APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1983, p. 1780–1784
0099-2240/83/061780-05$02.00/0

Vol. 45, No. 6

Isolation of Proteolytic Rumen Bacteria by Use of Selective Medium Containing Leaf Fraction 1 Protein (Ribulosebisphosphate Carboxylase)

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Received 1 November 1982/Accepted 28 March 1983

The principle proteolytic bacteria isolated from bovine rumen contents by virtue of the ability to obtain nitrogen from the proteolysis of leaf fraction 1 protein (ribulosebisphosphate carboxylase, EC 4.1.1.39) were identified as *Streptococcus bovis* and *Butyrivibrio* spp. Substitution of fresh fodder, rich in soluble protein, for a hay-concentrates diet resulted in enhanced ruminal proteolytic activity and a significant increase in the number of bacteria able to use fraction 1 protein as the sole nitrogen source. Isolated proteolytic bacteria degraded fraction 1 protein and casein readily. Bovine serum albumin was attacked by *Butyrivibrio* spp. but was resistant to proteolysis by the streptococci.

Early attempts to enumerate and isolate proteolytic rumen bacteria employed differential media containing casein. In general, proteolytic bacteria isolated from such media belonged to the major genera of rumen bacteria and utilized ammonia as the preferred nitrogen source (1, 2). Proteolytic bacteria surveyed by Fulghum and Moore (6) consisted largely of butyrivibrios. Among the known proteolytic rumen bacteria, *Bacteroides amylophilus* H18 has proved to be the most active and widely studied (see, for example, reference 3), even though its proteolytic activity represents only one of the range of proteinase activities normally found in the rumen (4).

There is now evidence that some proteolytic rumen bacteria may not utilize ammonia but may instead depend on the activity of their proteinases to generate the peptide nitrogen required for growth (8, 9). To date, no attempt has been made to enumerate or characterize proteolytic organisms of this type, so it is not known whether they contribute significantly to proteolytic activity in vivo. For the isolation of such bacteria, we have formulated a selective medium which contains leaf fraction 1 (F1) protein as the sole source of nitrogen. This medium was used to determine whether an increase in proteolytic bacteria resulting from the use of F1 protein as the N source could account for the increased rate of ruminal proteolysis, which occurrs when a fresh fodder like lucerne (which is rich in soluble proteins, including leaf F1 protein) is substituted for a hay-concentrates diet (18). We also used the medium to investigate a recent proposal (20) that *Streptococcus bovis* contributes significantly to proteolytic activity in vivo.

MATERIALS AND METHODS

Animals. Two Jersey cows (no. T10 and W24) with permanent rumen cannulae were fed twice daily (at 9:15 a.m. and 4:30 p.m.) and permitted ad libitum access to water. Dry rations comprised hay (2 kg) and concentrates (0.5 kg) for each feed. Substitution of a mixture of freshly cut lucerne (*Medicago sativa* L.) and cocksfoot (*Dactylis glomerata* L.) for dry rations was carried out in stages to avoid scouring. For a period of 2 days, hay (2 kg) and fresh fodder (4 kg) were administered together at each feed. The fresh fodder was then increased to 8 kg per cow (hay, 1 kg per cow) and after a further 2 days, to 13 kg per cow at each feed. Rumen fluid samples were withdrawn immediately before the morning feed.

Isolation and enumeration of proteolytic bacteria. Bacteria able to degrade soluble protein and utilize the liberated amino acids were isolated and enumerated in a low-N basal medium (9) containing glucose (4 g liter^{-1}) and F1 protein (0.5 g liter^{-1}) as the sole N source. Tubes (9 ml) of molten medium (agar, 20 g liter^{-1}; 45 to 50°C) were inoculated (1 ml) with whole rumen contents which had been serially diluted in the same medium minus agar, vitamins, and F1 protein. After mixing, the contents were allowed to solidify. A representative sample of rumen solids was taken with the initial sample by the use of a wide-bore pipette. For isolating bacteria, poured plates were prepared inside an anaerobic glove box (Coy Laboratory Products Inc., Ann Arbor, Mich.) under N_2-H_2 (95:5 [vol/vol]) and were incubated under CO_2 at 39°C. Counting and picking of colonies was carried out within 48 h of incubation. In the course of the study, countable numbers of proteolytic bacteria, utilizing F1 protein as

the N source, grew when rumen fluid diluted 10^{-6}, 10^{-7}, and sometimes 10^{-8} was plated. All isolations were made from well-separated colonies in this range of dilutions. Low-N basal medium without added F1 protein did not support bacterial growth during 48 h of incubation. To minimize the effects of fluctuations in the rumen flora, isolations were made from rumen contents, sampled immediately before the morning feed, only when the animals were well established on a particular diet.

Enumeration of *S. bovis*. *S. bovis* was enumerated by using the selective medium employed previously by Iverson and Millis (13) for culturing total *S. bovis* in rumen contents. Serial dilutions of rumen contents were inoculated (1 ml) into a selective medium (9 ml; agar, 20 g liter^{-1}) which contained nutrient broth, yeast extract, starch, nalidixic acid, and colistin sulfate. Colonies which developed consisted of, without exception, gram-positive cocci and were counted within 48 h of incubation at 39°C.

Proteins. F1 protein prepared from lucerne by the procedure of Jones and Mangan (14) and stored as a lyophilized powder at −20°C was dissolved in glass-distilled water, dialyzed at 4°C against three changes of 7.5 mM phosphate buffer (pH 6.8), and diluted to the required concentration by reference to a standard curve relating absorbance at 280 nm (A_{280}) to protein concentration. Bovine serum albumin (BSA; fraction V) and azocasein were obtained from Sigma Chemical Co., St. Louis, Mo., and casein (light, white, soluble) was obtained from BDH Biochemicals, Poole, England. Protein nitrogen was determined by a micro-Kjeldahl method (17). Protein solutions were filter sterilized before addition to the growth media.

Measurement of proteolytic activity. The activity of rumen proteinases was assayed at 39°C with azocasein (5 g liter^{-1}) as the substrate (5). Rumen contents were centrifuged (39,000 × *g*; 4°C; 20 min), and the pellet which contained 80 to 90% of the total proteolytic activity was washed once with 75 mM phosphate buffer (pH 6.8) containing 2 mM dithiothreitol before being resuspended to half the original volume in the same buffer. Incubations (3 ml of washed cells and 0.5 ml of azocasein [40 g liter^{-1}] under a gas phase of O_2-free N_2) were terminated after 20 min by rapid cooling in an ice bath, and after centrifugation (2,500 × *g*; 4°C; 20 min), the cell-free supernatant was deproteinized with trichloroacetic acid (TCA; final concentration, 10% [wt/vol]). TCA-soluble products (10,000 × *g*; 4°C; 20 min) were measured by adding NaOH (to 0.5 M) to the final supernatant and comparing the A_{440} of the solution with a standard curve for azocasein in 0.5 M NaOH; to ensure exclusion of fragments of the TCA pellet, the supernatant was passed through a glass filter (type A-E; Gelman Instrument Co.).

The ability of isolated bacteria to degrade different proteins under conditions of growth was determined by culturing each organism at 39°C for up to 48 h in the low-N basal medium (9) supplemented with Casitone (5 g liter^{-1}; Difco Laboratories, Detroit, Mich.) and the required protein (1 to 1.5 g liter^{-1}). Although all proteolytic isolates had demonstrated the ability to use F1 protein as the sole N source, casitone (which supported good growth of all the isolates) was added to the medium to promote active growth in the event of the added protein being only slowly degraded or poorly utilized. By using the open-tube technique (16), tubes (5 ml) of medium were inoculated (5 to 10% [vol/vol]) from a stock culture of each isolated organism in a nonselective rumen fluid-containing medium (medium 2 [10]). After 48 h of incubation, the cell-free culture supernatant (2,500 × *g*; 4°C; 10 min) was deproteinized with TCA, and the residual protein in the TCA pellet was dissolved in 0.5 M NaOH and measured by comparing the A_{280} of the solution to a standard curve relating the A_{280} of the purified protein in 0.5 M NaOH to its concentration. Control incubations were carried out to establish that protein could be quantitatively recovered and that determination of A_{280} was not significantly affected by growth of bacteria in the medium.

Characterization of isolates. Bacterial isolates were characterized according to the scheme described by Holdeman and Moore (11) and also by reference to other standard texts. The basal liquid medium for biochemical tests was medium 2 (10), to which required substrates were added at 0.1 to 0.5% (wt/vol). Volatile fatty acid production in medium 2 plus glucose (0.5% [wt/vol]) was measured chromatographically as described by Hazlewood and Dawson (7), except that neopentylglycoladipate (20% [wt/wt]) was substituted for Tween 80 as the stationary phase, and the operating temperature was 120°C.

RESULTS

Isolation of bacteria with F1 protein as the N source. Bacteria were isolated from the rumens of cows consuming either dry rations (hay-concentrates) or fresh fodder (lucerne-cocksfoot) by the use of a selective protein-containing medium devoid of other N sources. When screened for proteinase activity, organisms thus isolated were able to hydrolyze F1 protein and could be maintained in a medium containing that protein as the sole N source.

Identification of bacteria with F1 protein as the N source. Proteolytic bacteria isolated on selective F1 protein-containing medium from the rumens of cows fed either dry rations or fresh fodder were of two morphological types: nonmotile gram-positive cocci and motile gram-negative rods, the majority of which were distinctly curved. The ability of these proteolytic isolates to use ammonia as the N source was examined; only 12% of the gram-positive cocci tested grew on ammonia, and none were particularly vigorous. By comparison, 60% of the gram-negative rods tested were able to use ammonia as the N source. The gram-positive cocci in general produced lactate as a major fermentation product; hydrolyzed starch, raffinose, and melibiose; grew at 45°C; gave no reaction in the presence of potassium tellurite; did not use melezitose and mannitol; and did not grow at 10°C or in the presence of 6.5% (wt/vol) NaCl. These bacteria were identified as *S. bovis* with the exception of the few isolates which grew weakly at 10°C, failed to hydrolyze starch, but grew at the expense of melezitose and mannitol;

these organisms were assigned to the species *Streptococcus faecium*.

The gram-negative rods produced butyrate as a major fermentation product, and in some instances acetate also accumulated. Indole was not produced by any of the isolates, and esculin was hydrolyzed by all of them; mannose, lactose, raffinose, and starch were hydrolyzed by the majority of isolates, whereas mannitol and melezitose were not attacked. On the basis of these tests, we determined that the gram-negative rods probably belong to the genus *Butyrivibrio*, the majority being assignable to group 1 of Shane et al. (22), with the acetate producers characteristic of group 2.

Proteolytic activity of rumen contents. For both cows, substitution of fresh fodder for dry rations resulted in a significant increase (P = 0.01 to 0.001) in ruminal proteinase activity, with the average rate of azocasein proteolysis by washed rumen microorganisms some 2.5 times higher for animals on the fresh fodder diet (ca. 7.7 μg, compared with 3.0 μg of azocasein hydrolyzed ml of rumen contents^{-1} min^{-1}). An initial drop in rumen proteolytic activity occurred when one-third of the diet was replaced by fresh fodder; this could be due to an increase in rumen dilution rate resulting from the feeding of a fresh herbage with a lower dry matter content. Thereafter, proteolytic activity increased as the proportion of fresh fodder administered at each feed was increased.

Enumeration of bacteria with F1 protein as the N source. Proteolytic bacteria (34 strains) isolated from a cow on dry rations by the use of the F1 protein-containing selective medium comprised *S. bovis* (44% of isolates), *S. faecium* (6% of isolates), and *Butyrivibrio* spp. (50% of isolates). For both animals fed the dry diet, total numbers of *S. bovis* in the rumen exceeded the number of bacteria able to utilize F1 protein as the sole N source (Fig. 1), and it was concluded that under this feeding regime, although it is possible to isolate proteolytic bacteria which utilize F1 protein, the majority of *S. bovis* are not strongly proteolytic. Changing to a diet composed entirely of fresh fodder resulted in a significant increase (P = 0.01 to 0.001) in the number of bacteria able to utilize F1 protein: from $3.98 \times 10^7 \pm 2.81 \times 10^7$ to $27.32 \times 10^7 \pm 10.94 \times 10^7$ ml^{-1} (Fig. 1). At the same time, there was no significant increase in the total number of *S. bovis* cells present in the rumen contents of both cows. However, proteolytic bacteria (50 strains) isolated from animals on the fresh fodder diet through the ability to use F1 protein as the sole N source were composed of *S. bovis* (84% of isolates), *S. faecium* (6% of isolates), *Butyrivibrio* spp. (6% of isolates), and unidentified gram-negative rods (4% of isolates), suggesting that

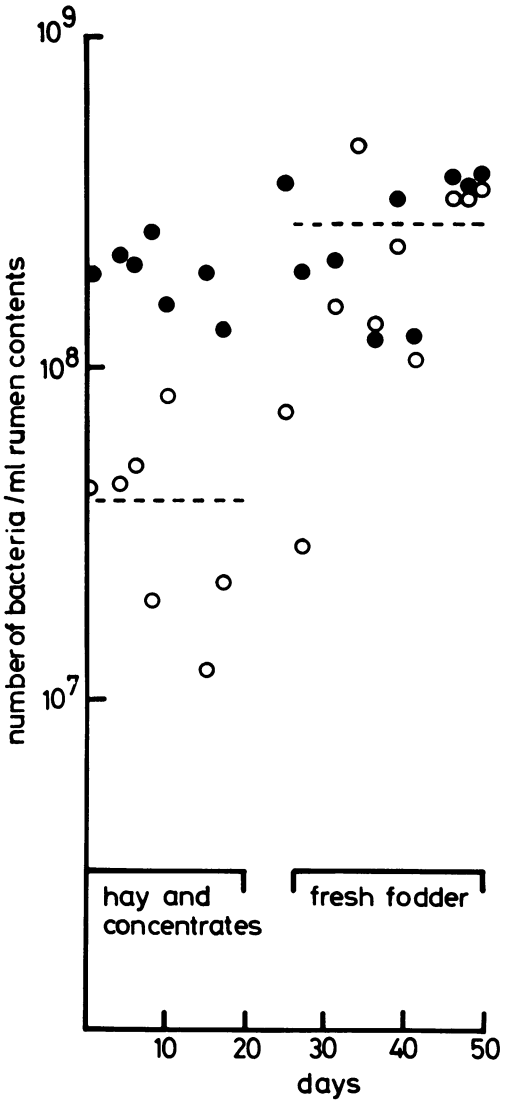

FIG. 1. Enumeration of F1 protein-utilizing proteolytic bacteria and total *S. bovis* cells in bovine rumen contents. Numbers of proteolytic bacteria (○) and *S. bovis* (●) were determined as described in the text for two cows initially fed hay-concentrates and subsequently fresh fodder (lucerne-cocksfoot). Each point is the average of two determinations. (----) Average populations of proteolytic F1 protein-utilizing bacteria in cows fed hay-concentrates or fresh fodder.

although total numbers of *S. bovis* cells had not been significantly affected by a fresh fodder feeding, the ability of the cells to degrade F1 protein had been greatly enhanced.

Substrate specificity of proteolytic bacteria. Proteolytic gram-positive cocci (17 strains) and gram-negative rods (18 strains), selected from isolates obtained by using the selective F1 protein-containing medium, were screened for their

TABLE 1. Proteolysis of three substrates by proteolytic rumen isolates[a]

Morphological type	F1 protein			Casein			BSA		
	No. of positive strains	% Proteolysis		No. of positive strains	% Proteolysis		No. of positive strains	% Proteolysis	
		Range	Mean		Range	Mean		Range	Mean
Gram-positive cocci	17	5–47	34	17	7–21	16	0		
Gram-negative rods	18	25–87	45	11	2–86	25	17	2–70	21

[a] Representative proteolytic gram-positive cocci (17 strains) and gram-negative rods (18 strains) were examined for their ability to degrade different proteins as described in the text.

ability to degrade F1 protein, casein, and BSA. All 35 isolates degraded F1 protein (Table 1), with the three most active isolates coming from the group of gram-negative rods. Casein was attacked by all the gram-positive cocci, but only 61% of the gram-negative rods hydrolyzed it. BSA was resistant to attack by the gram-positive cocci but was degraded to various extents by 17 of the 18 gram-negative rods. The three most active isolates degraded F1 protein and casein to similar extents and BSA only slightly less readily. For the remaining isolates, F1 protein appeared to be the preferred substrate, being broken down more rapidly and completely than either casein or BSA.

DISCUSSION

It is clear from our results that the F1 protein-containing selective medium will only support the growth of those proteolytic bacteria which can utilize F1 protein as the sole N source. It is possible that during prolonged incubation, NH_3 or amino acids liberated from the F1 protein by the action of proteolytic bacteria could support growth of nonproteolytic organisms. This problem did not arise when the medium was incubated for 48 h or less since all isolates were subsequently shown to be proteolytic. Our failure to isolate the proteolytic bacterium *Bacteroides ruminicola* R8/4 (8, 9) during this investigation may be due in part to this comparatively short incubation period, since we have observed that pure cultures of this bacterium will grow in the selective medium, but somewhat slower than the proteolytic butyrivibrios and streptococci. Alternatively, it is possible that this and other slower-growing proteolytic organisms are not numerically important in cows receiving fresh forage. Although F1 protein is probably the major soluble protein entering the rumens of animals consuming fresh forage, it is not the only protein, and it is therefore possible that our selective medium does not support the growth of all rumen bacteria which directly use protein N. In addition, the medium clearly cannot be used to culture proteolytic bacteria which degrade protein but do not use the products.

It is well established that both the types and the numbers of rumen microorganisms can vary with diet (12, 21), and it has been postulated that the increase in ruminal proteolytic activity observed after a change in diet results from an increase in total rumen bacteria (23), or more specifically, an increase in total proteolytic bacteria (18). In this context, after studying the effects of diet on the rumen microflora, Pietraszek (19) concluded that the high soluble-protein content of lucerne favors the proliferation of proteolytic rumen bacteria. Results presented here confirm that an increase in ruminal proteolytic activity occurs when fresh fodder is substituted for dry rations. Under similar circumstances, Nugent and Mangan (18) observed a greater increase in rumen proteolytic activity, but it may be significant that we, for convenience, used azocasein hydrolysis as a monitor of proteolytic activity, whereas they used lucerne F1 protein as the substrate for their proteinase assays. A significant increase in the number of proteolytic bacteria capable of utilizing F1 protein as the N source accompanied this increase in ruminal proteolytic activity. In view of the earlier observation (18) that the switch from a hay-concentrates diet to fresh fodder (lucerne) was not accompanied by any large increase in total bacterial N, it is likely that the increase in numbers of proteolytic bacteria is a selective phenomenon and is not due to a major increase in the total number of bacteria. For both diets, proteolytic bacteria isolated from the selective medium belonged to the genera *Streptococcus* and *Butyrivibrio; S. bovis* accounted for 50% of all such proteolytic isolates when dry rations were fed. When fresh fodder was fed, total numbers of *S. bovis* did not increase significantly, but the species accounted for 84% of the total F1-protein-utilizing proteolytic isolates. This would indicate that in streptococci, proteinase activity may be inducible by feeding herbage with a high soluble-protein content or is disseminated through the rumen population by the passage of a plasmid. Work is in progress to examine these two possibilities.

Substrate specificity of isolated proteolytic bacteria was, in general, similar to that of whole rumen contents (J. H. A. Nugent and J. L.

Mangan, Proc. Nutr. Soc., p. 48, 1978). Although BSA was extensively degraded by the three most active isolates, it was, probably by virtue of its tightly folded globular structure, the most recalcitrant of the three proteins tested.

In mixed in vitro incubations of rumen contents, in which conditions favored its growth, *S. bovis* was identified as an important proteolytic bacterium (20). Our results confirm that many strains of *S. bovis* are proteolytic, utilizing F1 protein as the sole N source, and further indicate that the species may make a major contribution to proteolytic activity in vivo. Thus, the high level of proteolytic activity in rumen contents of animals fed a cereal (23) or starch-supplemented diet (24) may be attributable in part to the large numbers of *S. bovis* cells which are known to proliferate under this type of feeding regime (12).

It has been proposed (15) that rumen proteolytic activity may be attributable to gram-negative bacteria alone. Our results indicate that appreciable numbers of proteolytic gram-positive bacteria can be isolated from the rumen contents of cows on either a hay-concentrates diet or fresh forage.

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