JOURNAL OF BACTERIOLOGY
Vol. 87, No. 6, pp. 1304-1308 June, 1964

VARIATION IN RUMEN *BUTYRIVIBRIO* STRAINS

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Received for publication 6 December 1963

ABSTRACT

MARGHERITA, S. S. (University of California, Davis), R. E. HUNGATE, AND HANNELORE STORZ. Variation in rumen *Butyrivibrio* strains. J. Bacteriol. **87**:1304-1308. 1964.—Five strains of *Butyrivibrio* isolated from the rumen of a single animal on an alfalfa hay ration were tested for serological relationships by agglutination and immunofluorescence. The main finding was a serological monospecificity of the strains. A cross-reaction between two strains was detected by agglutination and a second cross-reaction by immunofluorescence, but the cross-reacting pairs were different. Two years after the strains were isolated, fluorescein-conjugated antisera against three of them were used to test rumen contents of the same animal for homologous cell types. None was found. The findings indicate great variability in the serological characteristics of rumen butyrivibrios.

Identification *in situ* of rumen bacteria by immunofluorescence was demonstrated by Hobson and Mann (1957). This could provide a useful means for quantitative counts of cells belonging to various rumen species, provided serotypes persist in the rumen for long periods. Evidence of antigenic variation has been observed among certain species of the rumen flora (E. Hall, *unpublished data*). Utilization of immunofluorescence techniques to determine the numbers of cells of a given species in the rumen requires knowledge of rumen serotypes and the variation in the serological composition of the rumen population with time. Previously reported experiments (Margherita and Hungate, 1963) analyzed antigenic relationships in *Butyrivibrio* strains cultured from different geographic areas. Many strain differences were found.

In this paper, we report the results of immunofluorescence tests on the incidence of serotypes in the rumen of a single animal at different times.

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MATERIALS AND METHODS

Strains. All strains studied belonged to the genus *Butyrivibrio*. Strains L2-1, L2-2, and LW5-2 were isolated at Davis, Calif., 14 September 1959 from a Jersey heifer fed only alfalfa hay. Strains S7-1, S7-4, and S8-2-1 were isolated from the same animal on 15 April 1960. Immunofluorescence tests were made in the summer of 1962 on bacteria from the rumen of this same animal, the animal having been fed only alfalfa hay during the interval between the initial isolations and the tests with fluorescent antisera.

Each strain was initially selected because the colony exhibited a distinctive morphology. Isolates were maintained in the laboratory under anaerobic conditions on an agar medium (Hungate, 1957) containing alfalfa extract [33% (v/v) of a solution obtained by boiling 5% (w/v) alfalfa hay in distilled water], rumen fluid (33%, v/v), and mineral salts solution (33%, v/v). Bicarbonate (0.5%, w/v) and cysteine (0.025%, w/v) were also included in the medium. Routine transfers were made at approximately 6-week intervals.

The biochemical characteristics of the five strains studied most extensively are shown in Table 1. The characteristics of other strains mentioned were reported previously (Margherita and Hungate, 1963).

Preparation and conjugation of antisera. Cells to be used as antigen were grown anaerobically in a liquid medium, the composition of which was essentially that used for routine transfers, but with rumen fluid omitted. Antisera were prepared by injecting rabbits with cells washed and suspended in formalin-saline [final concentration of formalin, 0.3% (v/v)] and standardized by comparison with MacFarland standards (Zinsser and Bayne-Jones, 1934). Comparison was made photometrically by adjustment to an optical density corresponding to that of the standard.

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

Strain	Substrate fermentation																	Fermentation product					
	L-Arabinose	D-Xylose	Glucose	Mannose	Galactose	Rhamnose	Mannitol	Lactose	Trehalose	Melezitose	Sucrose	Dextrin	Starch	Inulin	Xylan	Pectin	Nitrate red	Sulfide production	Hydrogen	Formic acid	Acetic acid	Butyric acid	Lactic acid
L2-1	−	−	+	+	+	+	−	+	+	−	+	+	+	+	+	+	−	−	+	+	+	+	−
L2-2	+	−	+	+	+	+	−	+	−	−	+	−	±	+	+	−	+	+	+	+	+	+	−
S8-2-1	−	−	+	+	−	−	−	±	+	−	+	−	±	+	−	−	−	−	+	+	Uptake	+	+
S7-1	−	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+
LW5-2	−	−	−	−	+	−	−	+	+	−	−	−	−	−	−	−	−	−	+	+	Uptake	+	+

* All strains fermented salicin, esculin, cellobiose, and maltose, but not cellulose. None liquefied gelatin or produced indole.

The immunization schedule consisted of six intravenous injections given on alternate days. Increasing doses were given, with the final injection 20-fold more concentrated than the first. Blood was obtained by cardiac puncture after a rest period of 5 to 7 days. All experiments were done with pooled sera from several rabbits.

γ-Globulins were separated by isoelectric precipitation, accomplished by layering 1 volume of antiserum over 4 volumes of a salt solution of pH 6.4, containing 23.1% (w/v) $(NH_4)_2SO_4$ and 3.6% (w/v) NaCl (Friedman, 1958), and shaking to a maximal turbidity. Electrophoretic comparison of the fraction with whole serum indicated a high degree of purification of the γ-globulin. After application of this testing procedure, the agglutinin titer was utilized to measure persistence of the antibodies in the purified fraction.

Fluorescein isothiocyanate was coupled to the globulin by the method of Riggs et al. (1958). The protein was first dialyzed against 0.9% NaCl and standardized by a micro-Kjeldahl nitrogen determination. Powdered isothiocyanate was added, in a proportion of 1 mg per 25 mg of protein, to an aqueous mixture of globulin (1 to 2.5%, w/v), acetone (10%, v/v), and 0.05 M carbonate-bicarbonate buffer, pH 9.0 (10%, v/v). The mixture was held at 4 C for 18 hr, and then dialyzed against buffered saline (0.01 M phosphate, pH 7.2). Dialysis was continued until the dialysate contained no visible dye.

Prior to use, nonspecific fluorescence of the conjugates was reduced either by absorption with mouse liver powder or by passage through diethylaminoethyl (DEAE)-cellulose columns according to the method of Riggs, Loh, and Eveland (1960). As in that study, the fraction from cellulose showing the greatest reactivity and specificity was obtained by elution from the column with a solution of 0.125 M NaCl buffered at pH 6.3 with 0.0175 M phosphates. The treatments caused a fourfold decrease in agglutinin titer, but the loss in titer could be partially offset by pervaporation of the eluted fraction.

Fluorochroming. Cells from pure cultures grown in alfalfa hay extract broth were examined for fluorescence after incubating 1 volume of washed-cell suspension with 1 volume of purified conjugated antibodies and 2 volumes of 0.01 M phosphate buffer (pH 7.0) in a water bath (37 C) for 20 to 30 min (Hobson, Mann, and Smith, 1957). Wet mounts were prepared, and the cover slips were rimmed with Vaspar to prevent streaming. Background fluorescence was not a problem if air bubbles were carefully excluded and very thin layers of the liquid suspension of cells were used.

For fluorescence microscopy of cells obtained directly from the animal, a sample of rumen fluid was filtered through cotton to remove coarse particles, and centrifuged at 4 C to sediment the microorganisms. The sediment was washed twice with buffered saline, and after resuspension was diluted with saline to the turbidity of a #4 MacFarland nephelometer standard. Subsequent treatment was the same as that used for the pure cultures.

The sedimentation of the rumen fluid increased the number of cells per field, improving detection of rare serotypes, and diminished any

reactions with the rumen fluid itself. Occasionally, untreated rumen fluid was shaken in phosphate buffer before filtration through cotton, to release organisms adsorbed on digested particles.

The wet mounts were examined with a Leitz microscope equipped with dark-field condenser, exciter, and barrier filters, and an illuminator providing ultraviolet or white light.

RESULTS

With one exception, the five strains were monospecific in the agglutination test, the exception being the reciprocal cross-agglutination between strains S8-2-1 and L2-2 (Table 2). The fluorescent-antibody test, using a pure culture of L2-2 and a conjugate of S8-2-1 antiserum, did not reveal cross-reactivity of these strains (Table 3), even though the heterologous agglutinin titer of the conjugated globulin was of the order of 320. Conversely, L2-2 and L2-1, strains closely related biochemically, did not cross-agglutinate but did show a cross-immunofluorescence (Table 3). These findings may be explained by postulating a superficial layer over the immunofluorescent sites. The layer would contain the agglutinogens shared by S8-2-1 and would be permeable to the tagged globulins. Similar antibody tests were reported previously (Eldering, Eveland, and Kendrick, 1962).

Cells of the serotypes tested by immunofluorescence were not present in significantly high numbers in the rumen contents of the animal from which the strains had been obtained more than 2 years previously (Table 4). That the rumen fluid did not contain substances inhibit-

TABLE 2. *Agglutination reactions of Butyrivibrio strains with homologous and heterologous antisera*

Antigen	Antiserum				
	L2-1	L2-2	LW5-2	S7-1	S8-2-1
S8-2-1	—	640–1,280*	—	—	>1,280
S7-1	—	—	—	>1,280	—
L2-2	—	>1,280	—	—	160–320
LW5-2	—	—	1,280	—	—
L2-1	640–1,280	—	—	—	—

* Highest dilution of antiserum showing complete agglutination.

TABLE 3. *Fluorescence of Butyrivibrio cultures after incubation with fluorescein-conjugated antisera**

Strain	Source of strain	Conjugated antiserum to			
		L2-1	D_1	S8-2-1	Normal rabbit serum
N1C2	African zebu 1	—	—	—	
N1C3	African zebu 1	—	—	—	
N1C8	African zebu 1	—	—	—	
N1C11	African zebu 1	—	—	—	
N2C1	African zebu 2	—	—	—	
N2C2	African zebu 2	—	—	—	
T28	Steer at Pullman, Wash.	—	—	—	
8/3-42	Steer at Pullman, Wash.	—	—	—	
8/4-46	Steer at Pullman, Wash.	—	—	—	
7/5-42	Steer at Pullman, Wash.	—	—	—	
D_1†	Beltsville, Md.	—	++	—	—
S7/4	Heifer at Davis, Calif.	—	—	—	
L2-1	Heifer at Davis, Calif.	++++	—	—	—
L2-2	Heifer at Davis, Calif.	+	—	—	
LW5-2	Heifer at Davis, Calif.	—	—	—	
S7-1	Heifer at Davis, Calif.	—	—	—	
S8-2-1	Heifer at Davis, Calif.	—	—	++++	—

* Fluorescence intensity determined by direct visualization interpreted on an arbitrary scale.

† This strain was kindly supplied by M. P. Bryant.

ing the reaction was shown by the specific fluorescence of homologous cells when these were added to the suspension of rumen cells. That the fluorescence was specific was further demonstrated by the failure of conjugated normal sera to stain the added cells. The results were the same regardless of the time of day at which the ruminal samples were taken and the method of preparing them for examination.

TABLE 4. *Identification of Butyrivibrio in rumen contents incubated with fluorescein conjugate*

Antigen	Conjugate			
	L2-1	S8-2-1	D_1	Normal
Ruminal contents (RC)	−	−	−	−
RC plus L2-1 cells*	+	−	−	−
RC plus S8-2-1 cells	−	+	−	−
RC plus D_1 cells	−	−	+	−

* Washed cells added to a sample of rumen fluid.

DISCUSSION

The results indicate that the antigenic composition of the rumen *Butyrivibrio* population changed with time. Antisera against strains isolated in 1959 and 1960 failed to react with an appreciable number of cells in the same rumen in 1962.

It cannot be excluded that the antigenic nature of the isolated *Butyrivibrio* strains changed during the period of laboratory culture, losing antigens retained in the rumen population. If serotypes did change during cultivation, they did not change to a common type, since all cultured strains showed differences in their antigenic composition. The laboratory culture methods, similar for all, did not select identical types. The results, however interpreted, indicate a great capacity for serological variation in the genus *Butyrivibrio*.

The sugar fermentation pattern of *Butyrivibrio* strains also shows great variability (Table 1). This variability in sugar fermentation pattern has been noted in many *Butyrivibrio* strains not included in the present report. Variation with time of the sugar pattern of a given strain has also been observed. These observations support the conclusion from the present investigation that strains assigned to this genus on the basis of morphology and formation of butyric acid exhibit great diversity in other characters.

The greater sharing of precipitinogens in *Butyrivibrio* found in the antigenic analyses (Margherita and Hungate, 1963) suggests that precipitin tests are preferable for detecting common antigens. The precipitin test, however, is unsuited to identification of whole cells in the rumen contents.

The results with *Butyrivibrio* suggest a greater antigenic variety in the genus than is indicated for *Selenomonas* by the results of Hobson et al. (1962). They detected homologous *Selenomonas* cells in sheep rumen contents, by use of fluorescent antibodies from a *Selenomonas* strain isolated several years earlier. These workers also found a significant cross-reactivity of *Selenomonas* antisera with *Butyrivibrio*. This combination was not tested in the present investigation, but *Cillobacterium* and *Succinivibrio* strains were tested and found to be serologically distinct from *Butyrivibrio*. The difference in results with *Butyrivibrio* as compared with *Selenomonas* may also be due to differences in the rate of change of microbial serotypes in different hosts.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-01266-06 from the National Institute of Allergy and Infectious Diseases.

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