SEROLOGICAL ANALYSIS OF *BUTYRIVIBRIO* FROM THE BOVINE RUMEN

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ABSTRACT

MARGHERITA, S. S. (University of California, Davis) AND R. E. HUNGATE. Serological analysis of *Butyrivibrio* from the bovine rumen. J. Bacteriol. **86**:855–860. 1963.—The cultural and fermentation characteristics of a number of strains of *Butyrivibrio fibrisolvens* isolated from the bovine rumen of cattle from different areas were determined, and the strains were subjected to serological analysis by the techniques of agglutination, immunodiffusion, and indirect hemolysis. In general, the results of the three methods agreed fairly well, but some variation was shown according to the method of preparation of the antigen. Much serological heterogeneity was disclosed. The greatest degree of agglutinating cross-reactivity was observed with strains isolated simultaneously from two animals in the same herd of African zebu cattle and with a Pullman strain. These cross-reactions were confirmed by immunodiffusion and indirect hemolysis tests. Agglutinating cross-reactions at low titers were observed between additional strains. The African isolates were shown to possess unique, as well as shared, antigens.

Numerous studies have employed morphological and cultural features to characterize rumen bacteria, but the serological relationships within bacterial groups have not been analyzed. Many rumen investigations would be greatly facilitated if the bacteria could be identified easily, quickly, and quantitatively. A serological analysis might provide a means for such identification through immunofluorescence (Hobson and Mann, 1957).

Serotypes of the strains inhabiting the rumen must be surveyed before the usefulness of immunofluorescence for identification of a species can be evaluated. A conjugated fluorescent antiserum capable of detecting only a single strain of many present would be of little use in determining the contribution of the entire species to a particular biochemical function.

The present investigation was undertaken to obtain information on the serological relationships in the genus *Butyrivibrio*, using strains isolated from several different localities.

MATERIALS AND METHODS

Organisms. All strains except N2C3 (Table 1) were classified as *B. fibrisolvens* (Bryant and Small, 1956), and were gram-negative curved rods, producing butyric acid as an end product of metabolism. Although *B. fibrisolvens* was described as motile by monotrichous flagella, motility was not observed in T28 with the cultivation methods employed. The African strains were not examined for motility. All strains with the exception of D1, received from M. P. Bryant, were cellulolytic. N2C3, a cellulolytic gram-negative rod isolated from an African zebu steer, did not produce butyric acid and is not a *Butyrivibrio*, but was tested for possible serological relationships.

Colonies of the *Butyrivibrio* cultures in roll-tubes with sugar substrates were predominantly smooth and lenticular; the one exception, D1, was rough and filamentous. The geographical areas from which the strains were isolated are as follows.

N strains were obtained from Archer's Post, Northern Frontier District, Kenya. The N1 isolates were from a dilution series of rumen contents of one zebu; the N2 strains were from a second. Both animals were semistarved owing to poor grazing conditions. Strain D1 was isolated in Beltsville, Md. Strains T28, 7/5-42, 8/3-42, and 8/4-46, isolated in Pullman, Wash., were obtained from Hereford cows on a timothy hay ration plus some concentrates. Each strain was from a different animal except the 42 cultures,

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which were isolated 1 month apart from the same animal. The other strains were isolated on different days. Strain S7-4 was isolated in Davis, Calif., from a fistulated Jersey heifer fed alfalfa hay.

Characteristics of the Kenya, Pullman, and Davis strains have not previously been reported and are summarized in Table 1. The characteristics of D1 were reported by Bryant and Small (1956).

Stock strains were maintained on solid media under anaerobic conditions (Hungate, 1950); the cellulolytic strains were maintained on rumen fluid-cellulose medium, and the noncellulolytic D1 on rumen fluid-alfalfa hay extract medium. The latter medium contained 40 volumes of an aqueous extract prepared by heating 4 g of alfalfa hay in 100 ml of water, 21.6 volumes of rumen fluid filtered through cotton, and 33.3 volumes of inorganic salt solutions [percentage composition (w/v): KH_2PO_4, 0.15; NaCl, 0.3; NH_4Cl, 0.15; $MgSO_4$, 0.03; $CaCl_2$, 0.03; and K_2HPO_4, 0.15]. Resazurin (0.0001%, w/v) and 1.5% (w/v) agar were included, and sodium bicarbonate (0.5%, w/v) was added to increase the buffering capacity and to allow the use of carbon dioxide gas to exclude oxygen. Anaerobiosis was produced initially by the rumen fluid and, after the tubes were inoculated, was re-established by adding 0.7% cysteine hydrochloride. Equal parts of glucose and cellobiose were added just prior to inoculation to give a final concentration of 0.1% supplementary carbohydrate.

Preparation of antigens. Cells from broth cultures were used as a source of antigens. The batch method was used to culture small numbers of cells, and a continuous-flow system was used when larger quantities were needed. The bacteria used as antigens were grown in a liquid medium similar to that used for maintenance of noncellulolytic strains, except that it lacked rumen fluid. In the batch method, 6 liters of sterilized medium in an anaerobic flask were inoculated by injecting 50 ml of a young culture through the rubber stopper. The cultures were then incubated at 39 C for 12 to 18 hr.

In the continuous method, the medium was fed by an infusion pump (Harvard Apparatus Co., Inc., Dover, Mass.) to the culture vessel immersed in a water bath at 39 C. The overflow emptied into a large carboy into which formalinized saline dripped slowly from a separatory funnel. The reservoir of sterile medium was kept anaerobic by constant flushing with a slow stream of carbon dioxide. The feed rate for the culture was determined from the growth curve of a

TABLE 1. *Fermentation characteristics of the Butyrivibrio strains and N2C3**

Strain	Mannose	Rhamnose	Mannitol	Esculin	Salicin	Lactose	Trehalose	Raffinose	Starch	Inulin	Xylan	Pectin	Nitrate reduction	Hydrogen	Ethanol	Formic acid	Acetic acid	Butyric acid	Lactic acid
S7-4	+	+	+	+	+	+	+		+	+	+	+	−	68†	2	135	−12	139	53
8/4-46	−	−				+	−	−	+	−			−	765	0	436	113	404	61
7/5-42	+	−				+	+	+	+	−				807	24	836	209	444	31
8/3-42	−	±				+	−	−	+	±									
T28	±	−				+	−	+		−				492	0	166	−25	380	304
N1C2	+	+	−	+	+	+	−		+	+	+	+	−	9	0	194	−69	132	115
N1C3	+	+	−	+	+	+	−		+	+	+	+	+						
N1C8	+	+	−	+	+	+	−		−	−	+	+	−	19	0	198	−59	125	194
N1C11	+	+	−	+	+	−	−		−	+	+	−	−	21	0	170	−80	135	132
N2C1	+	+	−	+	+	+	−		+	+	+	+	−	17	0	167	101	156	163
N2C2	+	+	−	+		+	−		−	+	+	+	−	14	2	184	−52	139	160
N2C3	−	−	+	−	−	+	−		−	−	++	+	−	73	7	0	35	0	0

* All *Butyrivibrio* strains fermented D-xylose, L-arabinose, glucose, fructose, galactose, sucrose, cellobiose, maltose, and cellulose. N2C3 fermented all these except D-xylose and galactose. No strain produced acetoin or H_2S.

† Fermentation products formed by the various strains are given in moles. The amount of substrate fermented was not determined. The minus sign before the amount of acetate denotes that acetate was utilized rather than produced.

batch culture by calculating the cycle time from the number of organisms and the growth rate at a chosen point on the growth curve (Adams and Hungate, 1950). Cells were collected by centrifugation and washed three times with saline or formalinized saline; the suspension was standardized turbidimetrically A yield of 300 mg (dry weight) of cells per liter of medium was obtained in the continuous culture.

Antisera. Antisera were produced in New Zealand White rabbits by intravenous injection of washed-cell suspensions. Six injections of increasing dosage were usually sufficient to produce antisera of high agglutinin titer; only rarely was it necessary to give a second series. For each bacterial strain, antisera from three animals were pooled and stored at −20 C.

Serological tests. To determine agglutination, antisera, serially diluted in 0.85% saline, and equal volumes of standardized cell suspensions were incubated at 45 C for 2 hr. The results are reported as the highest dilution of antiserum showing complete agglutination.

Agglutinin-absorption tests were conducted as follows. To the sediment obtained by centrifuging a formalinized cell suspension (standardized to contain eight times the equivalent of a #4 MacFarland nephelometer tube) was added a volume of undiluted antiserum equal to the original volume of antigen suspension. The tubes were incubated at 45 C and recentrifuged. The supernatant fluid was decanted and either used directly in the agglutination tests or absorbed further.

Immunodiffusion tests were performed by the Ouchterlony double-diffusion method, in a medium containing 7.5% (w/v) glycine, in saline buffered with 0.07 M phosphate to pH 7.4, and with 0.7% (w/v) Ionagar (Consolidated Laboratories, Chicago Heights, Ill.) as the solidifying agent. The molten medium was clarified by centrifugation before it was poured into petri dishes. Wells 7 mm apart were cut into the agar with a cork borer.

Filtrates of sonically disrupted cell suspensions or extracts of whole cells served as precipitinogens. A 55-min period of sonic oscillation at 10 kc, carried out in an atmosphere of nitrogen or hydrogen to prevent protein denaturation, was adequate to disrupt cells and comminute cell envelopes. The limpid suspensions were clarified by filtration and standardized by Kjeldahl nitrogen determination. The cell extracts were prepared by two methods. Fraction A was obtained by a phosphate buffer (pH 7.2) extraction of cells previously treated with butanol (Morton, 1950); fraction B was obtained by aqueous extraction of deproteinized cells (Palmer and Gerlough, 1940).

With the concentrations selected, it was not necessary to refill the wells to obtain bands of precipitation. Plates were usually kept at room temperature, but, to obtain the more intense bands required for photography, refrigeration was necessary.

For indirect hemolysis tests, washed sheep red blood cells (2.5%) were sensitized by incubation at 37 C for 2 hr with a concentration of fraction B previously found to produce optimal sensitization. The sensitized cells were washed in normal serum-saline, diluted to 0.5%, and were added to serial dilutions of antiserum in the presence of excess guinea pig serum. Prior to use in the tests, the antisera were absorbed twice with normal sheep red blood cells and inactivated at 56 C for 30 min. The hemolysis titer was determined by inspection after incubation at 37 C for 30 min.

RESULTS

Cross-agglutination tests revealed much serological heterogeneity (Table 2). The N2C3 antiserum did not agglutinate any of the *Butyrivibrio* strains, but some indications of cross-reactions were shown by all *Butyrivibrio* antisera. Much cross-reactivity occurred among strains isolated from Kenya. For example, the N1C11 antiserum agglutinated all Kenya strains except N1C8; the N1C3 antiserum agglutinated all except N1C2. A similar degree of sharing was not evident among Pullman strains.

The closest similarity on agglutination cross-reactions was between T28 and N1C2, strains from widely distant geographical localities. T28 also reacted with low dilutions of N2C2, N1C8, and N1C11 antisera. D1 and S7-4 showed some cross-reactions with a few African strains.

Although the type of agglutination observed was flocculent and easily dispersed, it was not of the H type. Treatment of immunizing or test preparations with heat or alcohol—procedures known to affect the flagellar antigens of enteric organisms—failed to influence either the titer of the test or the nature of the aggregates. It is possible that the tendency of these organisms to

TABLE 2. *Homologous and heterologous agglutinin titers* of the tested strains*

Antiserum	Antigen											
	S7-4	8/4-46	7/5-42	T28	N1C2	N1C3	N1C8	N1C11	N2C1	N2C2	N2C3	D1
S7-4	+++	−	−	−	+	+	+	−	−	−	±	±
8/4-46	−	+++	−	−	−	±	−	−	+	+	−	−
7/5-42	±	−	++	−	±	−	−	+	±	+	±	−
8/3-42†	−	+	−	−	−	±	−	−	−	−	−	−
T28	−	−	−	+++	+++	±	+	+	−	+	−	−
N1C2	−	−	−	+++	+++	±	−	++	−	−	−	−
N1C3	−	−	−	+	−	++++	+++	++	±	±	−	−
N1C8	−	−	−	−	−	+	+++	−	−	−	−	−
N1C11	−	−	−	+	++	±	−	+++	++	±	−	−
N2C1	−	−	−	−	−	+	+	+	++++	++	±	+
N2C2	±	−	−	+	−	+	−	++	++	+++	−	−
N2C3	−	−	−	−	−	−	−	−	−	−	++++	−
D1	−	−	−	−	−	+	−	±	−	−	−	++++

* Symbols: ±, 10 to 20; +, 40 to 80; ++, 160 to 320; +++, 640 to 1280; ++++ 1280 or more. Expressed as the highest dilution of serum showing complete agglutination.

† Strain 8/3-42 cells agglutinated spontaneously in saline.

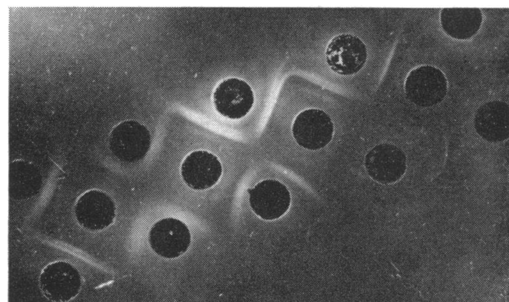
TABLE 3. *Comparison of serological results with T28 or T28 products as antigens and heterologous antisera*

Antiserum	Agglutination	Precipitation (Ouchterlony)		Hemolysis of fraction B T28
		Fraction A T28	Fraction B T28	
N1C2	+	+	+	+
N1C3	+	+	−	+
N1C8	−	+	+	−
N1C11	+	+	+	+
N2C1	±	+	−	+
N2C2	+	+	+	+
N2C3	−	−	−	−
7/5-42	−	−	−	−
8/3-42	−	−	−	−
8/4-46	−	−	−	−
D1	−	+	−	−
S7/4	−	−	−	−

form elongated cells was responsible for formation of these aggregates.

Strains 7/5-42 and 8/3-42, obtained from the same animal 1 month apart, differed in their agglutination reactions. Unfortunately, the cells of 8/3-42 agglutinated spontaneously and thus could not be tested against any of the sera.

The relationships revealed by the immunodiffusion technique (Table 3) were generally similar to those obtained by agglutination; however, there were some differences. The precipitation tests with the sonically prepared extracts showed broader relationships than were disclosed by agglutination, indicating a release of



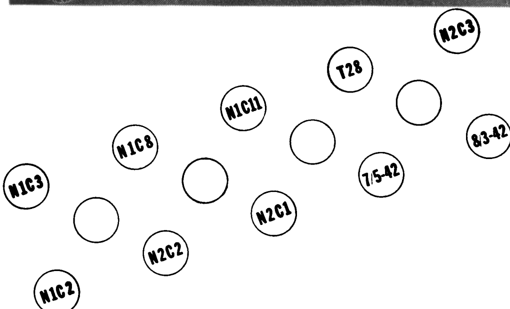


FIG. 1. *Precipitation of fraction A from T28 by homologous and heterologous antisera. Center wells contained antigen; outside wells contained antisera.*

subsurface, haptenic components by the treatment. Common antigens were indicated for T28 and all the African *Butyrivibrio* strains (Fig. 1). The postbutanol extract (fraction A) showed more extensive reactions than fraction B (Table 3), owing to a component shown by identity tests to be absent from the B fraction. Fraction A from T28 reacted with all the African strains (Fig. 1) and also with D1 (Table 3). When the same extraction procedure was applied to strain 8/4-46, the extracts gave no precipitation with antisera of T28, D1, and the African strains. Only the 8/4-46 antiserum reacted. The immunodiffusion tests thus disclosed more common antigens in the African, T28, and D1 strains than were detected by the agglutination tests, and fewer relationships with 8/4-46.

The indirect hemolysis tests, utilizing fraction B from T28 as sensitizing antigen, yielded data paralleling those obtained by agglutination, but with slightly more cross-reactions than were observed in the gel diffusion tests with the B fraction (Table 3). This may reflect the greater sensitivity of hemolysis. The parallelism of agglutination and indirect hemolysis supports the validity of the agglutination results.

That reciprocally agglutinating strains possessed some unique antigens was shown by agglutinin absorption. Agglutination by homologous strains occurred after heterologous absorption was complete. Strains N2C2 and N1C2 possessed both shared and unique antigens (Table 4). In a second test, N1C8 was shown to possess, in addition to antigens shared with N1C3, one or more antigens which were unique. The mirror absorption, however, did not reveal unshared antigens for N1C3.

TABLE 4. *Presence of shared and unique antigens revealed by mirror absorption tests with selected African strains*

Antiserum	Absorbing antigen	Test antigen	Preabsorption titer*	Titers after successive absorptions			
				1	2	3	4
N2C2	N1C3	N2C2	2560	640			
		N1C3	64	0			
N1C3	N2C2	N1C3	5120	640			
		N2C2	16	0			
N1C8	N1C3	N1C8	6400	1280			
		N1C3	100	0			
N1C3	N1C8	N1C3	1600	80	80	40	20
		N1C8	400	320	40	10	10

* Titers expressed as highest dilution of serum showing complete agglutination.

DISCUSSION

The results indicate that there are various degrees of serological relatedness among bacteria classified as *Butyrivibrio* from morphological and cultural characteristics. None of the 12 tested strains was identical to any other, in spite of the fact that most of them were isolated on a partially selective medium, cellulose being the substrate for initial detection of all strains except D1. If sugar had been the substrate for primary isolation, an even greater variability would presumably have been encountered.

The explanation for the variability in *Butyrivibrio* is not apparent. It may represent random variation with survival by chance, or the types may differ in characteristics of survival value and be selected by subtle unrecognized factors. Development of host antibodies might affect survival. Serum proteins are not vigorously attacked in the rumen (Lewis, 1961) and perhaps enter with the saliva. In the saliva of achlorhydric humans, hemagglutinins are presumably active in controlling the numbers and kinds of bacteria (Balázs, 1962). Similar selection by host antibodies in the rumen may occur, but there is no evidence on this point.

The similarity of T28 and N1C2 from widely separated localities encourages the hope that extensive serological similarities will be found if more isolates are examined, but the overall results suggest that elaboration of a system for serological identification of *B. fibrisolvens* will require study of a very large number of strains.

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