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Succession of *Streptococcus bovis* Strains with Differing Bacteriophage Sensitivities in the Rumens of Two Fistulated Sheep

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The bacteriophage sensitivity of the *Streptococcus bovis* population resident in the rumens of two fistulated sheep was monitored for 112 days. During this time, three changes in the bacteriophage sensitivity of *S. bovis* occurred in the absence of detectable bacteriophages. Identical changes in bacteriophage sensitivity occurred simultaneously in both animals and, except for the relatively short periods of changeover in phage sensitivity, the *S. bovis* population in the rumens of the two sheep was homogeneous with respect to phage sensitivity.

Rumen microorganisms have been the subject of intensive study because of their role in rumen physiology (11). Several authors examined rumen fluid by electron microscopy and reported the presence of large numbers of bacteriophages and bacteriophage-like particles (10, 20, 21). Bacteriophages (1, 2, 5, 14, 16) and lysogenic bacteria (15, 18, 22) have also been isolated from rumen fluid. In addition, Orpin and Munn (18) found bacteriophages associated with the rumen organism Eadie's Oval and reported that the disappearance of this organism from their experimental animals coincided with the presence of these phages. Nevertheless, the role of bacteriophages in rumen physiology is understood poorly.

It is possible that bacteriophages could influence rumen metabolism by lysing large numbers of bacteria while they are still in the rumen. After gaining experience with the bacteriophages of the common rumen organism *Streptococcus bovis* (14, 15), we decided to try to evaluate the effects of bacteriophages on the *S. bovis* population in the rumens of two fistulated sheep. Our plan was to isolate bacteriophages that lysed the *S. bovis* population in the rumens, add these bacteriophages to the rumens, and follow the interaction of bacteriophages and *S. bovis*. This would be done by measuring colony-forming units (CFU) of infected *S. bovis* and the plaque-forming units (PFU) of free bacteriophages (19).

One condition necessary for the success of this plan was that the bacteriophage sensitivity of the *S. bovis* population be stable in the absence of bacteriophages. Otherwise, one would never know if observed changes were due to the added bacteriophages or to some other factor. We found, in fact, that the bacteriophage sensitivity of the *S. bovis* population was not stable in the absence of bacteriophages. The details of these changes are reported in this paper.

MATERIALS AND METHODS

Media. The composition of the basal medium (NYE) was (grams per liter): Oxoid nutrient broth no. 2, 25; Oxoid yeast extract, 3; and, when desired, Davis agar, 12; the pH was 7.5. Two variations of this medium were used. They were NYESP, which was NYE containing phosphate buffer (0.02 M, pH 7.5), BDH potato starch (6.5 g/liter), nalidixic acid (15 mg/liter), and colistin sulfate (1 mg/liter), and NYEGP, which was NYE containing phosphate buffer, glucose (1 g/liter), nalidixic acid, and colistin sulfate. Soft-agar overlays consisted of 2.0 ml of NYE containing 4 g of Davis agar per liter.

Fistulated sheep. Two cross-bred wethers, about 12 months old, with rumen fistulae were fed ad lib a diet of chopped lucerne and wheaten chaff. The animals were housed individually in adjoining pens in an animal house that also contained about 20 other sheep and numerous other animals.

Procedure for sampling the rumen. The fistulated sheep were usually sampled twice a day. About 20 ml of rumen fluid was taken with wide-mouth pipettes from each animal, diluted in NYE, and plated within 20 min of collection.

Enumeration of *S. bovis* in rumen fluid. Portions (0.1 ml each) of appropriate dilutions of rumen fluid were added to soft-agar overlays, which were then poured over NYESP plates. The plates were incubated for 24 h at 37°C in an atmosphere of CO_2, and colonies surrounded by halos, an indication of starch hydrolysis (12), were counted as *S. bovis*. To confirm that colonies surrounded by halos were *S. bovis*, every week 10 such colonies from each sheep were checked to ensure that they were gram-positive, catalase-negative cocci that hydrolyzed starch,

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formed slime from sucrose, and gave a positive reaction on bile-esculin medium (3, 4, 6, 8, 9, 17).

Isolation of bacteriophages that lysed the resident *S. bovis* population. *S. bovis* isolates from the fistulated sheep were used as host strains, and bacteriophages that lysed these strains were isolated from abattoir wastes; high-titer phage stocks and phage antiserum were then prepared (14).

Enumeration of bacteriophage-resistant *S. bovis* in rumen fluid. Portions (0.1 ml each) of appropriate dilutions of rumen fluid and 0.1 ml of the appropriate bacteriophage, ca. 1×10^8 to 2×10^8 PFU, were added to soft-agar overlays. The overlays were poured over NYESP plates, which were then incubated and counted as described above.

Enumeration of bacteriophages of *S. bovis* in rumen fluid. Portions (0.1 ml each) of appropriate dilutions of rumen fluid and 0.1 ml of the appropriate indicator strain, ca. 10^8 CFU, were added to soft-agar overlays. The overlays were poured over NYEGP plates, and plaques were counted after an overnight incubation. The indicator strains were those used for the isolation of bacteriophages from abattoir wastes and had the same bacteriophage sensitivity as the resident *S. bovis* population. When the bacteriophage sensitivity of the resident *S. bovis* population changed, the indicator strain was changed accordingly.

RESULTS

Before studying the interaction of bacteriophages and the *S. bovis* population in the rumens, we tested the efficiency of the procedures for counting *S. bovis*, bacteriophage-resistant *S. bovis*, and bacteriophages of *S. bovis* in rumen fluid. This was done by adding known amounts of test bacteria and bacteriophages to a sample of rumen fluid and then subjecting this sample to the enumeration procedures. The added bacteria and bacteriophages were recovered quantitatively.

Initially, the two antibiotics were not included in media. However, without them, spreading organisms frequently overgrew the plates. The incorporation of nalidixic acid and colistin sulfate, based on the work of Ellner et al. (7), prevented the growth of the spreading organisms without altering the *S. bovis* count. However, the concentrations of nalidixic acid (15 mg/liter) and colistin sulfate (10 mg/liter) used by Ellner et al. (7) significantly reduced the colony size of *S. bovis*. By testing a concentration range of these two antibiotics, it was found that 15 mg of nalidixic acid per liter and 1 mg of colistin sulfate per liter prevented the growth of the spreading organisms without affecting the colony count or size of *S. bovis*.

The use of the two antibiotics also significantly reduced the background growth. When 0.3 ml of a 1:100 dilution of rumen fluid was plated on NYESP medium, the only colonies larger than 0.2 mm were those of *S. bovis*. With more concentrated rumen fluid, the background growth was much heavier and interfered with the enumeration procedures. This meant that the limits of detection were about 10^3 CFU/ml for both *S. bovis* and bacteriophage-resistant *S. bovis* and about 10^3 PFU/ml for *S. bovis* bacteriophages.

S. bovis isolates from each sheep were then used as host cells for the isolation of bacteriophages from abattoir wastes. Three bacteriophages were isolated, one of which was used to prepare a high-titer stock and phage antiserum. About 7 weeks were required for the isolation of the bacteriophages and for the preparation of the high-titer stock and phage antiserum, and during this time *S. bovis*, bacteriophage-resistant *S. bovis*, and bacteriophages of *S. bovis* in the rumen fluid were counted twice daily. The counts of *S. bovis* were quite constant, with most being between 1×10^6 and 4×10^6 CFU/ml (Fig. 1). Initially, no bacteriophage-resistant *S. bovis* and no bacteriophages of *S. bovis* were detected. However, after 37 days, a few bacteriophage-resistant colonies appeared, and after 43 days this bacteriophage-resistant strain had completely replaced the bacteriophage-sensitive strain in both rumens (Fig. 1). No bacteriophages had been added to the rumens, and no bacteriophages were detected in the rumens. During the changeover, the total *S. bovis* count remained at ca. 10^6 CFU/ml.

Undeterred by what was regarded as an unfortunate change in bacteriophage sensitivity,

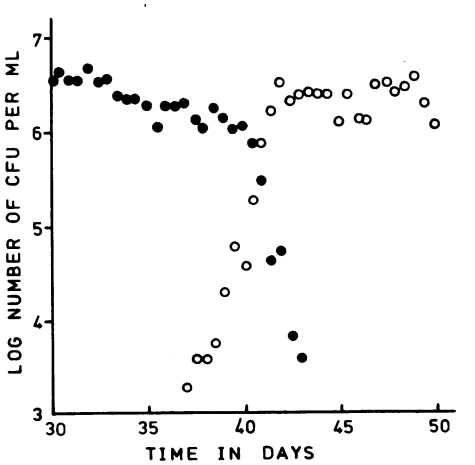

FIG. 1. *CFU of S. bovis per milliliter in the rumen fluid of a fistulated sheep. Symbols: ●, log CFU of S. bovis per milliliter sensitive to the first bacteriophage; ○, log CFU of S. bovis per milliliter resistant to the first bacteriophage but sensitive to the second bacteriophage.*

we isolated from abattoir wastes bacteriophages that lysed the new strain of *S. bovis;* these were then used to prepare a high-titer stock and phage antiserum. *S. bovis,* bacteriophage-resistant *S. bovis,* and bacteriophages of *S. bovis* in the rumen fluid continued to be counted. A second change in bacteriophage sensitivity occurred in both animals between days 66 and 69 and followed a pattern similar to that shown in Fig. 1. As before, no bacteriophages had been added to the rumens and none was detected.

These results suggested that changes in the bacteriophage sensitivity of the resident *S. bovis* population, in the absence of detectable phages, could be a regular phenomenon. The frequency of the changes made it impossible to prepare antiserum rapidly enough to carry out the experiments originally planned. These changes would also cloud the interpretation of the effects of added bacteriophages, since one could never be certain if the changes were due to the added bacteriophages or to some other factor.

To determine whether the bacteriophage sensitivity of the resident *S. bovis* population continued to change, *S. bovis,* bacteriophage-resistant *S. bovis,* and bacteriophages of *S. bovis* in the rumen fluid were monitored for another 43 days. Between days 81 and 84, a third change occurred; no more changes were observed in the next 28 days at which time these experiments were terminated.

DISCUSSION

In a period of 112 days, the bacteriophage sensitivity of the *S. bovis* population resident in the rumens of two fistulated sheep changed three times in the absence of detectable bacteriophages. It is possible that bacteriophages were present but were not detected. However, the methods used were successful in isolating bacteriophages from abattoir wastes and in quantitatively recovering test bacteriophages added to samples of rumen fluid. The new *S. bovis* strains may have been variants of the resident *S. bovis* population or may have been obtained from other animals. The other sheep in the animal house were frequently replaced, and new strains of *S. bovis* may have been brought into the animal house in this way. It has been shown that rumen bacteria can be transferred from one animal to another (11), and the simultaneous changes in bacteriophage sensitivity in both animals are consistent with this. Unfortunately, facilities for complete isolation of the sheep were not available, and this possibility could not be tested. The ability of one strain of *S. bovis* to replace another could be due to a growth advantage or to the inhibition of one strain by another. Bacteriocins of *S. bovis* have been reported (13) but were not detected among the strains of *S. bovis* isolated from these sheep.

Medrek and Barnes (17) reported that the serological specificity of the *S. bovis* strains in the feces of their cows changed with time; they also reported that the *S. bovis* population in the feces of their animals was serologically diverse. We found that, except for the relatively short periods of changeover in phage sensitivity, the *S. bovis* population in the two rumens was homogeneous with respect to phage sensitivity.

The procedure for the isolation and enumeration of *S. bovis* from rumen fluid was selective and efficient. The incorporation of nalidixic acid and colistin sulfate into the medium inhibited the growth of other bacteria, and the use of a medium that gave a direct visual indication of starch hydrolysis (12) obviated the need for indicator solutions. The isolation of bacteriophages from abattoir wastes which lysed four different strains of *S. bovis* emphasized that these wastes are an excellent source of *S. bovis* phages (14).

The frequency of the changes in the bacteriophage sensitivity of the *S. bovis* population resident in the rumens made it impossible to carry out the experiments originally planned and suggests that the flora of the rumen is in a highly dynamic state.

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