ISOLATION AND FERMENTATION CHARACTERISTICS OF STRAINS OF BUTYRIVIBRIO FROM RUMINAL INGESTA

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Anaerobic, gram-negative, butyric acid-producing, curved rods have been isolated from the rumen of cattle and sheep by Hungate (1950), Huhtanen and Gall (1952), Wilson (1953), Maki and Foster (1957), and Gill and King (1958). Bryant and Small (1956) established a new genus, *Butyrivibrio*, for monotrichous strains of these bacteria. Organisms of this genus probably contribute significantly to the rumen fermentation since they have been isolated by several investigators in different areas of the country, they occur in relatively high numbers in rumen ingesta, they are able to ferment a variety of substrates normally present in the rumen, and they produce considerable amounts of fatty acids.

The fermentation characteristics of the type culture in a rumen fluid-glucose medium were reported by Bryant and Small (1956). In view of the apparent importance of this genus an attempt was made, in the present investigation, to extend the observations of Branyt on the incidence of the genus and to determine the influence of environmental changes which are known to take place in the rumen upon the fermentation characteristics of this genus.

MATERIALS AND METHODS

The anaerobic techniques used to culture the rumen bacteria were those of Hungate (1950). Bryant and Burkey (1953) and Bryant and Small (1956) reported that unlike many organisms from the rumen, *Butyrivibrio* species grew well in glucose broth under a nitrogen atmosphere without the addition of sodium bicarbonate. These modifications in isolation techniques were made in an attempt to select for members of this genus.

Cultures were isolated from ruminal ingesta on Bryant and Burkey's (1953) isolation medium which was modified to contain, per 100 ml of medium: 15 ml each of their mineral solutions no. 1 and no. 2, 0.4 g of glucose, 0.1 ml of 0.1 per cent solution of resazurin, and 40 ml of rumen fluid. The pH was adjusted to 6.9 with sodium hydroxide and the total volume was brought up to 97.75 ml with water. Eight and eight-tenths ml of the medium was dispensed into tubes containing 0.18 g of agar. Two-tenths ml of freshly prepared cysteine hydrochloride solution containing 0.1 g of cysteine hydrochloride in 4.5 ml of water was then added to each tube, and the tubes were flushed with nitrogen. The tubes were then closed with rubber stoppers which were wired in place for sterilization at 121 C for 20 min. The final pH of this medium was between 6.8 and 6.9. All other media used in this study likewise contained cysteine hydrochloride and were prepared by the same method as for the isolation medium.

The rumen fluid used in these media was prepared by the method of Gill and King (1958), but the flasks were flushed with carbon dioxide before sterilization and storage in the refrigerator.

Samples of rumen fluid were diluted in the anaerobic dilution solution of Bryant and Burkey (1953); however, sodium carbonate was omitted. Sodium hydroxide was used to adjust the pH, and nitrogen was bubbled through the solution to replace any oxygen present.

Sample treatment, dilution series, and roll tube preparation were carried out according to the methods described by Bryant and Burkey (1953) except that nitrogen was used instead of carbon dioxide to maintain anaerobic conditions. The tubes were incubated at 39 C for 3 days.

All colonies from roll tube cultures inoculated with 1×10^{-8} ml of rumen samples were isolated. Pure cultures were obtained by serially diluting the well-isolated colonies twice in tubes of the isolation medium and preparing roll tube cultures. Macroscopic and microscopic observation of several colonies in each tube in both successive sub-

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cultures verified the presence of single morphotypes. Isolated cultures were maintained on the rumen fluid-glucose-cellobiose agar of Bryant and Burkey (1953).

Cultural characteristics. Media and methods for studying the morphological and physiological characteristics of the present strains were those of Bryant and Doetsch (1954) and Bryant and Small (1956). Fermentation characteristics were determined in the rumen fluid-glucose medium of Bryant and Small (1956) containing 20 per cent rumen fluid, minerals, 0.05 per cent cysteine hydrochloride, 0.4 per cent glucose, and 0.2 per cent sodium bicarbonate. The medium was modified to contain 0.0001 per cent resazurin and was adjusted to pH 6.9 before sterilization under a nitrogen atmosphere. After sterilization the pH of the medium was 7.0. In one experiment the rumen fluid of this medium was replaced by 1.5 per cent trypticase (BBL) and 0.5 per cent yeast extract (Difco).

For studies of the effect of pH on fatty acid production, 0.03 M K_2HPO_4 and the required amounts of syrupy H_3PO_4 were added to the rumen fluid-glucose medium. After sterilization the pH values of these media were 7.07, 6.59, 6.10, and 5.25.

One and eleven-hundredths milliequivalents of reagent grade acetic, *n*-butyric, propionic, and lactic acids were added per 100 ml of medium in experiments to determine the effect of product concentration upon fermentation activity. The acids were added, individually or mixed, to buffered rumen fluid medium. All of the media were adjusted to a pH of 6.5 before sterilization.

In order to study the fermentation in rumen fluid itself, a medium was prepared by centrifugation of fresh rumen fluid at 20,000 RCF for 30 min. One-half of one per cent casein hydrolyzate, 1.5 per cent glucose, 0.05 per cent cysteine hydrochloride, and 0.0001 per cent resazurin were added to the supernatant rumen fluid. The pH of this

TABLE 1

Morphological and colonial characteristics of 11 strains of Butyrivibrio grown on RGCA slants

Strain	Shape*	Arrangement of Cell at 18 Hr	Size	Flagella†		Appearance of Colonies	Diameter at 72 Hr‡
				Wave length	Amplitude		
			μ	μ	μ		mm
A4	C.R.	Single and short chains	0.3–0.4 × 1.4–2.7	2.11	0.38	Translucent; light tan	1.0
C5	S.C.R.	Single and short chains	0.2–0.4 × 1.4–2.4	2.41	0.53	Translucent; light tan	0.5
C7	V.S.C.R.	Single and short chains	0.2–0.4 × 1.4–2.4	1.98	0.33	Translucent; light tan	Irreg
C8	V.S.C.R.	Single and short chains	0.3–0.5 × 2.0–2.7	2.44	0.41	Translucent; light tan	0.5–1.0
C9	V.S.C.R.	Single and short chains	0.3–0.4 × 1.4–3.4	2.38	0.40	Translucent; light tan	Irreg
C13	V.S.C.R.	Single and short chains	0.2–0.3 × 1.7–2.4	1.98	0.36	Translucent; light tan	1.0–2.0
C15	V.S.C.R.	Long chains	0.6–0.7 × 2.7–5.4	1.98	0.33	Light tan	1.0–2.0
D3	V.S.C.R.	Single, short and long chains	0.6–0.7 × 2.4–4.0	2.64	0.61	Filamentous; tan	1.0
D5	V.S.C.R.	Short and long chains	0.3–0.4 × 2.0–2.7	1.98	0.33	Filamentous; tan	1.0–1.5
D11	V.S.C.R.	Long chains	0.3–0.5 × 5.7–6.7	1.65	0.26	Filamentous; tan	2.0–2.5
G	S.C.R.	Single and short chains	0.3–0.4 × 2.4–3.0	1.91	0.33	Translucent; light tan	Irreg

* C = curved, R = rods, S = slightly, V = very.

† Glucose concentration of the medium reduced to 0.1 per cent.

‡ Irreg = irregular outline.

medium was adjusted to 6.9 before sterilization. Nitrogen or carbon dioxide was used to flush the medium in separate experiments.

In all fermentation tests the tubes of medium were inoculated with one drop of a 24-hr culture grown in rumen fluid-glucose broth and were then incubated at 37 C for 14 days under a nitrogen atmosphere.

For identification of Butyrivibrio cultures, fermentation acids were determined by the chromatographic method of Langston (1955). For other fermentation experiments, the acids were determined by the method of Smith *et al.* (1956).

RESULTS

Duplicate samples of rumen ingesta were taken from the top and bottom of the rumen of a fistulated steer fed a winter ration of poor quality alfalfa hay and 4 lb of grain concentrate per day. Forty-nine colonies developed in 8 roll tubes inoculated with 1×10^{-8} ml of rumen fluid. Ten of the colonies were found to consist of anaerobic, gram-negative, monotrichous, curved rods which produced predominately butyric acid, and usually lesser amounts of formic, lactic, propionic, and succinic acids. Strains designated A and C were obtained from 2 top samples taken in the dorsal sac approximately 6 in below the surface of the hay mat, and D from 1 bottom sample taken approximately 4 in above the floor of the ventral sac of the rumen. Strain G was that isolated by Gill and King (1958). There was considerable variation in the morphological and physiological characteristics among the 11 isolates studied.

TABLE 2

Some variable characteristics among 11 strains of Butyrivibrio

	Strain										
	A4	C5	C7	C8	C9	C13	C15	D3	D5	D11	G
H_2S production	Sl*	−	+	−	−	−	−	−	−	−	−
Casein digestion	−	−	−	−	−	−	−	−	−	−	+
Nitrate reduction	+	−	−	−	−	−	−	−	−	−	−
Starch hydrolysis	+	−	−	−	−	−	+	+	+	+	+
Final pH of medium	5.07	5.30	5.25	5.27	5.20	5.30	5.25	5.16	5.13	5.14	5.40

* Sl = slight.

TABLE 3

Acid production in rumen fluid-glucose medium by strains of Butyrivibrio (14-day cultures)

Strain	Acid Produced (mEq/100 ml)						
	Butyric*	Propionic	Acetic	Formic	Succinic	Lactic	Total
A4	1.11	0.73	−0.84	0.64	0.05	0.05	1.74
C5	0.32	−0.09	−1.42	0.31	0.00	0.30	−0.58
C7	1.52	1.05	−0.82	0.85	0.15	−0.26	2.49
C8	0.32	−0.35	−1.43	0.71	0.00	0.10	−0.65
C9	1.21	−0.10	−1.50	0.10	0.02	0.89	0.62
C13	0.59	−0.25	−0.94	0.23	0.02	0.70	0.35
C15	0.74	0.04	−0.92	0.77	0.17	0.63	1.43
D3	0.59	−0.12	−0.46	0.61	0.00	0.28	0.90
D5	0.85	−0.25	−0.90	0.56	0.00	0.08	0.34
D11	1.44	−0.24	−1.05	0.78	0.04	0.01	0.98
G	0.39	0.12	−0.74	0.18	0.00	1.48	1.43
Avg	0.83	0.05	−1.00	0.52	0.04	0.38	0.82
Blank values for medium	0.95	0.98	2.13	0.75	0.21	0.82	

* Fraction contains butyric and higher fatty acids.

TABLE 4

Acids produced in buffered rumen fluid-glucose media by 11 strains of Butyrivibrio (14-day culture)

Strain	Buffered pH	Acid Produced (mEq/100 ml)			
		Butyric and higher	Propionic	Acetic plus formic	Succinic plus lactic
A4	7.07	0.60	0.95	0.65	1.41
	6.59	0.95	0.98	0.57	0.55
	6.10	1.36	1.12	−0.37	0.18
	5.25	0.18	0.10	−0.36	0.10
C5	7.07	0.52	−0.02	0.17	2.72
	6.59	0.60	−0.01	0.08	1.83
	6.10	0.49	0.03	−0.19	1.38
C7	7.07	0.43	1.34	0.74	1.53
	6.59	1.13	0.87	0.01	0.53
	6.10	1.80	1.19	−0.54	0.13
C8	7.07	0.52	−0.03	0.09	2.52
	6.59	0.72	−0.02	−0.42	3.01
	6.10	0.38	0.10	−0.11	1.25
C9	7.07	0.60	−0.04	−0.22	1.98
	6.59	1.39	0.06	−0.82	2.98
	6.10	0.80	0.08	−0.73	1.59
C13	7.07	0.54	0.01	0.18	2.46
	6.59	0.78	0.02	−0.21	2.26
	6.10	0.58	−0.02	−0.24	1.78
C15	7.07	1.00	0.10	0.39	3.22
	6.59	1.69	0.12	0.05	1.80
	6.10	1.52	0.08	−0.44	1.08
D3	7.07	1.16	0.01	0.40	1.88
	6.59	1.70	0.07	0.15	1.65
	6.10	1.23	−0.02	−0.35	1.35
D5	7.07	1.42	−0.01	0.43	1.41
	6.59	1.52	0.13	−0.58	1.13
	6.10	1.41	−0.02	−0.22	1.04
D11	7.07	1.02	−0.04	0.37	1.92
	6.59	1.79	−0.07	0.36	1.51
	6.10	1.22	0.09	−0.40	1.15
G	7.07	0.39	0.43	0.07	2.16
	6.59	0.71	0.13	−0.15	2.36
	6.10	0.70	0.14	−0.50	1.28

The morphological and colonial characteristics of the isolates are given in table 1.

The organisms grew well in a rumen fluid-glucose medium. Twenty-four-hour cultures of strains A4, C5, C7, C8, C9, C13, and G showed heavy turbidity. Strain C15 had flocculent sediment. Strains D3, D5, and D11 had granular sediments and adhered to the sides of the tubes. The organisms also grew well in rumen fluid-glucose medium with nitrogen in place of carbon dioxide and with bicarbonate omitted.

No visible growth or reduction of pH was found in any culture at 50 C or at 22 C. They all grew

TABLE 5

Acids produced on the addition of free acids in a buffered rumen fluid-glucose medium by strain A4 (14-day culture)*

Treatment	Buffered pH	Acid Produced (mEq/100 ml)			
		Butyric and higher	Propionic	Acetic plus formic	Succinic plus lactic
1	6.54	*1.20*	0.85	0.33	0.78
2	6.56	1.76	*0.25*	0.57	0.68
3	6.55	1.29	0.81	*0.19*	0.97
4	6.55	1.33	1.40	0.63	*−0.03*
5	6.35	*1.37*	*1.14*	*0.46*	*0.04*
Control..	6.35	1.23	0.90	0.21	0.75

* The value in italics indicates the acid added at the rate of 1.11 mEq/100 ml in each treatment.

TABLE 6

Acids produced on the addition of free acids in a buffered rumen fluid-glucose medium by strain C7 (14-day culture)*

Treatment	Buffered pH	Acid Produced (mEq/100 ml)			
		Butyric and higher	Propionic	Acetic plus formic	Succinic plus lactic
1	6.54	*1.29*	0.79	0.00	1.13
2	6.56	1.74	*1.00*	−0.39	0.38
3	6.55	1.52	1.18	*−0.45*	0.23
4	6.55	1.18	1.56	0.03	*−0.39*
5	6.35	*1.52*	*1.21*	*0.22*	*−0.33*
Control..	6.35	1.34	0.96	−0.12	0.67

* The value in italics indicates the acid added at the rate of 1.11 mEq/100 ml in each treatment.

well at 30, 37, and 39 C. Variable growth was obtained at 45 C.

None of the cultures produced acetylmethylcarbinol or indole or liquefied gelatin. Production of H_2S, digestion of casein and starch, and reduction of nitrate varied among strains as shown in table 2.

All of the cultures produced acid from xylose, glucose, fructose, sucrose, lactose, cellobiose, raffinose, and salicin, but none of them fermented sorbitol or mannitol with production of acid. No visible loss of cellulose was found from tubes of rumen fluid-cellulose broth after 1 week of incubation.

TABLE 7

Acids produced on the addition of free acids in a buffered rumen fluid-glucose medium by strain G (14-day culture)*

Treatment	Buffered pH	Acid Produced (mEq/100 ml)			
		Butyric and higher	Propionic	Acetic plus formic	Succinic plus lactic
1	6.54	*0.79*	0.02	−0.52	2.99
2	6.56	1.47	*0.12*	−0.44	1.75
3	6.55	1.21	0.02	*−0.65*	1.79
4	6.55	1.17	0.01	−0.62	*2.55*
5	6.35	*0.40*	*0.06*	*−0.45*	*2.76*
Control..	6.35	0.76	0.08	−0.93	1.70

* The value in italics indicates the acid added at the rate of 1.11 mEq/100 ml in each treatment.

TABLE 8

Acids produced on the addition of free acids in a buffered rumen fluid-glucose medium by strain C15 (14-day culture)*

Treatment	Buffered pH	Acid Produced (mEq/100 ml)			
		Butyric and higher	Propionic	Acetic plus formic	Succinic plus lactic
1	6.54	*1.46*	0.07	0.09	2.19
2	6.56	1.78	*−0.15*	0.06	1.41
3	6.55	1.91	0.02	*−0.77*	1.61
4	6.55	1.71	−0.04	−0.14	*2.80*
5	6.35	*1.96*	*0.06*	*−0.62*	*2.24*
Control..	6.35	1.55	0.02	−0.34	1.44

* The value in italics indicates the acid added at the rate of 1.11 mEq/100 ml in each treatment.

TABLE 9

Acids produced in a 98 per cent rumen fluid medium by strains of Butyrivibrio under nitrogen atmosphere (14-day culture)

Strain	Acid Produced (mEq/100 ml)				
	Butyric and higher	Propionic	Acetic plus formic	Succinic plus lactic	Total
A4	1.77	0.39	−1.05	0.77	2.93
C7	4.65	1.22	−4.15	−1.08	5.87
C15	3.47	0.57	−2.60	0.73	4.77
G	2.19	0.00	−1.12	1.46	3.65

TABLE 10

Acids produced in a 98 per cent rumen fluid medium by strains of Butyrivibrio under carbon dioxide atmosphere (14-day culture)

Strain	Acid Produced (mEq/100 ml)				
	Butyric and higher	Propionic	Acetic plus formic	Succinic plus lactic	Total
A4	3.38	0.15	−2.15	1.26	4.79
C7	5.40	1.38	−4.06	−0.88	6.78
C15	3.51	0.56	−1.11	2.41	6.48
G	3.32	0.32	−2.51	0.89	4.53

The organisms grew well when rumen fluid was replaced by yeast extract and trypticase in the glucose medium. Analysis of fermentation acids showed that without rumen fluid much larger quantities of butyric acid were produced, and that the acetic-formic chromatographic peak was increased rather than decreased as in the other media. The concentrations of fermentation acids produced by the 11 strains in rumen fluid-glucose medium are shown in table 3.

Table 4 shows fermentation acid production when the strains were cultured in rumen fluid-glucose medium buffered at graded pH values. None of the strains grew at pH 5.25 except strain A4.

Results of addition of acids to the buffered medium are recorded in tables 5 to 8. The added acids have already been subtracted from the results in these tables.

Strains A4, C7, C15, and G were cultured in the 98 per cent rumen fluid medium under a nitrogen atmosphere and under a carbon dioxide atmosphere. The results of fermentation acid anal-

yses from these cultures are given in tables 9 and 10.

Cultures of 4 strains were subsampled daily during 14-day incubation periods. Analyses of the fermentation acid products indicated that there was no consistent change in the ratio of acids produced, and that acid production in rumen fluid-glucose medium was most rapid during the first 24-hr period.

DISCUSSION

The 10 strains of Butyrivibrio isolated on a semiselective medium represented ⅕ of the organisms cultured from 10^{-8} dilutions of rumen fluid. Compared with the type culture described by Bryant and Small (1956), these strains had identical characteristics in that they were anaerobic, gram-negative, monotrichous, curved rods producing considerable amounts of butyric acid. They fermented xylose, fructose, glucose, cellobiose, sucrose, lactose, and salicin and did not ferment mannitol, inositol, or cellulose (after several laboratory transfers). The strains varied in other characteristics such as casein digestion, hydrogen sulfide production, and nitrate reduction. Similar variations also occurred among strains described by Bryant and Small (1956). As stated by these workers, in strains of Butyrivibrio, the flagellum is usually attached at the end of the cell but occasionally it appears to be attached laterally. Bennett and Edwards (1958) have suggested that polar and lateral flagella may be differentiated by comparison of their amplitude and wave length. Using *Vibrio percolans*, they found polar flagella to have an average amplitude of 0.6 μ and wave length of 2.4 μ, whereas lateral flagella had an average amplitude of 0.3 μ and a wave length of 1.0 μ. As can be seen in table 1, most of the present strains of Butyrivibrio possessed flagella with a different amplitude and wave length ratio from those which were found by Bennett and Edwards.

According to the results shown in tables 9 and 10, all tested strains produced more acid under a carbon dioxide atmosphere than under nitrogen in the same medium although the net increase was not greater in every case. This evidence of greater activity may simply reflect the high requirement for carbon dioxide as reported by Gill and King (1958). The satisfactory growth on a medium from which rumen fluid was omitted might indicate that *Butyrivibrio* species are not as fastidious as several other rumen organisms reported (Bryant and Doetsch, 1955). It would appear that a range of Butyrivibrio types exists in the rumen since no two strains were identical.

Fermentation characteristics of all the strains were very sensitive to environmental changes which are known to take place in the rumen (Smith *et al.*, 1956). From the data in table 4 it is evident that the ratio of fermentation acids was dependent upon the pH of the medium. Generally there was a tendency for cultures in relatively alkaline media to produce higher amounts of the acetic (plus formic) and lactic (plus succinic) acids than at lower pH values. Butyric and propionic acids were formed in higher proportions in acidic media. The total production of butyric acid was reduced in these media. It is possible that with some strains under some cultural conditions butyric acid production might not be evident.

The addition of fermentation acids to the medium at concentrations well below maximum physiological rumen levels modified the ratio of fermentation products. Strains A4 and C7 appeared to be able to reduce lactate to propionate. All 4 tested strains apparently converted either acetate or propionate to butyrate. This activity was evident in both the rumen fluid-glucose broth and in the 98 per cent rumen fluid medium (tables 5 to 8). Acid production by some of the strains was inhibited by increased concentrations of added fermentation acids; however, strain G and strain C15 produced increased amounts of lactic acid in the presence of added lactic acid. The production of acetic acid in its absence from the medium, and its utilization in its presence, indicate that this genus is probably not important in acetate production in the rumen since the production mechanism is so sensitive to the concentration of product.

The shift in the ratio of rumen fermentation products when rations of ruminants are changed (Briggs *et al.*, 1957; Reid *et al.*, 1957) may be caused by two mechanisms. As demonstrated by Pounden and Hibbs (1948) and Phillipson (1952) the types of rumen organisms may vary with the ration of the animal. A second mechanism is suggested by the present data. This mechanism involves a change in the ratio of fermentation products produced by the same individual rumen species. The sensitivity of the metabolism of Butyrivibrio to environmental changes, and the apparent interconversion of fermentation products by members of this genus demonstrate that in-

dividual organisms which occur in significant numbers in the rumen are capable of major changes in their fermentation. These fermentation changes take place under the influence of environmental changes which are compatible with physiological conditions in the rumen.

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SUMMARY

Ten strains of anaerobic, gram-negative, monotrichous, butyric acid-producing curved rods have been isolated from ingesta of the bovine rumen. These 10 strains of Butyrivibrio represented ⅓ of all isolates at 1×10^{-8} dilutions. Morphological and physiological characteristics of these strains and of one isolated by Gill and King (1958) have been studied. No two of the isolates were identical in all characteristics. Most of the organisms produced a large amount of butyric and some lactic, formic, propionic, and succinic acids with the utilization of acetic acid in a rumen fluid-glucose medium.

The fermentation carried on by these organisms was sensitive to most of the environmental changes tested. Studies with buffered rumen fluid-glucose media demonstrated a shift of the fermentation products with pH. Addition of fermentation acids to this medium indicated that these organisms were active in the conversion of acetate and possibly propionate to butyrate. Two strains apparently had the ability to produce propionate at the expense of lactate. The results of the fermentation tests in 98 per cent rumen fluid medium showed that the tested strains used acetic (plus formic) or lactic (plus succinic) to produce butyric or propionic acid and produced higher concentrations of acids under a carbon dioxide atmosphere than under nitrogen. When rumen fluid and acetic acid were absent, all strains had the ability to produce either formic or acetic acid.

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