Characteristics of *Ruminococcus* and Cellulolytic *Butyrivibrio* Species from the Rumens of Sheep Fed Differently Supplemented Teff (*Eragrostis tef*) Hay Diets

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SUMMARY

Twenty-four representative isolates of cellulolytic cocci and ten of cellulolytic rods from the rumens of sheep fed differently supplemented teff hay diets were characterized. Nine of the coccal isolates could be classified as *Ruminococcus albus* and twelve as *R. flavefaciens*. Three were classified as a new variety of *R. flavefaciens*, *R. flavefaciens* var. *lacticus*, on the basis of the preponderance of lactic acid among the products of cellulose fermentation. Two of the isolates did not fit into any of these groups. The rods were identified as *Butyrivibrio fibrisolvens*. Tests for the presumptive identification of cellulolytic bacteria from the rumen are discussed.

INTRODUCTION

The effect of variously supplementing low-protein teff hay on the numbers and types of predominant cellulolytic bacteria in the rumen was described in a previous paper (van Gylswyk, 1970), but only a presumptive identification of the bacteria was made. One group, *Cillobacterium cellulosolvens*, has since been described in detail (van Gylswyk & Hoffman, 1970). In the present communication the characteristics of bacteria presumptively identified as butyrivibrios, *Ruminococcus albus* and *R. flavefaciens* are reported in greater detail and compared with those of bacteria isolated by other workers from sheep and cattle fed on other diets (Hungate, 1950; Bryant & Small, 1956; Bryant, Small, Bouma & Robinson, 1958; Dehority, 1963; Kistner & Gouws, 1964; Jarvis & Annison, 1967; Shane, Gouws & Kistner, 1969).

METHODS

The culture techniques, media and identification tests used in this study were as described by van Gylswyk & Hoffman (1970) except that succinate was determined according to the method of Kmetec (1966). All determinations were done at least in duplicate, and data in Tables 1 and 2 represent mean values. Aggregation and cell dimensions were noted on at least nine occasions, using Gram-stained preparations.

RESULTS

Isolates 19, 20, 21, 22, 23, 24 and 41 were obtained from sheep fed on unsupplemented low-protein teff hay; 15, 17, 18, 28 and 46 from sheep fed on hay supplemented with urea; 12, 27, 38, 39 and 40 from sheep fed on hay supplemented with protein and the

remainder from sheep fed on hay supplemented with urea plus branched-chain volatile fatty acids. The diets were described in detail by van Gylswyk (1970).

All the isolates fermented cellobiose and cellulose (Whatman no. 1 filter paper). None grew at 22°, all grew at 30 and 38° and some growth of all was observed at 45° in at least one run. None produced catalase, indole or H₂S, and nitrite was never detected after growth in nitrate-containing medium. All the isolates gave a weakly positive Voges-Proskauer reaction in at least one run.

Cocci. The cocci were obligate anaerobes. Cell dimensions varied from 0.5 to 1.2 µm. (typically 0.6 to 1.0 µm.). All were Gram-variable except for 33 and 34 which were always Gram-negative. None was motile. On the basis of the types and proportions of end-products formed during the fermentation of cellulose the isolates were placed into four different groups (Table 1). Groups 1 and 2 were distinguished principally on the basis of their production of ethanol, succinate, lactate and hydrogen. Isolates 35 and 40 were anomalous in this grouping. In the case of strain 35, acetate, ethanol and hydrogen production were somewhat low while formate was relatively high for group 1. Isolate 40 produced more hydrogen and less acetate than was characteristic of its group. Group 3 comprised organisms that produced predominantly lactic acid. Group 4 consisted of only two strains that were very similar in fermentation products. They were isolated from the same animal on the same diet on two successive sampling dates. Fairly high figures for ethanol, no succinate for isolate 34, and very little for isolate 52 could perhaps have been criteria for inclusion in group 1. However, unusually high figures for lactate and low production of hydrogen made this less appropriate. Furthermore, the isolates showed higher production of formate and much lower production of acetate than group 1 except for isolate 35 which was similar in some respects to group 4.

All group 1 isolates, as well as isolates 25, 34, 40 and 52, grew well in medium without carbon dioxide (final pH 5.3 to 5.8). The remaining isolates grew poorly (final pH 6.0 or higher). All grew poorly in medium containing Trypticase and yeast extract instead of rumen fluid. That some growth occurred may have been due to transfer, with the inoculum of unwashed organisms, of growth factors from the cellobiose/rumen fluid agar slants on which the cells were grown. All the isolates fermented pectin and all except 39, 40, 41 and 52 fermented xylan. D-Galactose was fermented only by isolates 28, 34 and 40 and only isolates 39 and 43 fermented esculin. None of the isolates fermented L-rhamnose, maltose, trehalose, raffinose, dextrin, starch, inulin, salicin, glycerol, mannitol, inositol or DL-sodium lactate. Final pH of cultures showing a positive test was never below 5.4.

Other characteristics, in which both interstrain and interspecies differences were observed, are listed in Table 2.

Butyrivibrio-like rods. All the isolates (12, 15, 17, 18, 19, 20, 21, 22, 23 and 24) grew anaerobically but they often also showed considerable growth in the upper part of deep agar stab cultures, the surface of which was exposed to air containing 10 % CO₂ (van Gylswyk & Hoffman, 1970). In the case of isolates 12, 15, 20, 22, 23 and 24, growth was noticed up to the surface of the medium in at least one of the runs. The bacteria were slightly curved to curved, motile rods with a single polar or subpolar flagellum. They were 0.7 to 2.5 µm. long and 0.2 to 0.4 µm. wide (typically 0.8 to 2.0 µm. long and 0.3 to 0.4 µm. wide), occurring singly, in pairs or in short chains. The diameter of clearings in cellulose agar films after incubation for 2 to 4 weeks was

Table 1. *End-products of cellulose fermentation by isolates of cellulolytic cocci*

Group	Isolate no.	Compounds produced (+) or utilized (-)										
		Formate	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Lactate	Succinate	Ethanol	Hydrogen
1	27	+1.14	+1.99	-0.09	0	-0.03	0	-0.01	+0.11	+0.10	+3.66	+11.9
	28	+1.05	+2.23	-0.07	0	0	0	0	+0.09	+0.09	+3.32	+10.9
	29	+1.31	+2.68	-0.06	0	0	0	0	+0.09	+0.05	+3.63	+9.8
	30	+1.22	+2.65	-0.05	0	0	0	0	+0.10	0	+3.12	+13.6
	31	+1.22	+2.36	-0.10	-0.01	-0.01	0	0	+0.10	0	+3.54	+11.4
	32	+0.99	+2.50	-0.08	-0.01	-0.01	0	0	+0.11	+0.05	+3.65	+12.1
	33	+0.73	+2.84	-0.03	0	0	+0.01	0	+0.06	+0.05	+3.38	+10.7
	35	+1.71	+0.37	-0.04	-0.01	0	0	0	+0.16	0	+1.84	+3.3
2	36	+0.99	+2.48	+0.01	0	0	+0.01	0	+0.05	+0.06	+3.37	+11.6
	38	+1.41	+0.53	-0.12	0	-0.07	-0.04	-0.02	+0.10	+1.44	0	+2.0
	39	+0.86	+0.65	0	-0.03	0	-0.04	0	+1.09	+1.35	0	+1.7
	40	+0.37	+0.01	+0.01	-0.05	0	-0.05	0	+0.08	+1.97	0	+5.1
	41	+0.62	+1.08	+0.12	-0.02	0	-0.04	0	+0.05	+1.51	0	+2.2
	43	+0.21	+0.73	0	-0.03	0	-0.03	0	+1.98	+1.33	0	+2.2
	44	+0.70	+1.21	+0.01	-0.05	-0.02	-0.04	0	+1.25	+1.52	0	+1.6
	45	+0.73	+1.24	+0.01	-0.05	-0.02	-0.05	0	+0.23	+1.73	0	+1.9
3	46	+0.57	+0.90	-0.03	-0.05	-0.06	-0.05	0	+0.55	+1.49	0	+1.7
	47	+0.40	+1.12	+0.10	-0.03	0	-0.03	0	+0.36	+1.85	0	+1.6
	51	+0.57	+0.58	-0.10	-0.04	-0.07	-0.05	-0.02	+0.26	+1.48	0	+1.7
	25	0	-0.13	-0.07	-0.02	0	-0.03	-0.02	+2.81	+0.10	+0.47	+11.9
	37	+0.07	0	-0.07	-0.05	-0.04	-0.05	-0.01	+4.78	+0.05	+0.30	+1.3
	49	+0.14	+0.74	+0.06	-0.01	0	-0.02	0	+4.44	+0.03	0	+1.3
	34	+1.72	+0.34	-0.14	-0.02	-0.02	0	0	+3.17	0	+1.59	+1.9
	52	+1.35	+0.16	-0.16	-0.01	-0.05	-0.01	-0.01	+2.88	+0.11	+1.33	+1.6

Values expressed as mmole/100 ml. medium except in the case of hydrogen concentrations which are given as percentage by volume of the gas phase in the culture bottles. Mean values for the uninoculated medium were as follows (mmole/100 ml.): formate, 0.29; acetate, 4.24; propionate, 1.03; isobutyrate, 0.05; butyrate, 0.51; isovalerate, 0.05; valerate, 0.10; lactate, 0.07; succinate, 0; ethanol, 0; hydrogen (v/v % of gas phase), 0.03.

Table 2. *Some variable characteristics of isolates of cellulolytic cocci*

Group	Isolate no.	Approximate size of clearings on cellulose agar films (mm.)	Pigment production	Occurrence in chains (9 observations)	Gelatin liquefaction	Acid from						
						L-Arabinose	D-Xylose	D-Glucose	D-Mannose	D-Fructose	Lactose	Sucrose
1	27	1.0	-	-	+	+	+	±	+	+	+	+
	28	3.5	-	-*	+	+	+	+	+	+	+	+
	29	4.5	-	-	+	+	+	+	+	-	+	+
	30	3.0	-	-	-	+	+	-	+	+	+	-
	31	5.0	-	-	+	+	+	+	+	-	+	+
	32	4.0	-	-	+	+	+	+	+	-	+	+
	33	3.0	-	-	+	+	+	+	+	-	+	+
	35	1.0	-	-	+	-	+	-	+	-	+	+
	36	3.0	-	-	+	+	+	-	+	-	+	+
	38	2.0	+	+	+	-	-	-	-	-	-	-
2	39	1.0	-	+	-	+	-	+	-	-	-	+
	40	1.0	+	+	-	-	-	-	-	-	+	-
	41	2.0	+	+	-	-	-	-	-	-	-	-
	43	1.0	-	+	-	+	-	-	-	-	-	-
	44	1.5	+	+	-	+	-	+	-	-	-	-
	45	2.0	+	+	-	+	-	±	-	-	-	-
	46	1.0	+	+	-	+	-	+	-	-	-	-
	47	1.5	+	+	+	+	-	+	-	-	-	-
	51	2.0	+	+	-	+	-	±	-	-	-	-
	25	2.0	-	+	-	-	-	-	-	-	-	-
3	37	1.0	-	+	-	-	-	-	-	-	+	-
	49	2.0	-	+	-	-	-	-	-	-	+	-
4	34	2.0	-	+	+	+	+	+	-	-	+	+
	52	2.5	±	+	+	-	-	-	-	-	+	+

* Short chains noted in two cases. ± Variable.

usually less than 1 mm. but clearings were well defined. No pigment was found. All the isolates liquefied gelatin, grew in the absence of carbon dioxide (final pH 5.2 to 5.5) and in medium containing Trypticase and yeast extract instead of rumen fluid. All fermented the following sugars, the figures in parentheses indicating the mean final pH values; L-arabinose (5.3), D-xylose (5.3), D-glucose (5.2), D-galactose (5.5), D-mannose (5.7), D-fructose (5.2), maltose (5.2), lactose (5.3), sucrose (5.1), raffinose (5.2), dextrin (5.8), starch (5.2), esculin (5.9), salicin (5.5) and pectin (5.9). All gave weakly positive reactions in the case of inulin and xylan (final pH 6.2 or higher). In the case of glycerol isolate 22 gave negative tests in both runs while the others were weakly positive, often negative in one run and weakly positive to positive in the other (pH 6.5 to 5.7). None of the isolates except 15 (pH 5.5) fermented trehalose nor did any ferment L-rhamnose, mannitol, inositol or DL-sodium lactate.

Types and quantities of products formed (+) or utilized (−) during growth in medium containing filter-paper cellulose were as follows (mean and range in mmoles/100 ml. medium): formate, +0.44 (+0.25 to +1.09); acetate, −2.13 (−1.73 to −2.82); propionate, −0.17 (−0.13 to −0.27); isobutyrate, +0.01 (−0.01 to +0.03); butyrate, +3.70 (+2.23 to +5.28); isovalerate, +0.03 (+0.01 to +0.04); valerate, +0.01 (0 to +0.03); lactate, +1.14 (+0.85 to +1.63); hydrogen (percentage by volume of the gas phase in culture bottles), +6.9 (+5.9 to +8.0). None of the strains produced succinate or ethanol. The mean values for the uninoculated medium are the same as those given in Table 1. The quantities of the acid products of cellobiose fermentation in a medium containing 1.2 % (w/v) of this substrate were similar to those found when cellulose was used as substrate except that lactate production was almost three times as high.

DISCUSSION

All the coccal isolates were strict anaerobes, fermented cellulose and cellobiose, occurred singly, in pairs or in chains, were Gram-negative to Gram-variable and non-motile. No endospores were observed. According to these characteristics they belong to the genus *Ruminococcus* (Sijpesteijn, 1951), the definition of which was amended by Hungate (1957) and further amended by Bryant *et al.* (1958). The group 1 isolates of the current study clearly belong to the species *R. albus* (Hungate, 1957) in that they produced no pigment, did not occur in chains and produced hydrogen, ethanol, formate, acetate and lactate. Some of the isolates produced a little succinate but Bryant *et al.* (1958) and Jarvis & Annison (1967) also classified such isolates, which otherwise conformed to the characteristics of *R. albus*, as belonging to this species. The present isolates differed from previously described strains of *R. albus* principally in that most of them fermented L-arabinose, D-xylose, D-glucose, D-mannose, lactose and sucrose. Such organisms have been found infrequently by other workers. The group 2 isolates can be regarded as belonging to the species *R. flavefaciens* (Sijpesteijn, 1951) as redefined by Hungate (1957) and Bryant *et al.* (1958), on the basis of chain formation, production of succinate and lack of production of ethanol.

Five of the isolates could not, on the basis of fermentation products, be assigned to either of the two known species of the genus *Ruminococcus*. The three group 3 isolates produced mainly lactate. Bryant *et al.* (1958) also found a strain that produced mainly lactate, and said that this strain, B₁₃₃, could not be placed in either species. Jarvis & Annison (1967) found two strains, LY 8 and LY 13, that produced mainly lactate and

both were classified as 'intermediates similar to *Ruminococcus flavefaciens*'. On the basis of chain formation all six strains could be classified as *R. flavefaciens*, especially in the case of LY8 and LY13, which produced yellow pigment. The strains have been isolated from both sheep (present work and that of Jarvis & Annison, 1967) and cattle (Bryant *et al.* 1958) in three widely separated parts of the world, Australia, United States of America and South Africa. On these grounds it is proposed that the group 3 isolates as well as strains B₁33, LY8 and LY13 be named as a new variety of *R. flavefaciens*, *R. flavefaciens* var. *lacticus*. This variety differs from *R. flavefaciens* in that the principal end-product of cellulose or cellobiose fermentation is lactic acid. Small quantities of formate, succinate and ethanol may or not be produced and acetate may be formed in varying amounts.

The group 4 isolates also produced unusually large quantities of lactate. They formed considerable quantities of formate and ethanol and this together with low succinate excluded them from group 2. High production of lactate, low production of hydrogen and chain formation excluded them from group 1. Up to the present no strains with similar properties have been reported and an attempt to name these strains is considered premature.

The cellulolytic curved rods can be placed in the species *Butyrivibrio fibrisolvens* (Bryant & Small, 1956). However, they showed a greater resemblance to the 'less actively cellulolytic rumen rod' described by Hungate (1950) and to the group 1 curved rods of Shane *et al.* (1969) than to the type strain, especially in the case of cell dimensions. The dimensions of the type strain were given as 0.4 to 0.6 μm . wide and 2 to 5 μm . long. Other characteristics of our strains were rather similar to those of the type strain. It is noteworthy that *Butyrivibrio* strains similar to the group 2 strains of Shane *et al.* (1969) were never found. In the current study only isolates that had retained ability to degrade cellulose after repeated storing and culturing on cellobiose-containing agar slopes were used. In this way strains, similar to those of group 2 of Shane *et al.* (1969), that lost the ability to degrade cellulose could have been excluded.

The *Butyrivibrio* isolates described here all produced small amounts of isobutyrate and isovalerate. In the rumen the production of these acids is important for the growth of ruminococci (Bryant & Robinson, 1961) and isobutyrate and isovalerate were utilized by the majority of our *Ruminococcus* isolates. Whether these acids produced by our *Butyrivibrio* strains were synthesized from cellulose, or whether they were formed from proteinaceous material (in the rumen fluid of the medium) after hydrolysis and subsequent decarboxylation and deamination of the branched-chain amino acids (El-Shazly, 1952), remains to be determined.

The tests employed by van Gylswyk (1970) for the presumptive identification of a large number of cellulolytic isolates were found to be fairly reliable. Representative strains of the group described as 'Butyrivibrio-like rods' were subsequently all found to belong to the species *Butyrivibrio fibrisolvens* and those described as 'coccoid rods' were identified as *Cillobacterium cellulosolvens* (van Gylswyk & Hoffman, 1970). The ruminococci were easily recognized, but perfect agreement was not obtained in separating *Ruminococcus albus* from *R. flavefaciens*. The results could nevertheless be regarded as satisfactory. Of the 24 isolates the presumptive and final identifications agreed in 17 cases. Isolates 34 and 52, now placed in group 4, were presumptively identified as *R. albus* and atypical *R. flavefaciens* respectively. Isolates 25, 37 and 49, now assigned to the variety 'lacticus', were presumptively identified as *R. albus*,

atypical *R. flavefaciens* and *R. flavefaciens*. Isolates 46 and 47, presumptively identified as atypical *R. albus*, were shown by the more detailed testing to be *R. flavefaciens*.

Presumptive tests that proved valuable in differentiating between the different species included examination for cell morphology and arrangement, motility and the Gram-reaction. In the case of the ruminococci the quantitative test for ethanol and semi-quantitative test for succinate production from cellulose gave fairly reliable information on whether an isolate could be classed as *Ruminococcus albus* or as *R. flavefaciens*, and the production of yellow pigment provided additional information in confirming the identity of the latter species. Testing for ability to ferment glucose, xylose or xylan was found to be of little value. Further knowledge of the identity of ruminococci may be obtained by determining lactate in addition to succinate and ethanol. This would facilitate detection of *R. flavefaciens* var. *lacticus* and group 4-like isolates. A tendency to form chains is in itself a fairly good criterion for separating *R. albus* from *R. flavefaciens* as shown by the current work and that of Bryant *et al.* (1958) and Jarvis & Annison (1967).

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REFERENCES

- BRYANT, M. P. & ROBINSON, I. M. (1961). Some nutritional requirements of the genus *Ruminococcus*. *Applied Microbiology* **9**, 91-95.
- BRYANT, M. P. & SMALL, N. (1956). The anaerobic monotrichous butyric acid-producing curved rod-shaped bacteria of the rumen. *Journal of Bacteriology* **72**, 16-21.
- BRYANT, M. P., SMALL, N., BOUMA, C. & ROBINSON, I. M. (1958). Characteristics of ruminal anaerobic cellulolytic cocci and *Cillobacterium cellulosolvens* n.sp. *Journal of Bacteriology* **76**, 529-537.
- DEHORITY, B. A. (1963). Isolation and characterization of several cellulolytic bacteria from *in vitro* rumen fermentations. *Journal of Dairy Science* **46**, 217-222.
- EL-SHAZLY, K. (1952). Degradation of protein in the rumen of the sheep. 2. The action of rumen micro-organisms on amino acids. *Biochemical Journal* **51**, 647-653.
- VAN GYLSWYK, N. O. (1970). The effect of supplementing a low-protein hay on the cellulolytic bacteria in the rumen of sheep and on the digestibility of cellulose and hemicellulose. *Journal of Agricultural Science, Cambridge* **74**, 169-180.
- VAN GYLSWYK, N. O. & HOFFMAN, J. P. L. (1970). Characteristics of cellulolytic cillobacteria from the rumens of sheep fed teff (*Eragrostis tef*) hay diets. *Journal of General Microbiology* **60**, 381-386.
- HUNGATE, R. E. (1950). The anaerobic mesophilic cellulolytic bacteria. *Bacteriological Reviews* **14**, 1-49.
- HUNGATE, R. E. (1957). Micro-organisms in the rumen of cattle fed a constant ration. *Canadian Journal of Microbiology* **3**, 289-311.
- JARVIS, B. D. W. & ANNISON, E. F. (1967). Isolation, classification and nutritional requirements of cellulolytic cocci in the sheep rumen. *Journal of General Microbiology* **47**, 295-307.
- KISTNER, A. & GOUWS, L. (1964). Cellulolytic cocci occurring in the rumen of sheep conditioned to lucerne hay. *Journal of General Microbiology* **34**, 447-458.
- KMETEC, E. (1966). Spectrophotometric method for the enzymic microdetermination of succinic acid. *Analytical Biochemistry* **16**, 474-480.
- SHANE, B. S., GOUWS, L. & KISTNER, A. (1969). Cellulolytic bacteria occurring in the rumen of sheep conditioned to low-protein teff hay. *Journal of General Microbiology* **55**, 445-457.
- SUPESTEIN, A. K. (1951). On *Ruminococcus flavefaciens*, a cellulose-decomposing bacterium from the rumen of sheep and cattle. *Journal of General Microbiology* **5**, 869-879.