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Enumeration and Isolation of Lactate-Utilizing Bacteria from the Rumen of Sheep

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A highly specific medium was developed for the enumeration of lactate-utilizing bacteria in the rumen of sheep. This medium, which contained 2.0% lactate, 2.0% Trypticase, 0.2% yeast extract, and volatile fatty acids, hemin, and trace elements in place of rumen fluid, enabled high counts (42×10^7 to 190×10^7/g of ingesta) of lactate-utilizing bacteria to be made with a high degree of specificity (96%). The medium also supported the growth of all species of predominant lactate-utilizing bacteria reported to occur in the rumen and thus is of importance for ecological studies where the incidence and influence of the different species on lactate metabolism under changing conditions in the rumen cannot be predicted. The survival rate of isolates was increased from 60 to 96% by addition to the modified maintenance medium of 40% rumen fluid in place of the volatile fatty acids, hemin, and trace elements used in the counting medium. These results, together with the slow growth of colonies in roll bottles, showed that, although highly selective, the counting medium was not optimal for the types selected.

Lactate-utilizing bacteria occupy a special niche in the rumen ecosystem. They metabolize lactic acid, thereby controlling the accumulation of this less readily absorbed end product of ruminal fermentation, particularly when ruminants are fed diets containing large proportions of grain (17). The accumulation of lactic acid contributes greatly to the creation of acid conditions in the rumen, which are inhibitory for all but a few acid-tolerant species of bacteria and which, if sufficiently severe, can destroy the ecosystem (25). Thus, special attention has to be paid to the enumeration and isolation of bacteria utilizing lactic acid in ecological studies of the ruminal flora.

Several workers have attempted viable counts of lactate-utilizing bacteria by using either habitat-simulating media containing rumen fluid as a source of partially unknown growth factors and lactate as the only added energy source (11, 14), or semidefined media without rumen fluid but containing yeast extract, peptone, volatile fatty acids, trace elements, and hemin with lactate as energy substrate (10, 16). It was generally found that habitat-simulating media support the growth of larger numbers of bacteria but are not as specific as the semidefined media (6). More recently, Dehority and Grubb (7) used a preincubated, energy-depleted rumen fluid medium with lactate as the sole added energy substrate to reduce nonspecific counts. However, they did not determine actual specificity by testing bacteria isolated from this medium for ability to utilize lactate. Thus, no single medium has been universally accepted for viable counts of this group of bacteria.

In view of this, work was done on the development of a highly specific medium without rumen fluid which would support the growth of lactate-utilizing bacteria reported to occur in the rumen, namely *Megasphaera elsdenii*, *Veillonella alkalescens*, *Selenomonas ruminantium*, *Propionibacterium* spp. (5), and *Anaerovibrio lipolytica* (22). Varying concentrations of Trypticase and yeast extract were tested since the lactate-utilizing bacteria are known to require peptides, amino acids, and vitamins present in these medium components for growth. Increasing concentrations of lactate were used to inhibit growth of bacteria other than the lactate utilizers. Rumen fluid was excluded as a source of growth factors since it had been shown to reduce specificity. On the other hand, its inclusion in maintenance medium improved survival rate.

MATERIALS AND METHODS

Animals and management. Seven mature South African Mutton Merino wethers with large (ID, 83 mm) permanent ruminal cannulae (26) were used as a source of rumen ingesta. They were given the roughage

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diets listed in Table 1 and a series of diets (designated A, AE, E, EB, and B; see Table 5) increasing in amount of readily fermentable carbohydrate (18). All diets containing maize stover were fed in equal portions twice daily, whereas the lucerne and teff hay diets were fed once daily. Water was available at all times except on sampling days, when it was removed before the morning feeding and offered again after sampling at 2 to 2.5 h later. Diets F, G, and H were fed for at least 3 months before the first sampling; the numbers of days the sheep had been on diets A through B at the time of sampling are given in Table 5.

Sampling and processing of samples. The contents of the rumen and reticulum of sheep fed diets F, G, and H were mixed manually through the cannula while CO_2 was bubbled vigorously into the rumen ingesta. A pregassed, wide-mouthed bottle was inserted into the rumen, filled, withdrawn, stoppered, and immediately taken to the laboratory. By using a scoop, approximately 10 g of sample was weighed out accurately. This sample was diluted 10-fold with anaerobic diluent (see below) and processed in an Ultra-Turrax homogenizer (Janke & Kunkell KG, Staufen i. Br., Germany; 20,000 rpm) for 1 min with CO_2 bubbled through the fluid by means of a bent needle. The ingesta of sheep fed diets A through B were sampled and processed as described by Mackie et al. (18).

Media. All media were prepared and inoculated by using aseptic, anaerobic techniques based on those of Kistner (13). The gas phase was 98% CO_2–2% H_2, and the final pH of all media was 6.7 to 6.8. Clarified rumen fluid was obtained by sampling sheep 6 h after feeding lucerne hay, straining the ingesta through two layers of cheesecloth, and centrifuging at 1,500 × *g* for 30 min to yield a supernatant free of coarse particles. In this way several batches were collected and pooled as a single, uniform batch, which was used throughout the experiment. One volume of the cysteine-Na_2S reducing agent (3) was added to 44 volumes of molten medium. It was added to bottles of counting medium immediately before use and to the bulk medium before distribution into maintenance slopes. Anaerobic diluent contained 3.75 ml each of mineral solutions 1 and 2 (3), 1.0 ml of indigo carmine solution (0.05%, wt/vol), 7.0 ml of $NaHCO_3$ solution (9.1%, wt/vol), and 0.05 g of cysteine hydrochloride and was made up to 100 ml with boiled deionized water.

Several media without rumen fluid but differing in amounts of lactate, Trypticase, and yeast extract were tested during the development of the medium for enumerating lactate-utilizing bacteria (Table 2). The final medium selected contained 2% lactate, 2% Trypticase, and 0.2% yeast extract; the remaining constituents are given in Table 2. Two media were used to determine the total culturable count, one with rumen fluid and the other with rumen fluid replacement. Their compositions are given in Table 2. Maintenance slopes were made from 5 ml of LL, LH, or MLH medium (Table 2).

Roll bottles were inoculated in triplicate at each of three dilutions (10^{-7}, 10^{-8}, and 10^{-9}) and incubated at 39°C for 7 days. Colonies appearing in the agar films were counted after 3, 5, and 7 days of incubation for lactate-utilizing bacteria and after 7 days for total culturable bacteria.

TABLE 1. *Composition of roughage diets fed to sheep*[a]

Diet[b]	Roughage		Maize meal (g/day)	Nitrogen supplement	
	Type	Amt (g/day)		Type	Amt (g/day)
F	Teff hay	1,500	200	Biuret	20
G	Lucerne hay	2,000	0		0
H	Maize stover[c]	1,500	50	Urea	10

[a] One sheep was on each of the diets.

[b] Diets also contained 8 g of minerals (Kerolik no. 3; Cooper and Nephews, South Africa) and 8 g of NaCl (feed grade).

[c] Aerial portion of dry mature maize plants without ears or husks.

Isolation of lactate-utilizing bacteria. By using a bent platinum needle, colonies from LH and LL media were isolated quantitatively from roll bottles of the highest dilutions containing well-spaced colonies and stabbed into appropriate maintenance slopes. The slopes were incubated at 39°C and examined daily. When growth was sufficiently heavy, 0.5 ml of anaerobic diluent was injected onto the slope, and the growth was washed off and transferred onto two fresh maintenance slopes. The duplicate slopes were incubated until growth was heavy and then stored in solid CO_2 until required for fermentation tests.

Smears of isolates, made at the time of isolation and at each subsequent transfer, were stained by using the Gram method (modification of Jensen) and examined microscopically.

Lactate fermentation tests. Isolates obtained from LH and LL media were transferred into medium containing 1.0% lactate (Table 2) from which agar was omitted. The isolates were incubated for 7 days, and disappearance of D- and L-lactate was compared to control bottles inoculated with the same volume of sterile anaerobic diluent. D- and L-lactate were determined by specific enzymic methods (2), using biochemicals obtained from Boehringer Mannheim GmbH (West Germany).

Presumptive identification. The isolates were identified on the basis of cell morphology and size, Gram reaction, and ability to utilize lactate, with reference to *Bergey's Manual of Determinative Bacteriology* (5). It would not be possible to distinguish between *Selenomonas* and *Anaerovibrio* on the basis of these tests.

Chemical analysis. Starch and soluble sugars (see Table 5) were determined by the method of Gaillard and Van't Klooster (9).

RESULTS

Development of medium for enumeration and isolation of lactate-utilizing bacteria. The numbers of lactate-utilizing bacteria found in the rumen of sheep fed the three high-roughage diets tended to be highest on diet F, which contained added maize meal, lowest on lucerne hay (diet G), and intermediate on diet H, which contained maize stover (Table 3). This trend was apparent on all media tested, and hence the

TABLE 2. *Composition of media with rumen fluid and rumen fluid replacement for enumerating and/or isolating total culturable and lactate-utilizing bacteria*[a]

Component	% in:[b]							
	Media for total culturable bacteria		Media for counts of lactate-utilizing bacteria					
	RF[c]	RFR[c]	LH	LA	LM	LB	LL	MLH
Sodium DL-lactate (10% solution)[d]			20	20	10	3.5	3.5	20
Glucose	0.05	0.05						
Maltose	0.05	0.05						
Cellobiose	0.05	0.05						
Soluble starch	0.05	0.05						
Xylan	0.05	0.05						
Clarified rumen fluid[d]	40							40
Rumen fluid replacement:								
Trypticase[e]		0.2	2	0.2	1	2	0.2	2
Yeast extract[f]		0.05	0.2	0.05	0.1	0.2	0.05	0.2
Volatile fatty acids[d, g]		1	1	1	1	1	1	
Trace elements[d, h]		2	2	2	2	2	2	
Hemin (0.05% solution)[d]		2	2	2	2	2	2	

[a] In addition, all media contained 2 g of agar (Difco), 7.5 ml each of mineral solutions 1 and 2 (according to Bryant and Robinson [3]) 7 ml of $NaHCO_3$ (9.1% solution), and 1 ml of indigo carmine (0.05% solution).
[b] Percentages are weight/volume unless otherwise indicated.
[c] RF, Rumen fluid; RFR, rumen fluid replacement.
[d] Volume/volume additions to media.
[e] BBL Microbiology Systems, Cockeysville, Md.
[f] Difco Laboratories, Detroit, Mich.
[g] Final concentration of each acid in the medium according to Caldwell and Bryant (6).
[h] According to Kogut and Podoski (15).

TABLE 3. *Effect of varying the concentration of lactate, Trypticase, and yeast extract on numbers of lactate-utilizing bacteria from sheep fed three high-roughage diets*

Medium	Variable components in medium (%, wt/vol)	No. of lactate-utilizing bacteria ($\times 10^8$/g of ingesta) on different diets[a]			
		Diet F	Diet G	Diet H	For three diets
LH	Lactate, 2.0; Trypticase, 2.0; yeast extract, 0.2	1.0[b]	0.4[b]	0.6[b]	0.7 ± 0.4[c]
LA	Lactate, 2.0; Trypticase, 0.2; yeast extract, 0.05	1.1	0.3	0.6	0.7 ± 0.4
LB	Lactate, 0.35; Trypticase, 2.0; yeast extract, 0.2	2.2	1.7	2.8	2.2 ± 0.6
LM	Lactate, 1.0; Trypticase, 1.0; yeast extract, 0.1	2.5	1.1	3.8	2.5 ± 1.6
LL	Lactate, 0.35; Trypticase, 0.2; yeast extract, 0.05	10.0	3.3	4.3	5.9 ± 3.9

[a] The count on each diet was repeated twice.
[b] Mean.
[c] Mean ± standard deviation.

mean for all three diets was used to compare the different media. The mean counts on media LH and LA were the same; both media contained 2.0% lactate, although they differed in amounts of Trypticase and yeast extract. When the amounts of Trypticase and yeast extract were maintained at 2.0 and 0.2%, respectively, but the lactate was decreased to 0.35% (medium LB), the counts increased three-fold compared with LH medium. On medium LM, which contained 1.0% lactate, 1.0% Trypticase, and 0.1% yeast extract in the same ratio as medium LH (10:10:1) but in quantities half as large, the counts were also ca. three-fold higher compared with LH medium. When lactate, Trypticase, and yeast extract were kept at a low concentration (medium LL), the counts were ca. eight-fold higher than those on medium LH. Further work on these two media, which gave the highest and lowest counts, was done to determine which was the most specific and also enabled the maximum number of isolates to survive.

The survival rate of lactate-utilizing bacteria on first transfer to slopes of the same medium was higher on medium LH than on medium LL. The percentages of isolates which failed to grow

were 20% on LH and 45% on LL. Further losses occurred with each successive transfer, but those on LH medium were less than those on LL. After three transfers the surviving isolates were tested for ability to utilize more than 10% of the DL-lactate present in the medium. On LH medium 92% of the survivors were found to utilize lactate, whereas on LL only 78% of survivors utilized lactate. An analysis of the distribution of genera among surviving isolates after the first transfer showed that there was an almost complete absence of *Veillonella*- and *Megasphaera*-like organisms on medium LL (0 and 2.9%, respectively), compared with 14.8 and 16.0% on medium LH. The percentage of *Selenomonas*-like organisms was also much lower on LL (7.4%) than on LH (23.5%). Thus, of the two media, LH not only gave the more specific count but also enabled a greater number of isolates to survive than LL. In view of this, medium LH was selected for the enumeration of lactate-utilizing bacteria. Nevertheless, the loss of isolates on repeated transfer in medium LH demonstrated that even this medium, although highly specific, was not optimal. This substantiates the general principle that media which are selective are rarely optimal for the types selected (21).

It was possible that the rumen fluid replacement containing volatile fatty acids, hemin, trace elements, and a source of nonspecific growth factors (yeast extract and Trypticase) could not adequately replace rumen fluid for the growth of rumen bacteria. Thus, counts of total culturable bacteria were made in media containing rumen fluid or rumen fluid replacement at the same time that counts of the lactate-utilizing bacteria were made on media LH and LL (Table 4). Mean numbers of total culturable bacteria increased 2.2- to 3.2-fold when made in medium containing rumen fluid as compared with medium containing rumen fluid replacement. These results show clearly that rumen fluid is extremely difficult to replace even in semidefined media containing Trypticase and yeast extract as sources of nonspecific growth factors. Consequently, a third maintenance medium (MLH) based on medium LH but containing rumen fluid and omitting volatile fatty acids, trace elements, and hemin was formulated. All of the 176 colonies transferred from counting bottles of LH medium onto slopes of MLH grew. Only two failed to survive the next transfer in MLH medium. All 174 remaining isolates grew in fermentation medium (LM); of these, 168 proved to be lactate-utilizing bacteria. Thus, by using LH medium for counts and MLH for maintenance of isolates it was possible to obtain a specificity of 96% and a survival rate of 94%. This survival rate was not decreased when isolates were kept in solid CO_2 for at least 6 months. The effect of rumen fluid on the calculation of lactate-utilizing bacteria as a percentage of the total culturable bacteria is shown in Table 4. The numbers on LL medium were 9.3 to 35.4% of the total culturable bacteria in medium containing rumen fluid replacement but only 4.2 to 11.5% in medium containing rumen fluid. A similar decrease was found for the LH medium.

TABLE 4. *Mean numbers of lactate-utilizing bacteria calculated as a percentage of total culturable bacteria in media containing rumen fluid or rumen fluid replacement*[a]

Diet	No. of total culturable bacteria ($\times 10^9$/g of ingesta)		Lactate-utilizing bacteria as % of total culturable count			
			LL medium		LH medium	
	RF[b]	RFR[b]	RF	RFR	RF	RFR
F	3.4	1.5	11.5	26.0	2.4	5.3
G	4.1	1.3	4.2	9.3	0.8	1.9
H	6.0	2.7	11.2	35.4	1.2	3.8

[a] The count on each diet was repeated twice; the values given are means.

[b] RF, medium containing rumen fluid; RFR, medium containing rumen fluid replacement.

Application of medium for enumeration of lactate-utilizing bacteria. Medium LH was used to enumerate lactate-utilizing bacteria during the stepwise adaptation of sheep from a high-roughage to a high-concentrate diet (Table 5). On the final high-concentrate diet (diet B) the numbers of lactate-utilizing bacteria (42×10^7 to 190×10^7/g of ingesta) were greater than the highest numbers (3×10^7 to 8×10^7/g of ingesta) reported in the literature for this type of diet (16). Not only had the numbers of lactate-utilizing bacteria increased 84- to 379-fold compared with the high-roughage diet (diet A), but the relative increase was greater than the increase in numbers of total culturable bacteria (up to 12-fold). Thus, the lactate-utilizing bacteria formed 20.0 to 22.3% of the total culturable bacteria after 21 and 54 days on the final diet containing 48.7% starch and soluble sugars, when lactate production in the rumen would be greatest. In view of the fact that the medium used to enumerate the total culturable bacteria contained rumen fluid and the medium for the lactate utilizers did not, the values for the lactate-utilizing bacteria expressed as a percentage of total culturable bacteria are minimal (Table 4).

DISCUSSION

The medium chosen for enumerating lactate-utilizing bacteria (medium LH) has two outstanding characteristics. First, it enables high

TABLE 5. *Mean numbers of lactate-utilizing bacteria as a percentage of total culturable bacteria during the stepwise adaptation of sheep to a high-concentrate diet*

Diet	No. of days on diet	% of starch and soluble sugars in diet	No. ($\times 10^7$/g of ingesta) of:[a]		Lactate-utilizing bacteria as % of total culturable bacteria
			Lactate-utilizing bacteria grown on LH medium	Total culturable bacteria grown on medium containing rumen fluid	
A	17	5.6	0.5 ± 0.2[b]	262.5 ± 79.3[b]	0.2
AE	7	14.9	0.9 ± 0.2	205.0 ± 50.0	0.4
E	7	19.8	2.4 ± 2.0	322.5 ± 78.1	0.7
EB	7	44.3	6.5 ± 6.0	682.5 ± 748.0	1.0
B	7	48.7	112.3 ± 118.3	2825.0 ± 780.5	4.0
	21		42.0	210.0	20.0
	54		189.5 ± 307.6	850.0 ± 652.8	22.3

[a] Four sheep were sampled on each occasion except day 21 of diet B, when only two were sampled.
[b] Mean ± standard deviation.

counts (42×10^7 to 190×10^7/g of ingesta) of lactate-utilizing bacteria in the rumen to be made with a high degree of specificity (96%). This is largely due to the fact that lactic acid is known to suppress the growth of bacteria in general and non-lactate utilizers in particular (1, 8, 19, 23, 24). The long incubation period of 7 days required for most lactate-utilizing colonies to appear in the medium could be due to this suppression. These belonged to species other than those of *Propionibacterium*, which are intrinsically slow growing (11). Second, it supports the growth of all species of predominant lactate-utilizing bacteria reported to occur in the rumen, since in addition to *Megasphaera elsdenii*, *Veillonella alkalescens*, *Selenomonas ruminantium*, and *Propionibacterium* spp., *Anaerovibrio lipolytica* was also isolated from this medium during the stepwise adaptation experiment, as reported elsewhere (18). Thus, it gives an accurate representation of the types of lactate-utilizing bacteria present in the rumen, unlike medium LL which did not support the growth of the lactate-utilizing cocci. This is of importance in ecological studies, where the occurrence and potential contribution of the different species under changing environmental conditions is unknown and cannot be predetermined.

The loss of isolates on repeated transfer in counting medium LH was most likely due to the lack of adequate growth factors to meet their requirements, since it is known that isolated individual cells (present in a small inoculum) are often more exacting nutritionally than a dense population (20). Furthermore, any deficiencies or imbalances in the components of the medium would be exaggerated on repeated transfer. The fact that the survival rate improved from 60 to 96% after the addition to the modified maintenance medium (MLH) of 40% rumen fluid in place of the supplement of volatile fatty acids, hemin, and trace elements is evidence in favor of this hypothesis, since rumen fluid contains a balanced source of partially known growth factors normally present in the rumen habitat. The growth of *S. ruminantium*, *M. elsdenii*, and *A. lipolytica*, which are known to be stimulated by or have an absolute requirement for peptides, amino acids, and vitamins, was assured by the presence of Trypticase and yeast extract in addition to the rumen fluid (4, 12, 22).

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