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Natural Variability and Diurnal Fluctuations within the Bacteriophage Population of the Rumen

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To investigate the impact of nutritional and environmental factors on bacteriophage activity in the rumen, it is first valuable to determine the extent of natural variations and fluctuations in phage populations from different animal species, and from animals located together and separately, and variation in animals over time. Differences in phage populations between sheep on different diets, between sheep and goats, and within the rumen over time were investigated by using pulsed-field gel electrophoresis and comparing total phage DNA in ruminal fluid. It was found that no two individuals had similar DNA banding patterns, even when similarly fed and penned together, indicating there is considerable individual diversity in phage populations between animals. Despite these individual differences, the quantities, but not the banding patterns, of phage DNA were similar for animals within groups but varied between groups, suggesting that nutritional factors may influence overall phage activity in the rumen. In sheep fed once daily, a distinct diurnal variation in the phage population was observed. Two hours postfeeding, total phage DNA dropped to its lowest level. The phage population then increased, reaching a maximal level 8 to 10 h postfeeding before declining over the next 4 h to reach a stable concentration for the rest of the cycle. The general trend in phage DNA concentration appeared similar to previously recorded diurnal fluctuations in ruminal bacterial populations in cattle fed once daily.

Bacteriophages are obligate pathogens of bacteria, occur in dense populations in the rumen (3, 4, 8, 9), and lead to the eventual lysis of the bacterial host. Knowledge of the role of bacteriophages in bacterial lysis and nutrient cycling within the rumen is limited. However, the size of the population suggests that they could be responsible for considerable bacterial lysis and may be a factor in reducing the efficiency of feed utilization (2, 4, 6, 7).

The effect of changes in dietary components or intraruminal conditions on the levels of phage-mediated bacterial lysis in the rumen is unknown. To investigate the impact of these factors on ruminal fermentation, it is useful to know the extent of background, or natural, variation and fluctuations that occur in the ruminal phage population. Recently, Klieve and Swain (4) developed methodology using pulsed-field gel electrophoresis (PFGE) and laser densitometry to estimate the numbers and, by comparison of DNA banding patterns, diversity of phages in the rumen at any time. Preliminary data from this work suggested that the phage population within the rumen ecosystem was highly labile.

This report describes the use of PFGE to investigate differences in the phage population in different animal species, and in animals located together or separately, and variation in animals over time.

MATERIALS AND METHODS

Estimation of the size of the phage population. Techniques for the purification of phage particles, the isolation of phage DNA, conventional agarose gel electrophoresis, PFGE, and determination of phage density have been reported previously (4).

Variation in ruminal phage populations of animals penned individually and in small groups. We examined whether phage populations varied between species of ruminants, between animals held in close association with each other, or between animals on different feed by comparing the phage populations from the following groups of animals: group 1, four sheep in the same pen and sharing the same feeding troughs; group 2, four goats penned and fed as for the sheep in group 1; and group 3, three sheep on pasture. In addition, the ruminal phage populations of three sheep, kept in individual pens, that were defaunated (protozoa had been eliminated) were examined. All sheep used were mature merino wethers (approximately 28 kg [liveweight]), and penned animals received a daily ration of 1 kg of oaten chaff-lucerne (70:30). Ruminal contents from penned animals were taken via a ruminal cannula as previously described (4), whereas ruminal contents were obtained from the grazing sheep through a tube inserted into the rumen via the esophagus. All samples were strained through a layer of nylon gauze prior to phage purification and concentration. DNA from the phage fraction of each sample was separated by PFGE, and total phage DNA was determined (4).

Diurnal fluctuation in the ruminal phage population. The rate and extent of change over time of the ruminal phage population in an animal was examined by observing changes in total phage DNA and the DNA banding pattern on pulsed-field gels of the population. Changes were observed in a single animal at daily and then 2-h intervals. The animal had been on this diet for more than a month prior to sampling.

Ruminal contents were sampled, via a ruminal cannula, daily for 4 days from a sheep that was penned by itself and given a daily ration of 1 kg of oaten chaff-lucerne (70:30). When variation at 2-h intervals was examined, samples were initially taken over 48 h from 6 a.m. on the first day to 8 p.m. on the second. No samples were taken between 8 p.m. and 6 a.m. Feed was 1 kg of oaten chaff-lucerne (70:30) and was offered once daily at 8 a.m. This experiment was performed twice on the same animal to determine the reproducibility of observations at another point in time.

A further experiment with 13 rumen-cannulated sheep was then undertaken. Eleven animals were fed once daily (at 9 a.m.) 1 kg of oaten chaff-lucerne (70:30), and two had feed (oaten chaff-lucerne [70:30]) always available. Prior to the experiment, the animals had been adapted to the diet for 2 weeks. Samples were taken from each sheep at 2-h intervals over a 24-h period. Samples from three of the sheep fed once daily and two fed continuously were processed individually; samples from the remaining eight animals were combined by mixing equal amounts from each animal and processed as a single sample.

RESULTS

Natural variation. Considerable variation in phage DNA banding patterns on pulsed-field gels was observed between all

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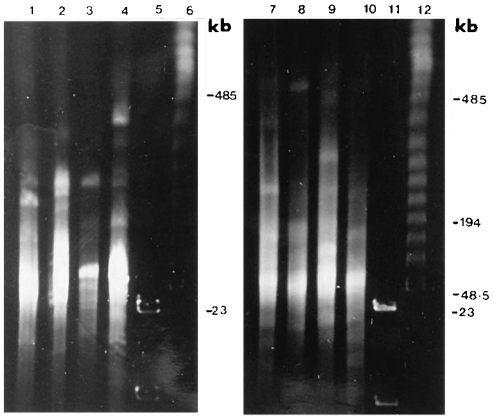

FIG. 1. DNA from ruminal phage samples from four sheep penned together and fed a once-daily ration of 1 kg of oaten chaff-lucerne (70:30) (lanes 1 to 4) and four goats held under similar conditions (lanes 7 to 10). DNA size markers were the *Hin*dIII digest of lambda phage DNA (lanes 5 and 11) and the lambda ladder (lanes 6 and 12).

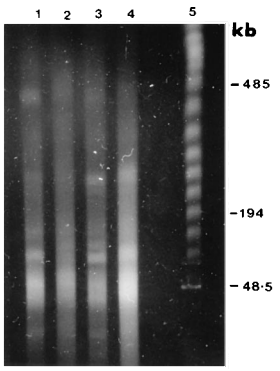

FIG. 2. DNA from ruminal phage samples from one sheep fed once daily a ration of 1 kg of oaten chaff-lucerne (70:30) and sampled on four consecutive days (lanes 1 to 4 respectively). DNA size marker was the lambda ladder (lane 5).

animals examined, including those penned together (Fig. 1). All animals had phage populations that gave a broad band of DNA between 30 and 200 kb in size. Discrete bands of DNA were evident between 10 and >500 kb. The exact positions of the discrete bands varied from animal to animal. No two animals had similar DNA banding patterns, indicating that there was diversity in phage populations between animals. Grazing sheep showed fewer and less dramatic differences in DNA banding patterns.

Despite the differences between individuals, the total phage DNA concentrations in samples were similar for animals within groups, but the mean concentrations varied between groups of animals (Table 1). An exception was the defaunated sheep: one individual had much less phage DNA present per milliliter than the other two in that group. Approximately four to five times the concentration of phage DNA was isolated from rumen fluid of grazing sheep than from their counterparts fed oaten chaff-lucerne (70:30).

Diurnal fluctuation. When observed on a daily basis (Fig. 2), the phage populations were found to be different on each day. While a broad band of DNA (30 to 100 kb) was always present, the positions of discrete bands varied considerably. The total phage DNA present was 0.69, 3.87, 2.00, and 1.98 μg/ml of ruminal fluid on days 1, 2, 3, and 4 respectively.

To determine how rapidly the day-to-day changes in the phage population occurred, an individual sheep was sampled at 2-h intervals over 48 h with a 10-h break between 8 p.m. and 6 a.m. The PFGE results are shown in Fig. 3a. Between some consecutive samples, the phage population was remarkably stable, with virtually no change in DNA banding pattern, the relative intensities of the bands, or total phage DNA present. Changes in pattern and intensity of discrete bands were gradual and occurred over a number of consecutive samples. In addition to discrete changes, an increase in total phage DNA

TABLE 1. Total phage DNA isolated from ruminal fluid samples from sheep and goats housed in group pens or penned individually and either grazing pasture or fed a roughage diet

Treatment[a]	Total phage DNA (μg/ml of ruminal fluid) for animal:				Group avg[b]
	1	2	3	4	
Goats penned together, OC-L	5.02	5.59	6.02	5.40	5.51a
Sheep penned together, OC-L	3.60	2.88	1.26	1.58	2.33a
Defaunated sheep penned individually, OC-L	4.92	16.74	13.95		11.87b
Sheep grazing pasture	13.50	14.23	12.30		13.34b

[a] OC-L, oaten chaff-lucerne (70:30).
[b] Means not followed by the same letter differ significantly ($P < 0.05$).

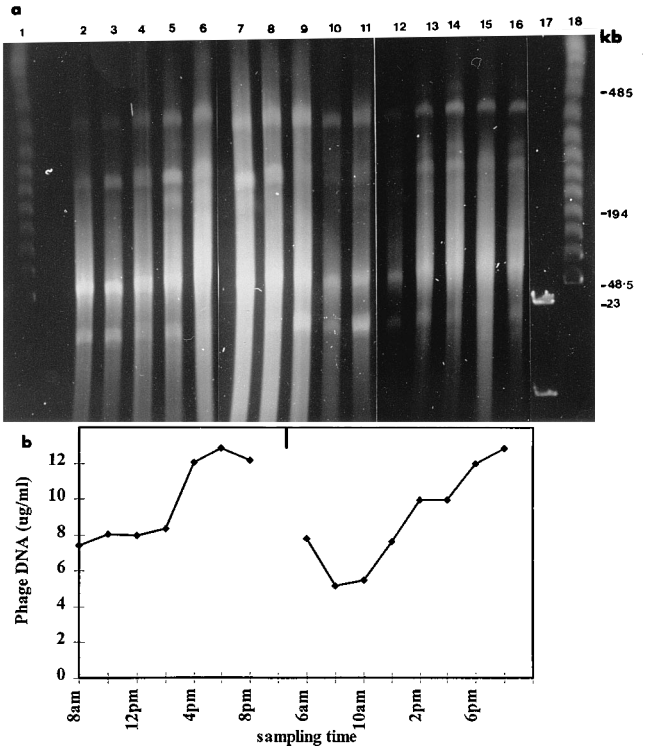

FIG. 3. DNA from ruminal phage samples from one sheep fed once daily (8:00 a.m.) a ration of 1 kg of oaten chaff-lucerne (70:30) and sampled at 2-h intervals over 48 h, from 8:00 a.m. to 8:00 p.m. on the first day (lanes 2 to 8) and 6:00 a.m. to 8:00 p.m. on the second day (lanes 9 to 16). (a) DNA banding patterns of the samples as separated by PFGE; (b) total phage DNA present in the samples. DNA size markers were the *Hin*dIII digest of lambda phage DNA (lane 17) and the lambda ladder (lanes 1 and 18).

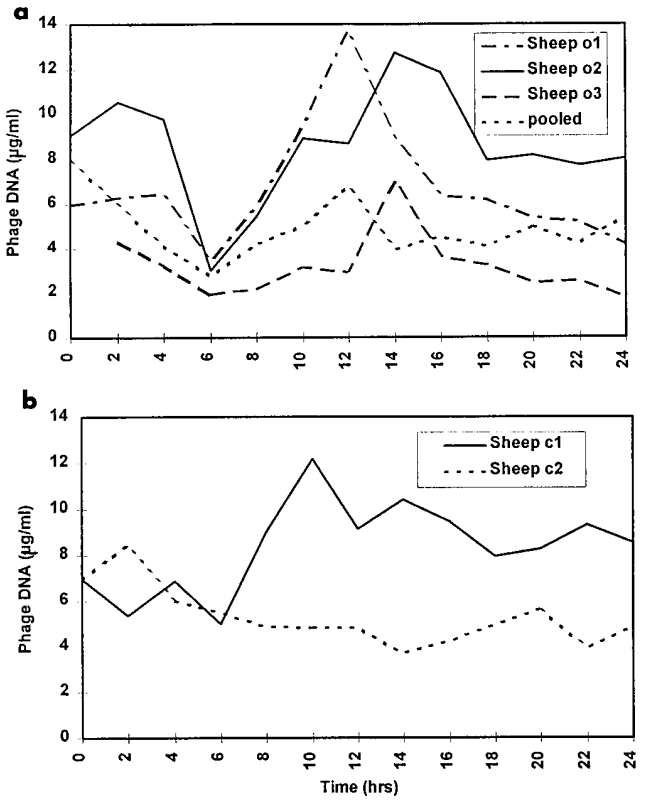

FIG. 4. Diurnal fluctuations in total phage DNA from ruminal fluid samples from (a) 11 sheep fed once daily (3 sheep were sampled and the ruminal fluids were processed individually; the ruminal fluid samples from the other 8 sheep were pooled and processed as a single sample) and (b) 2 sheep for which feed was continuously available. Samples were taken at 2-h intervals from 5:00 a.m. (time zero) for 24 h. The animals fed once daily were fed at 9:00 a.m. (4-h mark).

occurred 6 to 8 h after feeding (Fig. 3b). Total phage DNA remained stable for the first 6 h, at approximately 8 μg of DNA per ml of ruminal fluid, then rose to 12 μg of DNA per ml. The trend on the second day appeared similar. From PFGE results (Fig. 3a), the increase was across the entire population. Similar results were obtained when this experiment was repeated with the same animal.

These experiments indicated that a diurnal variation in the phage population may occur in animals fed once daily. A further experiment was carried out with 11 once-daily-fed sheep and 2 continuously fed sheep. This experiment was conducted over a 24-h period, and there was no break in the sampling. A diurnal variation in total phage DNA was seen in the animals fed once daily (Fig. 4). The three animals from which phage populations were individually determined showed similar trends. Following feeding, phage numbers dropped and reached the low point of the cycle 2 h postfeeding. For the next 6 to 8 h, phage activity increased and peaked 8 to 10 h after feeding. Phage numbers dropped rapidly, and then stabilized, some 4 h after they had peaked and remained steady for the remainder of the 24 h at levels (in terms of total DNA) similar to those found at the start of sampling. Phage populations from the pooled sample showed a similar trend, although not as pronounced.

The two continuously fed sheep had different patterns of phage variability over the 24-h period. One showed a trend in changing total phage DNA concentration similar to that for the once-daily-fed sheep, while the other showed little if any diurnal variation and phage activity appeared to be relatively stable throughout the sampling period.

Pulsed-field gels showed that increases in total phage DNA occurred across the spectrum of the DNA banding profile in a manner similar to that shown in Fig. 3.

DISCUSSION

Phage populations differed markedly between animals. The individuality of phage DNA patterns on pulsed-field gels was such that animals on the same diet and penned together, so that exchange of microflora and fauna between animals could occur, did not have similar phage populations. This work not only confirms preliminary findings (4) that the phage population of the rumen is highly labile but also shows that the phage population in an animal at any given time is unique to that animal.

As the phage population and bacterial population are linked, the results suggest that the bacterial and other microbial populations are as diverse and dynamic between individual animals as is the phage population.

Despite the differences in specific phage populations between animals, the total phage DNAs isolated from animals within a group were similar. These results will need to be confirmed by further research, as the number of animals in a group was low and the number of different dietary regimens was very limited. However, it is possible that differences between groups (e.g., sheep on pasture versus penned sheep given roughages) are due to differences in the diet and that dietary components affect phage activity. If this hypothesis is correct, it may be possible to manipulate diet to reduce the level of phage-induced bacterial lysis. To this end, we are currently investigating the possible impact of secondary plant compounds on phage activity in the rumen.

Not only does phage activity vary between individual animals and groups of animals, but there is distinct diurnal variation within animals fed once daily. Three features were apparent: a drop in phage numbers shortly after feeding, followed by a continuous rise to a peak in phage numbers some 8 to 10 h postfeeding, after which phage numbers dropped back to a fairly stable level that was maintained through the rest of the cycle. The reasons for these changes are currently unknown but appear to be linked to feeding. The rise in phage numbers is likely to be due to either feed components inducing temperate phages to enter the lytic phase or an increase in host numbers becoming available for lytic infection, or both. The decrease in phage numbers 2 h postfeeding appears more difficult to explain. One possibility is that there is an increase in attachment of phage to bacteria resulting from an increase in receptor sites on the bacterial surface. Some phages, such as lambda of *Escherichia coli*, use sugar transport receptors (maltose) to infect bacteria (1). Only when this substrate is plentiful are receptors produced by the bacterium. When the substrate is low in concentration, or nonexistent, few phage will be able to adsorb to the cell and initiate an infection. If this were the situation in the rumen, feeding would trigger an increase in metabolic activity and production of substrate receptors in many rumen bacteria. This would in turn increase the rate of phage adsorption, thus lowering their numbers in the fluid phase. Further research is required to validate this hypothesis, but if correct it implies that the major limitation to lytic phage reproduction in the rumen may be availability of receptor sites. Such a mechanism could maintain pseudolysogenic or carrier states between bacteria and phages in the rumen.

As the increase in phage DNA was across the spectrum of the size classes observed in ruminal fluid and not limited to one

type or group of phages, the increase in total phage population is probably due to a combination of the factors described above.

The trend in diurnal fluctuation of the phage population shows a striking similarity to that observed by Leedle et al. (5) in ruminal bacterial populations of once-daily-fed dairy cattle. Both dipped sharply 2 h postfeeding, rose to a maximum level, and then declined slowly until the next feeding. While the bacterial populations peaked at 16 h postfeeding, the phage populations in our study peaked at 8 to 10 h postfeeding. However, in the present study, changes in the bacterial populations were not measured, and therefore the point at which they peaked may have been different from that in the cattle used by Leedle et al. (5).

Diurnal variation was not apparent in one animal that was continuously fed but appeared similar to that in the animals fed once daily in the other. Less variation would be expected in continuously fed animals, and it is possible that the anomaly was due to differences in feeding behavior; i.e., one animal may have been consuming one large meal in the day. The feeding behavior of the animals was not investigated.

The diurnal fluctuation in animals fed once daily does have implications for sampling. For comparative studies of different diets and dietary components, samples should always be taken at the same time of day relative to feeding and, to avoid differences in individual cycles, preferably in the stable period prior to feeding. It may become necessary to take a number of samples over a 24-h period to gauge the full effect of dietary changes on bacteriophage activity in the rumen, as the impact may be greater at one point in the diurnal cycle than at others. Continuous feeding may overcome this need, although more data are required to confirm this.

Currently, it is not possible to determine whether the presence of phage in the rumen is disadvantageous or advantageous. As phage lyse their bacterial hosts within the rumen, they would be a factor involved in protein recycling within the rumen. This has been identified as reducing the efficiency of feed utilization (2, 4, 6, 7) and would be disadvantageous. However, it is also possible that phage maintain bacterial population diversity and balance, which could be to the advantage of the ecosystem, allowing it to rapidly adapt to changing conditions such as dietary changes. Phage may also play a role in recycling limiting nutrients within the rumen.

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REFERENCES

1. **Arber, W.** 1983. A beginner's guide to lambda biology, p. 381–394. *In* R. W. Hendrix, J. W. Roberts, F. W. Stahl, and R. A. Weisberg (ed.), Lambda II. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. **Firkins, J. L., W. P. Weiss, and E. J. Piwonka.** 1992. Quantification of intraruminal recycling of microbial nitrogen using nitrogen-15. J. Anim. Sci. **70:** 3223–3233.
3. **Klieve, A. V., and T. Bauchop.** 1988. Morphological diversity of ruminal bacteriophages from sheep and cattle. Appl. Environ. Microbiol. **54:**1637–1641.
4. **Klieve, A. V., and R. A. Swain.** 1993. Estimation of ruminal bacteriophage numbers by pulsed-field gel electrophoresis and laser densitometry. Appl. Environ. Microbiol. **59:**2299–2303.
5. **Leedle, J. A., M. P. Bryant, and R. B. Hespell.** 1982. Diurnal variations in bacterial numbers and fluid parameters in ruminal contents of animals fed low- or high-forage diets. Appl. Environ. Microbiol. **44:**402–412.
6. **Nolan, J. V., and R. A. Leng.** 1972. Dynamic aspects of ammonia and urea metabolism in sheep. Br. J. Nutr. **27:**177–194.
7. **Nolan, J. V., and S. Stachiw.** 1979. Fermentation and nitrogen dynamics in merino sheep given a low quality roughage diet. Br. J. Nutr. **42:**63–80.
8. **Paynter, M. J. B., D. L. Ewert, and W. Chalupa.** 1969. Some morphological types of bacteriophages in bovine rumen contents. Appl. Microbiol. **18:**942–943.
9. **Ritchie, A. E., I. M. Robinson, and M. J. Allison.** 1970. Rumen bacteriophage: survey of morphological types, p. 333–334. *In* P. Favard (ed.), Microscopie electronique, 1970, vol. 3. Societe Francaise de Microscopie Electronique, Paris.