CHARACTERISTICS OF RUMINAL ANAEROBIC CELLULOLYTIC COCCI AND *CILLOBACTERIUM CELLULOSOLVENS* N. SP.

M. P. BRYANT, NOLA SMALL,[1] CECELIA BOUMA, AND I. M. ROBINSON

Dairy Cattle Research Branch, U. S. Department of Agriculture, Beltsville, Maryland

Received for publication June 1, 1958

Many studies have indicated that cocci are among the more important cellulolytic agents in the rumen. Their importance was first suggested by the use of direct microscopic methods with which they were observed to be present in enzymatic cavities in fibrous feed particles of ruminal contents and in purified celluloses incubated in ruminal contents (Baker and Harriss, 1947).

Although several earlier workers had cultured cellulolytic sporeforming anaerobes from the rumen (Sijpesteijn, 1948), cellulolytic bacteria were first isolated and cultured in large enough numbers to indicate that the types isolated were of significance in the rumen by Hungate (1947). One of the groups cultured was composed of anaerobic iodophilic cocci.

Hungate (1947, 1950) isolated the cocci using an agar medium containing cellulose, a carbonic acid-bicarbonate buffer, minerals, reducing agent, and, usually, rumen fluid as a source of growth factors, and an anaerobic technique which involved the use of rubber-stoppered roll tubes containing a gaseous phase of carbon dioxide.

Subsequent to the first work reported by Hungate, many workers using similar cultural techniques have found anaerobic cocci among the more numerous cellulolytic bacteria in the rumen (Sijpesteijn, 1948, 1951; Bryant and Burkey, 1953*a*, *b*; Kitts *et al.*, 1954; King and Smith, 1955; Maki, 1955). Hall (1952) isolated similar organisms from rabbits. Gall *et al.* (1947) isolated cellulolytic cocci using a rich organic medium radically different from that of Hungate. The rate of cellulose digestion was extremely slow on this medium and subsequent comparative studies of the techniques indicated that the technique of Hungate was far superior for cocci as well as other ruminal cellulolytic bacteria (King and Smith, 1955).

[1] Present address: Second Army Medical Laboratory, Veterinary Section, Fort George Meade, Maryland.

Sijpesteijn (1948, 1951) described the characteristics of two species of cellulolytic cocci, *Ruminococcus flavefaciens* and *Ruminobacter parvum*. The latter species was not isolated in pure culture. The genus *Ruminococcus* Sijpesteijn (1951), based on the characteristics of two strains from cattle and one from sheep, included gram-positive, nonmotile, nonsporeforming, anaerobic cocci that fermented cellulose and other carbohydrates with the production of large amounts of succinic acid. All strains were placed in the type species, *R. flavefaciens* Sijpesteijn, which included cocci, in chains or pairs, that produced a yellow pigment on cellulose, fermented cellulose and cellobiose, varied in glucose fermentation, and did not ferment maltose, lactose, xylose, or starch. They were catalase negative and mesophilic.

The genus *Ruminococcus* Sijpesteijn excluded ruminal cellulolytic cocci described by Hungate (1947, 1950). Hungate recognized two groups, colorless cocci and yellow cocci, based on some characteristics of five strains. They differed from *R. flavefaciens* Sijpesteijn in being gram-variable and in producing little or no succinate and the colorless cocci did not produce the yellow pigment. Hungate's groups were similar in being strictly anaerobic, gram-variable cocci that occurred as singles, diplos, and sometimes in chains. They fermented cellulose and cellobiose and, sometimes, glucose, and produced carbon dioxide, hydrogen, ethanol, acetate, lactate, and intracellular iodine-staining material from cellulose. The colorless cocci fermented cellulose more rapidly; formed a less compact, spreading colony with no yellow pigment in cellulose agar; produced less lactate and some formate; and grew well only in media containing rumen fluid.

The cellulolytic cocci isolated from rabbit caecal contents by Hall (1952) fit the description of *R. flavefaciens* Sijpesteijn except that no pigment was observed.

Cellulolytic cocci isolated by other workers were not described in detail. However, those of

Maki (1955) had fermentation acids identical with *R. flavefaciens* Sijpesteijn.

It was evident that all cellulolytic cocci described were similar in some characteristics such as being strictly anaerobic, iodophilic cocci that divide on only one plane and produce acetic acid, but they appeared to vary in many characteristics including Gram reaction, ability to form chains and ferment glucose, nutritional requirements, rate of growth and colony appearance in cellulose medium, and production of succinate, formate, and ethanol in the fermentation of carbohydrate.

Most workers have isolated cellulolytic bacteria using media in which cellulose served as the main carbon source. The earlier studies of Hungate (1947) suggested that the cellulolytic bacteria in the rumen were outnumbered by other bacteria to an extent that it would be difficult to isolate them or obtain an estimate of their numbers using a medium containing a relatively nonselective carbon source such as cellobiose. However, during a study of "predominant" bacteria cultured from the rumen with a medium similar to Hungate's rumen fluid-cellulose agar except that glucose and cellobiose were substituted for cellulose, Bryant and Burkey (1953*a*, *b*) found that cellulolytic organisms accounted for a significant proportion of the total strains cultured from tubes of medium inoculated with dilutions of rumen fluid (usually 10^{-8}) which allowed the development of well isolated colonies. Maki (1955) confirmed this finding.

The few characteristics determined suggested that the groups of cellulolytic bacteria isolated by Bryant and Burkey (1953*a*) corresponded to those isolated by Hungate (1950). Detailed studies indicated that one group was *Bacteroides succinogenes* Hungate (Bryant and Doetsch, 1954). Another group was identical with the less actively cellulolytic rod of Hungate and was named *Butyrivibrio fibrisolvens* Bryant and Small (1956). The third and usually most numerous cellulolytic group, included anaerobic cellulolytic cocci. One strain of an apparently different group of cellulolytic bacteria was isolated but later lost. This was a gram-positive, motile, anaerobic, coccoid to lancet-shaped organism.

The purpose of the present work was to determine further characteristics of a large number of strains of cellulolytic cocci to gain knowledge of the species involved. Another strain of the motile organism was isolated and included in the study.

EXPERIMENTAL METHODS

The 28 strains of cellulolytic cocci selected for study were isolated using the rumen fluid-glucose-cellobiose agar and the anaerobic roll tube method of Hungate (1950) as modified by Bryant and Burkey (1953*a*). The strains had been placed in the CeC group. This group included strictly anaerobic, nonmotile, gram-positive to gram-variable cocci that produced acid from cellobiose and hydrolyzed cellulose as indicated by the visual disappearance of 0.2 per cent of filter paper from liquid rumen fluid medium. They were

TABLE 1

Sources of strains of cellulolytic cocci

Strain No.	Date Isolated	Rumen Content Dilution	Cow*	Ration
7	4/9/51	1–100,000,000	817	Alfalfa hay-grain
C94	1/11/52	1–500,000,000	817	Grain mixture
20, 30, 52	12/18/52	1–100,000,000	817	Alfalfa hay-grain
D2, D50	1/5/53	1–100,000,000	817	Alfalfa hay-grain
D70	1/19/53	1–100,000,000	817	Alfalfa hay-grain
D89, D94, D95, D101	1/29/53	1–100,000,000	817	Alfalfa hay-grain
D117, D127, D154, D157	2/4/53	1–100,000,000	817	Alfalfa hay-grain
FD1	2/4/53	1–10,000		Pill containing ruminal organisms
$B_1$13, $B_1$15, $B_1$33, $B_1$46	4/23/53	1–100,000,000	280	Alfalfa hay
B_1C7, B_1C8, B_1C9, B_1C43, B_1C45	5/5/53		293	Alfalfa hay
$B_3$36, $B_3$37	9/29/53	1–500,000,000	SX233	Clover pasture

* All cows were mature Holsteins except for SX233 which was a Sindi (¾)-Jersey (¼) crossbred heifer.

nonmotile and did not produce hydrogen sulfide, liquefy gelatin, or hydrolyze starch in one week of incubation. They varied in pigment production and arrangement of cells as singles and diplococci or in chains and a few strains produced acid from glucose and D-xylose. The CeC group included all of 61 cellulolytic coccoid organisms isolated from the rumen of cattle on rations as divergent as a grain mixture only, wheat straw, or alfalfa hay (Bryant and Burkey, 1953*b*) except for the one motile lancet strain that was subsequently lost.

Table 1 shows the source of strains selected for study.

Methods of study were those of Bryant and Small (1956) with modifications used by Bryant *et al.* (1958). As most strains of cellulolytic cocci will not utilize glucose, an equal weight of cellobiose was substituted for glucose in all media except those used to determine carbohydrates fermented. To determine if reducing sugars were produced when grown in excess cellulose, the strains were inoculated into the rumen fluid-cellulose medium containing one per cent of filter paper. After one month of incubation, Benedict's qualitative reagent was used to test for reducing sugar.

An anaerobic, cellulolytic, motile, gram-positive, lancet-shaped organism was isolated from the sample from the animal on clover pasture (table 1). The organism fermented glucose and cellobiose but did not ferment xylose, hydrolyze starch, liquefy gelatin or produce hydrogen sulfide. This strain, $B_3$48, was studied in the same manner as the cellulolytic cocci.

RESULTS

Characteristics of cellulolytic cocci. The 28 strains had the following characteristics. Morphological characteristics were determined using Gram stains and wet mounts of the water of syneresis of 18 to 24 hr slant cultures. The only morphological basis found which was quite consistent for the separation of strains into groups was chain length. One group consisted of 16 strains which always showed some long chains of 8 to 50 or more cells. When morphology was studied on primary isolation and again 3 months to 3 years later, chain formation was consistent although some strains forming extremely long chains at one time might have considerably shorter chains at another time. Some strains have been checked 4 to 6 years after primary isolation and chain formation was still consistent. Another group of 10 strains always showed arrangements of singles and diplococci. Occasionally arrangements of cells which may have been chains of 3 or 4 cells were seen in these cultures.

One strain ($B_3$37) was arranged as singles and diplococci on primary isolation but shorter chains of 6 to 8 cells were found later. Another strain (D2) showed short chains on primary isolation but, later, chains were not evident.

Cells of both groups varied from spherical to somewhat elongated in shape. The ends of elongated cells were always rounded or flat with little tendency toward pointed ends or lancet-shapes. The sides of cells were often flattened where they were in contact. This was especially so in the diplococci which were similar in shape to neisseria and pediococci.

Strains varied in average size from about 0.7 to 1.2 μ with most strains 0.8 to 1.0 μ. Some cells of all strains contained iodine-staining material. Some cells of all strains showed refractile bodies when viewed with a phase-contrast microscope. The refractile bodies varied in size from minute to slightly smaller than the size of the cell. In gram-negative cells the bodies did not stain as deeply as the rest of the cell. It is possible that the iodine-staining intracellular material corresponded to the refractile bodies.

Some cultures of all strains showed many gram-positive cells but at other times cultures handled in the same way appeared to be gram-negative.

All strains were nonmotile.

Surface colonies formed after incubation for 3 days in rumen fluid-glucose-cellobiose agar were entire, smooth, and slightly convex. When viewed by transmitted light they were translucent to opaque and some colonies showed a fluorescent to "frosted-glass" appearance. The colonies were white to light tan in color with the exception of strain D2 which formed yellow colonies. Colonies of most strains were 2 to 4 mm in diameter. Deep colonies were invariably lenticular.

Growth in rumen fluid-cellobiose broth was evenly turbid in 18 to 24 hr.

All strains grew well at 37 and 30 C and none grew at 15 or 50 C. Only two strains grew at 22 C and three strains grew at 45 C.

The final pH in slightly buffered rumen fluid-cellobiose medium was 5.0 to 5.2 for 4 strains,

TABLE 2

Some variable characteristics of representative strains of cellulolytic cocci isolated from rumen contents of cattle

Strain	Group I					Group II							D157	B133
	FD1	D101	B1C45	C94	B146	B337	B1C7	B113	D89	B336	7	20		
Occurrence in chains	+	+	+	+	+	±	−	−	−	−	−	−	+	+
Yellow pigment	+	+	+	+	−	−	?	+	+	−	−	−	−	−
Growth at 22 C	−	−	−	−	−	−	−	−	−	+	+	−	−	−
Growth at 45 C	−	−	−	+	+	−	−	−	−	−	−	−	−	−
Voges-Proskauer test	+	w*	−	−	−	w*	+	+	+	−	+	+	−	−
Gelatin liquefaction	−	−	−	−	−	+	−	−	−	+	−	−	+	+
Gas-cellobiose agar	−	−	−	−	−	+	+	+	+	+	+	−	−	−
Final pH-cellobiose	5.3	5.4	5.4	5.6	5.0	5.5	5.5	5.3	5.3	5.1	5.4	5.5	5.6	5.5
Acid from:														
Glucose	−	−	+	−	−	+	−	−	−	−	+	+	−	−
D-Xylose	−	−	+	−	−	−	−	−	−	−	−	+	−	−
L-Arabinose	−	−	−	−	−	−	−	−	−	−	−	+	−	−
Fructose	−	−	−	−	−	−	−	−	−	−	−	+	+	−
Sucrose	−	−	−	−	−	+	−	−	−	−	+	−	−	−
Lactose	−	−	−	−	−	w	−	−	−	−	−	+	−	−
Xylan	+	+	+	+	+	+	+	+	+	−	+	+	+	+
Esculin	−	−	+	−	−	+	−	−	−	−	+	−	−	−
Products in rumen fluid-cellobiose medium, mM per 100 ml†														
Hydrogen	0	0	0	0	0	0.25	0.57	0.91	0.55	0.14	0.74	0	0	0
Ethanol	0	0	0	0	0.11	3.71	1.41	3.32	3.62	1.80	3.65	1.85	0.43	0.24
Acetic acid	2.38	1.90	2.18	2.32	2.30	1.14	1.36	2.70	2.22	0.66	3.46	1.38	0.46	0.22
Formic acid	3.12	1.72	2.50	2.72	2.66	1.90	0.80	3.90	3.12	0.56	3.74	3.14	0	0.46
Succinic acid	1.76	1.21	2.15	1.85	2.93	0	0.11	0	0	0	0.11	0	0	0
Lactic acid	0.14	0.14	0.10	0.52	0.32	0	0.28	0	0.12	0	0	0.34	0.64	3.02

* Refers to a weak reaction.

† No methane or propionic and butyric or longer-chained volatile fatty acids were detected.

5.3 to 5.5 for 19 strains, and 5.6 to 5.9 for 5 strains.

All of the strains were strict anaerobes and none produced indole, hydrogen sulfide, or catalase; hydrolyzed starch; or reduced nitrate.

In the Voges-Proskauer reaction 12 strains were positive, 3 strains were weakly positive, and 13 strains were negative.

Four strains liquefied gelatin.

All 28 strains produced acid from cellobiose. The number of strains that produced acid from other carbon sources was as follows: xylan, 24; glucose, 8; D-xylose, 5; esculin, 4; fructose, 3; sucrose, 3; lactose, 3; and L-arabinose, 2. None of the strains produced acid from maltose, glycerol, mannitol, dextrin, inulin, salicin, or gum arabic.

All strains produced reducing sugar when grown in medium containing excess cellulose. As noted above, 1 strain produced a colony with a yellow pigment. When grown in the liquid medium containing excess cellulose, 14 strains produced a definite yellow to orange pigment. Two strains produced what appeared to be a small amount of pigment and none was detected in the other 12 strains.

With two exceptions, the strains could be grouped as chain-formers and non-chain-formers. However, this characteristic could not be well correlated with other characteristics listed above so that well differentiated groups could be defined. Fourteen strains were selected for further study. The characteristics of these 14 strains are shown in table 2.

On the basis of fermentation products, three main groups occurred. One group of five strains produced mainly acetic, formic and succinic acids, and little or no ethanol or gas. A second group of 6 strains produced mainly acetic and formic acids, ethanol, and gas which included hydrogen. Strain 20 belonged to this group except that gas was not detected. Two strains did not fit these groups. Strain $B_1$33 produced mainly lactic acid (86 per cent of the carbon of products recovered) with small amounts of ethanol, and acetic and formic acids. Strain D157 produced lactic acid (51 per cent of the carbon) and ethanol and acetic acid but only small amounts of products were recovered.

It is possible that the strains that were negative for gas production in cellobiose agar produced some noncombustible gas such as carbon dioxide. These organisms grew very poorly in this medium, undoubtedly because of the low level of carbon dioxide and bicarbonate in the medium. The only addition of these substances in this medium was from the inoculum (0.2 ml) which came from rumen fluid-cellobiose medium with 0.4 per cent of Na_2CO_3 and a carbon dioxide gaseous phase.

The medium in which specific products were determined contained Na_2CO_3 and the carbon dioxide gaseous phase so that determination of carbon dioxide produced or used could not be determined. The fact that the products recovered from the first group were highly oxidized compared to cellobiose and the products recovered from the second group were highly reduced suggests that the first group may have fixed carbon dioxide and the second group may have produced carbon dioxide in addition to hydrogen. The oxidation-reduction balances of products recovered from strain $B_1$33 (0.96) and strain 20 (0.85) were much closer to that of cellobiose than other strains.

Cillobacterium cellulosolvens n. sp. Type strain $B_3$48 is a motile coccoid to lancet to rod-shaped bacterium with pointed ends and is 0.5 to 0.7 μ wide by 1 to 2 μ long. When freshly isolated it appeared more coccoid than later. It was gram-positive but weakly so, as shown by the fact that many gram-negative and gram-variable cells were present in most smears. The gram-variable cells showed gram-positive ends with a bipolar effect. Some chains of about 8 to 10 cells were usually present. Many cells showed single flagella but some cells with 3 or 4 peritrichous flagella were found. The cells contained iodine-staining material.

Surface colonies were entire, translucent, flat to slightly convex, and light tan in color. They were 3 to 5 mm in diameter. Deep colonies were lenticular.

Growth in liquid cellobiose medium was heavily and evenly turbid.

Good growth occurred at 30 to 37 C but no growth occurred at 45 or 22 C.

Good growth occurred in cellobiose medium in which trypticase and yeast extract replaced rumen fluid.

The final pH in cellobiose medium was 4.8.

The organism was a strict anaerobe. It would not grow in media in which resazurin was oxidized.

It did not produce indole, H_2S, or catalase.

Starch was not hydrolyzed.

Nitrate was not reduced.

The Voges-Proskauer reaction was negative.

Gelatin was not liquefied.

It fermented glucose, cellobiose, maltose, sucrose, fructose, inulin, salicin, esculin, and cellulose but not D-xylose, L-arabinose, lactose, glycerol, mannitol, dextrin, gum arabic, xylan, or lactate.

It did not form gas splits in cellobiose agar shake cultures.

Reducing sugar was formed in medium containing excess cellulose but no pigment was formed on this medium.

Fermentation products recovered from cellobiose medium were lactic, acetic, and formic acids in amounts of 6.42, 0.42, and 0.26 mm per 100 ml of medium, respectively. No butyrate, propionate, succinate, hydrogen, or ethanol were detected. The oxidation-reduction balance of the products recovered plus the fact that no gas splits occurred in cellobiose agar suggests that no carbon dioxide was produced.

DISCUSSION

During the course of the present study, Hungate (1957) reported the characteristics of seven strains of cellulolytic cocci isolated from cows and three isolated from sheep. On the basis of the characteristics of these strains and those reported by other workers, it was concluded that all cellulolytic cocci described should be placed in one genus. The description of the genus *Ruminococcus* Sijpesteijn was broadened to include gram-negative or variable cocci that ferment carbohydrate

to form acetate, at least traces of hydrogen, and various combinations of ethanol, formate, lactate, and succinate. Other characteristics of the genus are that it includes nonmotile, nonspore-forming, strict anaerobes that ferment cellulose

All of the strains of the present study fit the genus *Ruminococcus* (Sijpesteijn) Hungate except for the fact that hydrogen could not be detected in many of the cultures even though very good growth occurred in the highly buffered medium. Also, no gas splits could be detected in agar shake tubes. It is believed that the description of this genus should be amended to include organisms that do not produce gas.

It is evident that most strains of the genus *Ruminococcus* can be included in the genus *Peptostreptococcus* (Kluyver and van Niel) Smith (Breed *et al.*, 1957). However, this genus includes species of such diverse characteristics that it is probable that when more detailed comparisons of species are made under comparable conditions some species will be placed in new genera or subgenera.

On the basis of the occurrence of chains, production of hydrogen and the amount of ethanol and succinic acid produced, 12 of the 14 cultures studied in detail could be placed in two groups as shown in table 2. They differed in that group I produced large amounts of succinic acid and group II produced little or none. The reverse was true with ethanol production. Group I always formed long chains and group II, with the exception of one strain which showed some short chains, occurred as singles and diplococci. Group I did not produce gas and group II, with the exception of one strain, produced gas that included hydrogen.

All group I strains, in our opinion, should be included in the species *R. flavefaciens* Sijpesteijn on the basis of the fermentation of cellulose and cellobiose and production of a large amount of succinic acid. The description of the species by Sijpesteijn (1951) would exclude strain $B_1$46 because the yellow pigment was not detected. However, both Hall (1952) and Hungate (1957) studied strains that fit Sijpesteijn's description except for pigment, and Hungate concluded that these strains should be included in the species.

R. flavefaciens of Sijpesteijn fermented cellulose, cellobiose, varied in glucose fermentation, and did not ferment maltose, lactose, xylose, or starch. Strain B_1C45 would also be excluded from the species on this basis. However, Hungate included one strain in the species that fermented lactose and one that fermented sucrose weakly. It seems best to include organisms that ferment a few other substances in this species. On the basis of strains studied to date (Sijpesteijn, 1951; Hall, 1952; and Hungate, 1957) and the present group I, the carbohydrates fermented by the species should be as follows: cellulose, cellobiose, and, usually, xylan are fermented; glucose, lactose, D-xylose, sucrose, and esculin are usually not fermented; maltose, L-arabinose, D-arabinose, mannose, fructose, L-xylose, galactose, raffinose, inulin, trehalose, gum arabic, salicin, rhamnose, dextrin, mannitol, glycerol, sorbitol, dulcitol, and inositol are not fermented.

In addition to variations in pigment production and carbohydrates fermented, the species varies in growth at 45 C, the Voges-Proskauer reaction and production of hydrogen, carbon dioxide, and ethanol.

The species seems to be constant in being iodophilic; growing at 30 or 32 C; failing to grow at 22 C; not producing indole or H_2S, reducing NO_3 or liquefying gelatin; producing reducing sugars when grown in excess cellulose; and producing succinic and acetic acids and at least traces of formic and lactic acids from carbohydrate.

Group II strains shown in table 2 undoubtedly belong to the species *Ruminococcus albus* Hungate (1957). They fit the description of the species in all characteristics except that two strains produced a very small amount of succinic acid and Hungate stated that no succinic acid was produced.

The group II strains are similar to the five strains of Hungate in the following characteristics. Both groups were composed of cocci that usually occurred as singles and in twos but included strains that formed chains. Both groups included some strains that produced yellow pigment. Both groups produced ethanol, acetic acid, and formic acid with the exception of one strain of Hungate, and hydrogen with the exception of one strain of group II. Both groups fermented cellobiose and some strains in both groups fermented L-arabinose, lactose, sucrose, and fructose. None of either group fermented inulin, salicin, or starch.

Strains of *R. albus* of Hungate and group II seem to differ somewhat in the following characteristics. All of the group II strains produced intracellular iodine-staining material. *R. albus*

Hungate (1957) usually does not. The colorless cocci (Hungate 1947, 1950) some of which are included in the species *R. albus* did produce this material. *R. albus* produced no succinic acid and three strains produced relatively large amounts of lactic acid while the group II strains produced little or none of either of these acids.

The strains of *R. albus* Hungate did not ferment glucose, D-xylose, or esculin and one strain weakly fermented maltose whereas three of the group II strains fermented glucose, one fermented D-xylose, two fermented esculin, and none fermented maltose.

On the basis of the characteristics of the group II cocci, the description of the species *R. albus* Hungate (1957) should be amended to include organisms that produce a small amount of succinic acid. Xylan is usually fermented. Yellow pigment may or may not be produced and strains may or may not be iodophilic. Some strains grow at 22 C but none grow at 45 C. The Voges-Proskauer test is usually positive and some strains liquefy gelatin.

Strains D157 and $B_1$33 shown in table 2 do not fit well into either species of the genus *Ruminococcus*. They seem to be more closely related to *R. flavefaciens* in forming long chains, being negative in the Voges-Proskauer reaction, and in failing to produce hydrogen. However, they seem to be more closely related to *R. albus* in their failure to produce yellow pigment, in liquefying gelatin, and in producing no succinic acid. They differ from all other strains in the present study in that lactic acid was the most prominent fermentation product recovered.

Hungate (1957) included strains producing large amounts of lactic acid in both species of the genus. Only one of these, strain A, was considered a variety of *R. flavefaciens* because a trace of succinic acid was detected and yellow pigment and chains were produced. Three of five strains placed in the species *R. albus* produced lactic acid in larger amounts than any other fermentation product. Strains U and 46-1 appear to fit the species quite well in other characteristics but strain 56-2 produced long chains and yellow pigment. Another strain was excluded from both species. The reasoning for excluding it seems to be that it produced yellow pigment but only short chains and only a small amount of acetic acid. The fact that no succinic acid was produced definitely excluded it from *R. flavefaciens*.

It seems best to exclude strains 157 and $B_1$33 of the present study from either species of the genus *Ruminococcus*.

Hungate (1950, 1957) stated that the nutrient requirements of the species *R. albus* were not met by usual ingredients of bacteriological culture media but could be met by rumen fluid, extracts of feces or other media containing metabolic products of other microorganisms.

Recently, Allison *et al.* (1958) showed that strains of the present study, C94, FD1, and $B_1$46 of *R. flavefaciens*, and 7 and 20 of *R. albus* required, or were greatly stimulated by, the volatile acid fraction of rumen fluid or volatile fatty acids similar to those present in rumen fluid when grown in a medium containing B-vitamins, acid hydrolyzed casein, minerals, cellobiose, and Tween 80. Good growth was obtained with only isovaleric and acetic acids added. More detailed work with strain C94 indicated that isovaleric or isobutyric acids were required for growth whereas acetate shortened the lag phase of growth.

The observation of Sijpesteijn (1951), that sterile filtrates of *Clostridium sporogenes* cultures stimulated growth of *R. flavefaciens*, suggests that volatile fatty acids may have been a growth factor for her strains. It is well known that *C. sporogenes* produces volatile fatty acids, including the branched-chain acids, from amino acids.

These studies suggest that members of the two species of ruminococci are closely related in nutritional requirements. However, Fletcher (1956), working with the type strain 69 of *R. albus*, purified a growth factor from rumen fluid which appeared to be yellow colored, nonvolatile, carboxylic acids with neutralization equivalents of about 223. Chromatographic analysis suggested that two similar acids were involved. The results of Allison *et al.* (1958) and Fletcher (1956) indicate that individual strains of the species *R. albus* may have quite different growth requirements.

The fermentation of xylan by 24 of the 28 strains of cellulolytic cocci studied further stresses the importance of these bacteria in ruminant digestion. Many workers have shown that xylans or pentosans that appear to be mainly xylan are quantitatively among the more important constituents of ruminant feeds and are readily digested in the rumen (Heald, 1953).

As xylan, but usually not xylose, is fermented by most strains, it is suggested that a situation analogous to that with cellulose and its hydrolytic

products occurs. Cellulose and cellobiose are fermented but usually not glucose. It would be interesting to determine if xylobiose is fermented by these organisms. Howard (1955) showed that this disaccharide is an intermediate in the fermentation of xylan by the ruminal flora.

The present study substantiates the observation of Hungate (1947) that there is considerable variability in the characteristics between strains of cellulolytic cocci. It is of interest that widely different strains were isolated from the same sample of rumen contents. For example, $B_1$13 is *R. albus*, $B_1$46 is *R. flavefaciens*; and $B_1$33 does not fit either species. Also, very similar strains were isolated from widely differing samples of rumen contents. There seems to be no correlation between characteristics of cellulolytic cocci isolated and the ration consumed by the animal.

The characteristics of type strain $B_3$48 of *Cillobacterium cellulosolvens* do not fit well into any known genus of bacteria. The organism has some similarities to the genus *Cellulomonas* (Bergey *et al.*) Clark (Breed *et al.*, 1957) in being cellulolytic, weakly gram-positive, rather small rods, with a single or a few peritrichous flagella, and producing acid but no gas from carbohydrates. However, *Cellulomonas* species do not have pointed ends and are catalase-positive, facultative anaerobes that liquefy gelatin and grow well at room temperature.

The strain also has some similarities to motile species of the genus *Brevibacterium* Breed (Breed *et al.*, 1957) but differs from all of these in being a cellulolytic, strict anaerobe. It differs from most species in its failure to produce a pigment and from some in having peritrichous flagella.

Being a motile, peritrichous, gram-positive, strict anerobe, the most obvious genus is *Cillobacterium* Prevot (Breed *et al.*, 1957). Strain $B_3$48 differs from all species of this genus as the latter produce gas and usually, butyric acid and protein decomposition products among their fermentation products whereas the strain seems to have a homofermentative lactic acid fermentation. Ninety-four per cent of the carbon of the products recovered was lactic acid.

This strain is provisionally placed in the genus *Cillobacterium* although placing it in this genus may place too much emphasis on its morphology and character of being a strict anaerobe and not enough emphasis on fermentation products. The products suggest that this organism is more closely related to other genera of the family *Lactobacillaceae* than presently recognized species of *Cillobacterium*.

The facts that only one strain has been found among the more numerous bacteria grown from rumen contents using the nonselective rumen fluid-glucose-cellobiose agar and that other workers have not isolated similar bacteria using relatively selective cellulose media (Hungate, 1950, 1957; Sijpesteijn, 1951) suggest that this bacterium is not an important ruminal species. However, a similar strain was isolated but lost before a detailed study of characteristics could be made and it is possible that under conditions as yet not studied this organism could be among the more numerous ruminal species.

SUMMARY

A study was made of the characteristics of 28 strains of cellulolytic cocci isolated from rumen contents of five different cows fed five different rations. All strains were strictly anaerobic, nonmotile, iodophilic, produced reducing sugar from excess cellulose, and fermented cellobiose. None of the strains produced indole, hydrogen sulfide, and catalase; hydrolyzed starch; reduced nitrate; fermented maltose, glycerol, mannitol, dextrin, inulin, salicin, and gum arabic. There was variation between strains in many characteristics including chain formation (16 strains formed long chains and 10 did not), production of yellow pigment (14 positive), Voges-Proskauer reaction (15 positive), gelatin liquefaction (4 positive), acid from xylan (24 positive), glucose (8 positive), D-xylose (5 positive), esculin (4 positive), fructose (3 positive), sucrose (3 positive), lactose (3 positive), and L-arabinose (2 positive), and final pH (5.0 to 5.9) in cellobiose medium. These characteristics could not be used to separate the strains into well defined species.

When fermentation products from cellobiose were determined on 14 representative strains, 12 of the cultures were placed in 2 groups. Five strains, placed in the species *Ruminococcus flavefaciens* Sijpesteijn, formed long chains and produced mainly succinic, acetic, and formic acids. Seven strains, placed in the species *Ruminococcus albus* Hungate, usually occurred as singles and diplococci and produced mainly acetic and formic acids, ethanol, and, usually, gas that included hydrogen. Two strains producing a large

amount of lactic acid did not fit well into either group. There seemed to be no correlation between the characteristics of cellulolytic cocci isolated and the sample of rumen contents from which they were obtained.

Cillobacterium cellulosolvens n. sp. is a new species of anaerobic, cellulolytic, gram-positive, peritrichous rod with pointed ends that produces predominantly lactic acid in rumen fluid-cellobiose medium. As far as is known, it seems to be relatively unimportant in the rumen under conditions so far studied.

REFERENCES

ALLISON, M. J., BRYANT, M. P., AND DOETSCH, R. N. 1958 A volatile fatty acid growth factor for cellulolytic cocci of the bovine rumen. Science, **128,** 474–475.

BAKER, F. AND HARRISS, S. T. 1947 Microbial digestion in the rumen (and caecum), with special reference to the decomposition of structural cellulose. Nutrition Abstr. & Revs., **17,** 3–12.

BREED, R. S., MURRAY, E. G. D., AND SMITH, N. R. 1957 *Bergey's manual of determinative bacteriology,* 7th ed. The Williams & Wilkins Co., Baltimore.

BRYANT, M. P. AND BURKEY, L. A. 1953*a* Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci., **36,** 205–217.

BRYANT, M. P. AND BURKEY, L. A. 1953*b* Numbers and some predominant groups of bacteria in the rumen of cows fed different rations. J. Dairy Sci., **36,** 218–224.

BRYANT, M. P. AND DOETSCH, R. N. 1954 A study of actively cellulolytic rod-shaped bacteria of the bovine rumen. J. Dairy Sci., **37,** 1176–1183.

BRYANT, M. P. AND SMALL, N. 1956 The anaerobic monotrichous butyric acid-producing curved rod-shaped bacteria of the rumen. J. Bacteriol., **72,** 16–21.

BRYANT, M. P., SMALL, N., BOUMA, C., AND CHU, H. 1958 *Bacteroides ruminicola* n. sp. and *Succinimonas amylolytica* n. gen., succinic acid producing bacteria of the bovine rumen. J. Bacteriol., **76,** 15–23.

FLETCHER, D. W. 1956 Studies on the growth requirements of a cellulolytic coccus from the bovine rumen. Thesis, State College of Washington, Pullman.

GALL, L. S., STARK, C. N., AND LOOSLI, J. K. 1947 The isolation and preliminary study of some of the physiological characteristics of the predominating flora from the rumen of cattle and sheep. J. Dairy Sci., **30,** 891–899.

HALL, E. R. 1952 Investigations on the microbiology of cellulose utilization in domestic rabbits. J. Gen. Microbiol., **7,** 350–357.

HEALD, P. J. 1953 The fermentation of xylans in the rumen of the sheep. Brit. J. Nutrition, **7,** 124–130.

HOWARD, B. H. 1955 Ruminal fermentation of pentosan. Biochem. J. (London), **60,** i.

HUNGATE, R. E. 1947 Studies on cellulose fermentation III. The culture and isolation of cellulose-decomposing bacteria from the rumen of cattle. J. Bacteriol., **53,** 631–645.

HUNGATE, R. E. 1950 The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Revs., **14,** 1–49.

HUNGATE, R. E. 1957 Microorganisms in the rumen of cattle fed a constant ration. Can. J. Microbiol., **3,** 289–311.

KING, K. W. AND SMITH, P. H. 1955 Comparisons of two media proposed for the isolation of bacteria from the rumen. J. Bacteriol., **70,** 726–729.

KITTS, W. D., CARR, P. H., AND UNDERKOFLER, L. A. 1954 Isolation of cellulose-digesting microorganisms of the rumen. Iowa State Coll. J. Sci., **29,** 129–131.

MAKI, L. R. 1955 Characteristics of rumen bacteria isolated from cattle fed high and low roughage rations. Thesis, University of Wisconsin.

SIJPESTEIJN, A. K. 1948 Cellulose-decomposing bacteria from the rumen of cattle. Thesis, Leiden University, Holland.

SIJPESTEIJN, A. K. 1951 On *Ruminococcus flavefaciens,* a cellulose decomposing bacterium from the rumen of sheep and cattle. J. Gen. Microbiol., **5,** 869–879.