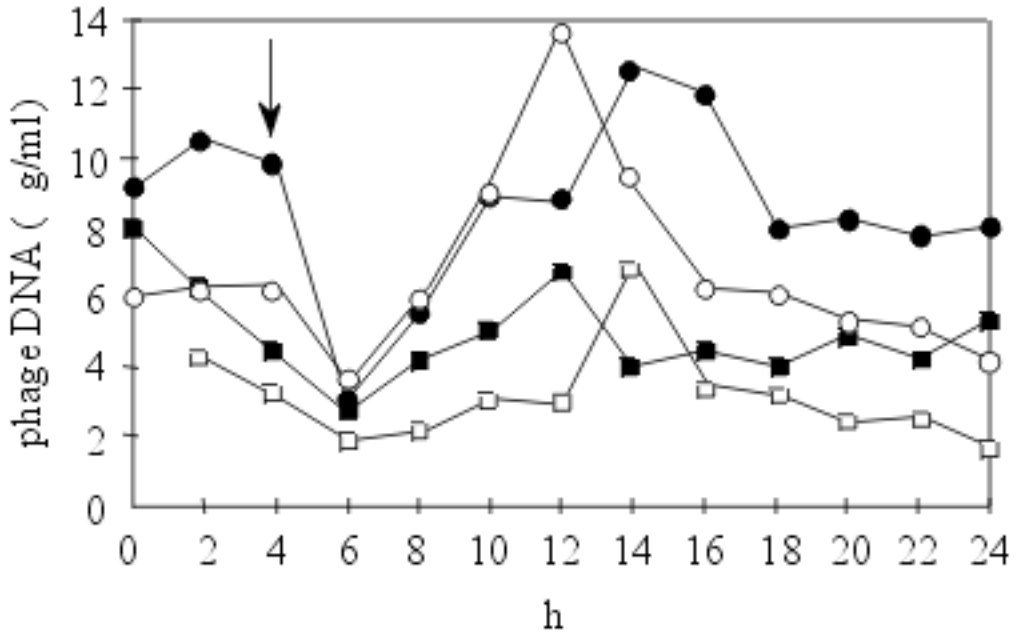


Fig. 4. Diurnal fluctuations in ruminal phage DNA concentrations. Rumen fluid was collected from 11 fistulated sheep which were fed once daily (time of feeding indicated by arrow) an oaten chaff-lucerne (70:30) ration [20]. Three of the 11 sheep were sampled individually (○, ●, □) every two hours over a period of 24 hr while the remaining 8 sheep samples were pooled and processed as a single sample (■).

available and cells would express receptor proteins to facilitate their uptake. Phage would absorb to these receptor sites thereby reducing the free ruminal phage population, which would be reflected by recovery of lower amounts of phage DNA. After absorption, phage would infect the bacterial cells, multiply, lyse the cell and be released in large numbers causing the peak of phage seen at 8 to 10 hours post feeding. The slow decline of phage populations thereafter may be due to absorption of phage to fresh receptor sites or slow degradation of free phage particles.

Concluding comments

To gain a better appreciation of rumen microbial ecology there are several outstanding problems which must be addressed. Firstly, we must be able to gain access to all of the organisms present before we can attempt to define how they interact in the ecosystem.

However, we cannot be sure that we have detected every organism until we know every extant microbe. There is no solution to this problem, but an iterative approach, in which microbial diversity is continually reassessed using new probes nested at different taxonomic levels seems the best alternative. Secondly, we must be able to accurately differentiate between organisms and enumerate them at a level which is relevant to both the organisms themselves and the ecosystem in which they reside. For example, minor changes in a single bacterial population may seem insignificant, but if that population carries out a central metabolic process or produces a compound which affects a large number of other organisms, then it may have profound effects on the ecosystem in general. Therefore resolution of microbial populations will need to be defined also by their functional contribution to the community. The improved sensitivity of the cPCR technique described here may be useful in this regard. Finally, we must account for biological factors, such as bacteriophage, broad host range plasmids and transposons, which have significant influences on the dynamics of bacterial populations in the rumen, but which cannot be assessed using ssrRNAs. The PFGE/laser densitometry technique for quantitative determination of ruminal phage DNA is a step in this direction and hopefully will provide fresh insights into phage population dynamics and their influence on rumen microbial ecology.

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