APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1982, p. 686–693
0099-2240/82/030686-08$02.00/0

Vol. 43, No. 3

Physiological Diversity of Rumen Spirochetes

B. J. PASTER† AND E. CANALE-PAROLA*

Department of Microbiology, University of Massachusetts, Amherst, Massachusetts 01003

Received 31 August 1981/Accepted 28 November 1981

Bovine rumen fluid contained relatively large numbers of spirochetes capable of fermenting polymers commonly present in plant materials. Polymers such as xylan, pectin, and arabinogalactan served as fermentable substrates for the spirochetes, whereas cellulose did not. Furthermore, spirochetes cultured from rumen fluid utilized as growth substrates hydrolysis products of plant polymers (e.g., D-xylose, L-arabinose, D-galacturonic acid, D-glucuronic acid, cellobiose), but did not ferment amino acids. Viable cell counts of spirochetes capable of fermenting individual plant polymers or their hydrolysis products yielded minimum values ranging from 0.2×10^6 to 4×10^6 cells per ml of rumen fluid. Thirteen strains of rumen spirochetes were characterized in terms of their fermentation products from glucose, the guanine plus cytosine content of their DNA, their ultrastructure, and their ability to ferment pectin, starch, or arabinogalactan. Of the 13 strains, 6 fermented glucose mainly to formate, acetate, and succinate, whereas the remaining 7 strains did not produce succinate, but instead formed ethanol, in addition to formate and acetate. The succinate-forming strains had two periplasmic (axial) fibrils per cell, measured 0.2 to 0.3 by 5 to 8 μm, had a guanine plus cytosine content of the DNA ranging from 36 to 38 mol%, and lacked the ability to ferment pectin, starch, or arabinogalactan. The ethanol-forming strains had from 8 to more than 32 periplasmic fibrils per cell, tended to be larger in cell size than the succinate-forming strains, and had a guanine plus cytosine content of the DNA ranging from 41 to 54 mol%. Some of the ethanol-forming strains fermented pectin, starch, or arabinogalactan. The results of this study indicate that the bovine rumen is inhabited by a physiologically and morphologically diverse population of spirochetes. It is likely that these spirochetes contribute significantly to the degradation of plant materials ingested by the ruminants.

Recent studies have shown that large numbers of morphologically different spirochetes are present in the bovine rumen (16). However, only a limited amount of information is available on the metabolic activities of these spirochetes. Stanton and Canale-Parola have isolated and described a species of rumen spirochetes that interacts with cellulolytic bacteria (17). This species, named *Treponema bryantii*, does not break down cellulose, but utilizes glucose and cellobiose released into the external environment by the metabolic activities of rumen bacteria that degrade cellulose (17). Furthermore, it has been shown that other rumen spirochetes are capable of catabolizing pectin (19, 20).

One of the major objectives of the studies we report here was to determine whether spirochetes contribute significantly to the breakdown of various polymers present in the plant materials ingested by ruminants and of other organic molecules commonly found in the rumen. Thus, we enumerated spirochetes present in bovine rumen fluid that were capable of fermenting selected plant polymers and the hydrolysis products of such polymers. Polymers used in the enumeration studies included pectin, xylan, arabinogalactan, and cellulose. In addition, we isolated strains of different rumen spirochetes and characterized them with regard to key physiological properties, their ultrastructure, and the guanine plus cytosine (G+C) content of their DNA.

Our investigations were aimed at obtaining a greater understanding of (i) the extent of physiological diversity that occurs among rumen spirochetes, (ii) the roles played by spirochetes in the complex metabolic processes that occur in the rumen, and (iii) physiological interactions between spirochetes and other rumen bacteria.

MATERIALS AND METHODS

Bacterial strains and culture media. Strains of rumen spirochetes characterized in this study included: (i) seven strains (GL, GP, XA, PA, LB, LU, and PB) which were isolated during this investigation; (ii) strain 606 isolated by Ziolecki (19); and (iii) six strains (6M,

† Present address: Department of Dairy Science, University of Illinois, Urbana, IL 61801.

6A, 8G, CA, DA, and *T. bryantii* RUS-1) isolated previously by Stanton and Canale-Parola (16, 17).

The strains listed above, except for strain 606, were cultured in medium RFG and medium MVTY. The composition of medium RFG, a rumen fluid-containing medium, was described by Stanton and Canale-Parola (17). Medium MVTY contained a supplement of fatty acids, yeast extract, and Trypticase Peptone (BBL Microbiology Systems, Cockeysville, Md.) instead of rumen fluid. This medium was identical to medium VTY described previously (17), except that it did not contain acetic and propionic acids. Other cultural conditions were identical to those described by Stanton and Canale-Parola (16). Strain 606 did not grow in RFG or MVTY media and was cultured in a pectin-containing medium described by Tomerska (18).

In experiments involving enumeration of spirochetes, a modified medium MVTY was used. This medium, called enumeration medium, differed from medium MVTY because it contained 0.02 g of yeast extract per 100 ml of medium (instead of 0.2 g/100 ml).

Media were solidified by adding 0.7 g of agar per 100 ml of medium. Agar media (RFG, MVTY, and enumeration medium), to which potential growth substrates (0.2 g per 100 ml of medium) had been added in place of glucose, were used in the isolation and enumeration of rumen spirochetes. In most of the isolation and enumeration experiments, rifampin (1 μg per ml of medium) (16) was added to these media. Rifampin served as a selective agent because the growth of rumen spirochetes is not inhibited by concentrations of this antibiotic that inhibit the growth of many other rumen bacteria (16). Substrates tested in these experiments included Casamino Acids, ball-milled cellulose (17), carboxymethyl cellulose (sodium salt, low viscosity; Sigma Chemical Co., St. Louis, Mo.), pectin (polygalacturonic acid methyl ester, grade I; Sigma Chemical Co.), polygalacturonic acid, xylan, arabinogalactan, D-xylose, L-arabinose, L-rhamnose, D-galactose, D-glucuronic acid, D-galacturonic acid, cellobiose, and glucose. D-Glucuronic acid and D-galacturonic acid solutions (adjusted to pH 7.0 with 2 N KOH) were filter-sterilized and then were added aseptically to the media before inoculation. Pectin, polygalacturonic acid, and xylan were added to agar media before autoclaving. Solutions of the other substrates tested were sterilized separately by autoclaving and then were added aseptically to agar media before inoculation. Pectin, polygalacturonic acid, xylan, arabinogalactan, and starch preparations used in this study were washed three times in 70% ethyl alcohol or dialyzed against distilled water before addition to media to remove contaminating low-molecular-weight sugars. Growth substrates used in the isolation media were: pectin for strains GP, PA, and PB; D-xylose for strain XA; D-glucuronic acid for strains GL, LB, and LU. However, strains GP and PA were not pectinolytic, and strain GL did not utilize D-glucuronic acid as a growth substrate.

Rumen fluid samples. Rumen fluid samples for viable counts and for isolation of spirochetes were obtained from four fistulated cows housed at the University of Massachusetts Dairy Facility, South Deerfield, Mass. These cows (Holstein breed) were fed a mixed diet of hay and corn silage. Rumen fluid samples were collected at random times, i.e., without consideration of the cows' feeding times.

Methods used for collecting and transporting rumen fluid samples were described earlier (16). Rumen fluid is defined as described previously (16).

Viable cell counts. Viable cell counts were performed to determine the numbers of spirochetes in rumen fluid that were capable of fermenting plant polymers, the hydrolysis products of these polymers, and other compounds. Viable cell counts of other bacteria were also determined. Rumen fluid samples were serially diluted (30 to 60 min after collection) into agar deeps of enumeration medium or RFG medium to which potential substrates were added in place of glucose. In most experiments, rifampin (1 μg per ml of medium) was added as a selective agent. After 24 to 72 h of incubation at 39°C, the colonies that developed in these agar media were counted. Spirochete colonies were readily recognizable because of their characteristic spreading appearance, which results from the ability of spirochete cells to migrate through agar media (4). The different morphological types of spirochete colonies that were observed are described below.

Agar medium test tube cultures (deeps), each containing approximately 10 to 60 spirochete colonies, were used to estimate the numbers of viable spirochetes in the rumen fluid samples.

Not all colonies that had the appearance of spirochete colonies consisted of spirochetes. Thus, when performing viable counts, it was necessary to determine whether the colonies were indeed spirochete colonies. This was accomplished by examining the cells in each colony by light microscopy when the agar deep cultures contained 30 or fewer spirochete-like colonies. When the cultures contained more than 30 of these colonies, an estimation of the number of spirochetes was performed, as described by Stanton and Canale-Parola (16).

Selective isolation of rumen spirochetes. Rumen spirochetes were isolated as follows. Rumen fluid samples were serially diluted into deeps of MVTY or RFG agar media that contained pectin, xylose, or glucuronic acid instead of glucose as growth substrate. Rifampin (1 μg per ml of medium) was added to the medium as a selective agent. Spirochetes were isolated and cloned from the colonies that developed in these media by methods described earlier (16). The isolates were cultured routinely in MVTY or RFG agar media.

To determine whether the isolates were saccharolytic, we measured growth in MVTY broth media with and without the addition of carbohydrate. Spirochete cell yields were determined by means of direct counts in a Petroff-Hausser counting chamber. Counts were performed after three transfers in any given liquid medium.

Pectinolytic, cellulolytic, amylolytic, and arabinogalactan-hydrolyzing activities. Pectinolytic activity was determined by testing the ability of growing spirochete cells to liquefy pectin (1.0 g per 100 ml of medium) present in RFG or MVTY broth from which glucose had been omitted. Cellulolytic or arabinogalactan-hydrolyzing activities were determined by measuring the growth of spirochetes in MVTY broth that contained ball-milled cellulose or larchwood arabinogalactan, respectively (0.2 g per 100 ml of medium) instead of glucose as growth substrate. Furthermore, cellulolytic activity was determined by observing the disappearance of the cellulose from the medium. Larchwood arabinogalactan (a gift from A. A. Salyers) was

used in this investigation primarily because it has a structure comparable to that of many other polysaccharides present in plant cell walls (15). Starch hydrolysis was determined by adding Lugol's iodine solution to 24-h stab cultures in enumeration medium to which soluble starch (0.2 g per 100 ml of medium) was added in place of glucose. A clear zone around the line of growth indicated starch hydrolysis (amylolytic activity).

Analysis of end products of glucose fermentation. For qualitative analysis of fermentation end products, spirochete strains were grown in MVTY broth medium. Formate was assayed colorimetrically as described by Lang and Lang (12). Other nongaseous fermentation end products were determined by gas-liquid chromatography with a Varian 3700 gas chromatograph (Varian Instrument Division, Palo Alto, Calif.) equipped with a flame ionization detector. Methylation of nonvolatile fatty acids and ethyl ether extraction of volatile fatty acids and alcohols for gas-liquid chromatography were performed as described by Holdeman et al. (7). Products were separated on a glass column (6 ft [ca. 1.83 m] by ¼ in [ca. 0.635 cm] outer diameter) packed with SP (Supelco Phase)-1000-H_3PO_4 (10/1%) on 100/200 Chromosorb WAW (Supelco, Inc., Bellefonte, Pa.). Detector and injector temperatures were 200°C. Sample injection volumes were 5 μl, and carrier gas (N_2) flow rate was 30 ml/min. Nonvolatile fatty acids were separated isothermally at 120°C. In the separations of volatile fatty acids and alcohols, the oven temperature was maintained at 70°C after sample injection and was increased after 7 min to 130°C at a rate of 44°C per min.

Uninoculated MVTY broth was analyzed to determine the background level of fermentation products.

G+C content of the DNA. The G+C content of the DNA of rumen spirochetes was determined by thermal denaturation analysis as described by Breznak and Canale-Parola (2) and calculated by using the equation of DeLey (5). DNA isolated from *Escherichia coli* K-12 was used as a control

Electron microscopy. Samples for scanning electron microscopy were prepared as follows. One-milliliter samples of rumen fluid were centrifuged at 3,000 × *g* for 10 min, and the resulting pellets were fixed in freshly prepared 2% (wt/vol) glutaraldehyde in 0.2 M phosphate buffer (pH 7.0) for 2 h at 5°C. The fixed samples were washed three times in buffer and then were suspended in 1 ml of buffer. Samples of the suspension (0.2 ml) were filtered through Nuclepore filter disks (pore diameter, 0.1 μm; disk diameter, 13 mm). The filters, impregnated with cells and debris, were then dehydrated through a graded ethyl alcohol series consisting of 20, 50, 75, 95, and 100% ethyl alcohol for 10 min, twice at each concentration. Samples were immediately critical-point dried, mounted on copper stubs, and vacuum coated as previously described (9).

Negatively stained specimens for electron microscopy were prepared as follows. Spirochetes were cultured to late logarithmic growth phase in MVTY or RFG broth. A drop of culture was placed on a Formvar-coated, carbon-reinforced copper grid (300 mesh), and the excess fluid was drawn off with tissue paper after 90 s. Then the cells on the grid were stained with 1% (wt/vol) phosphotungstic acid (pH 6.5) for 10 to 15 s.

Thin sections of spirochetes were prepared by a modification of the method of Sabatini et al. (14). Five-milliliter cultures were centrifuged as described above, and the cell pellets were fixed in 1 ml of 2% (wt/vol) glutaraldehyde in Millonig's buffer (pH 7.2) for 1 h at 5°C. The fixed cells were washed five times in Millonig's buffer (pH 7.2) by centrifugation and then were fixed in 1% (wt/vol) osmium tetroxide in buffer for 1.5 h at 5°C. After the fixed cells were washed three times in buffer, the samples were embedded in Noble agar (2 g per 100 ml of Millonig's buffer) (8). The agar gel was cut into blocks, and the specimens were dehydrated through a graded ethyl alcohol series as described above. The samples were impregnated with propylene oxide and with Epon 812 as described by Holt and Leadbetter (8). Ultrathin sections were cut with a Sorvall Porter Blum MT-2 Ultramicrotome and stained for 10 min with 2.5% (wt/vol) uranyl acetate and then for 10 min with Reynolds' lead citrate solution.

Some samples were examined with a Philips EM 200 transmission electron microscope operating at 60 kV and photographed on Kodak fine-grain release positive film 5302. Other samples were examined with a JEOL 100S transmission electron microscope operating at 80 kV and photographed on Kodak electron image film 4463 photograph emulsion. Liquid nitrogen cold fingers at the level of the specimen were used to reduce contamination. Samples for scanning electron microscopy were examined with a JEOL 25S scanning electron microscope operating at 15 kV and photographed on Polaroid type 665 positive-negative Land film.

RESULTS

Enumeration of rumen spirochetes. Substrate concentration had a marked effect on the morphology of the spirochete colonies that developed in agar medium deeps. For example, when an individual strain of rumen spirochetes was inoculated in enumeration medium to which no growth substrate had been added, the colonies that developed were spherical and almost transparent (Fig. 1a). Growth of the cells in these colonies was supported by substrates present at very low concentrations in some of the complex constituents of the medium (e.g., yeast extract or agar). When a growth substrate was added at a low final concentration (e.g., 0.02% [wt/vol]), the spirochetes formed smaller, less transparent colonies that had well-defined edges (Fig. 1b). At higher substrate concentrations (e.g., 0.2% [wt/vol]), the colonies that developed had a denser center and diffuse edges ("cotton-ball-like" colonies [16]; Fig. 1c). The cells in these colonies utilized for growth the substrate being tested and thus attained relatively high population densities within the colonies.

When determining the numbers of spirochetes in rumen fluid that were capable of utilizing a given substrate, the final concentration of the potential growth substrate added to the enumeration medium was 0.2% (wt/vol). When the enumeration medium containing this concentration of substrate was inoculated with rumen

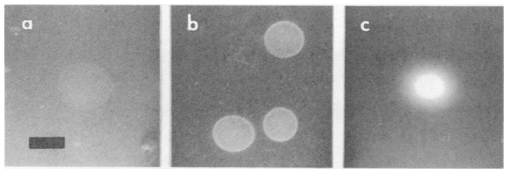

FIG. 1. Photographs of subsurface colonies of rumen spirochete strain PB grown for 24 h in enumeration media at three different substrate concentrations. (a) No glucose; (b) 0.02% glucose; (c) 0.2% glucose. All micrographs are at the same magnification. Bar = 2 mm. Colonies were photographed on Kodak Tri-X film at ASA rating 400. Exposures were made by dark-field illumination in a Leica R3 camera equipped with a macro 60 Elmarit lens.

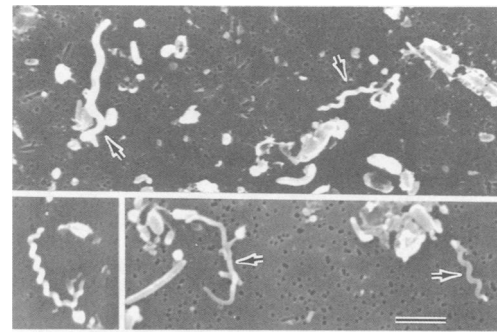

FIG. 2. Scanning electron micrographs of a rumen fluid sample demonstrating various morphological types of rumen spirochetes. Most of the spirochetes are indicated by the arrows. All micrographs are at the same magnification. Bar = 2 μm.

fluid, usually all three types of spirochete colonies (Fig. 1a through c), as well as other bacterial colonies, developed. However, of the three types of spirochete colonies present in the agar medium deeps, only the cotton-ball-like colonies (Fig. 1c) were counted. In addition to the cotton-ball-like colonies, we counted another type of spirochete colony that developed in enumeration medium to which either pectin or polygalacturonic acid was added as growth substrate. These colonies, formed by cells utilizing pectin or polygalacturonic acid for growth, were similar in appearance to those shown in Fig. 1b, but had a much denser central portion.

Spirochetes capable of utilizing amino acids or cellulose as fermentable substrates were not detected in rumen fluid (Table 1). On the other hand, relatively high population densities (0.2×10^6 to 4×10^6 cells per ml of rumen fluid) of spirochetes that fermented xylan, pectin, arabinogalactan, as well as hydrolysis products of these polysaccharides, were present in the rumen fluid samples that we tested. Furthermore, as reported previously (16), products of cellulose hydrolysis, such as cellobiose and glucose, supported the growth of relatively large numbers of rumen spirochetes (Table 1).

When media that contained rumen fluid (e.g., medium RFG) were used for the enumeration of rumen spirochetes capable of utilizing individual growth substrates, it was difficult to differentiate colonies on the basis of their morphology because the media were turbid as a result of the presence of rumen fluid. However, rough estimates of the number of spirochete colonies growing in these rumen fluid-containing media yielded viable count values two to five times higher than viable count values obtained with the enumeration medium. These higher viable counts probably resulted because rumen fluid contained growth factors that allowed the growth of spirochetes that were not able to grow in the enumeration medium. Regardless of the medium employed, spirochetes represented from 0.3 to 15% of the total number of rumen bacteria that grew under the experimental conditions used.

Viable counts of spirochetes varied by as much as twofold from sample to sample of rumen fluid from the same cow and by as much as fivefold from samples from different cows. These variations, which have been reported previously (10, 16), may be due to different times of sampling or to changes in the feeding habits of the animal (10). The effect of these factors on the number of spirochetes in rumen fluid was not determined since this line of inquiry was not within the scope of the investigations reported here.

Characterization of spirochetes. The enumeration studies showed that spirochetes present in rumen fluid possess considerable nutritional versatility. Furthermore, spirochetes differing in cell size and cell coiling patterns were observed commonly in rumen fluid (Fig. 2). Thus, it appeared that pronounced physiological and morphological heterogeneity existed among spirochetes indigenous to the rumen. To understand more clearly the nature of this heterogeneity, we investigated key physiological and morphological characteristics of 13 strains of spirochetes isolated from rumen fluid. These strains were studied with respect to their fermentation end products, the G+C content of their DNA, their ultrastructure, and their ability to utilize pectin, arabinogalactan, and starch as growth substrates.

All 13 strains were obligately anaerobic and used carbohydrates, but not amino acids, as fermentable growth substrates. The strains fermented glucose and could be divided into two groups on the basis of the fermentation end products that they formed from this sugar. Strains in one of the two groups produced for-

TABLE 1. Enumeration of rumen spirochetes that utilize different substrates

Growth substrate	Total no. of spirochetes[a] (10^6)	No. of spirochetes that utilized substrate[a] (10^6)	% of spirochetes[b]
None	3.0	ND[c]	—
Amino acids[d]	3.0	ND	—
Cellulose	NC[e]	ND	—
Carboxymethyl cellulose	2.2	ND	—
Pectin	3.2	0.6	0.4
Polygalacturonic acid	4.5	1.5	2.5
Xylan	11.0	0.8	2.7
Arabinogalactan	10.0	0.4	0.3
D-Xylose	5.0	4.0	3.0
L-Arabinose	6.4	4.0	10.0
L-Rhamnose	2.0	0.2	0.7
D-Galactose	4.3	2.5	5.0
D-Glucuronic acid	1.3	0.5	0.6
D-Galacturonic acid	3.0	0.5	1.0
Cellobiose	2.5	1.5	3.8
Glucose	4.5	3.5	6.0

[a] Viable counts of spirochetes per ml of rumen fluid serially diluted into enumeration agar media containing the substrate indicated in the table and rifampin. Total number of spirochetes was obtained by counting all morphological types of spirochete colonies (see the text). The numbers of spirochetes that utilized the substrates indicated were obtained by counting only cotton-ball-like colonies (see the text). However, when the substrates were pectin or polygalacturonic acid, colonies similar to those shown in Fig. 1b were also counted, in addition to the cotton-ball-like colonies (see the text). The numbers represent the average of two determinations performed in triplicate with rumen fluid samples from the same cow. Determinations carried out with rumen fluid from three other cows yielded similar results.

[b] Percentage of spirochetes among all bacteria that utilized the substrate being tested. Total numbers of bacteria were determined by subtracting the number of bacteria grown in enumeration media that contained no added substrate from the number of bacteria grown in media that contained the substrate under test. These total viable counts were obtained with agar media that did not contain rifampin. —, None.

[c] ND, Not detected. The lower limit of resolution was 10^4 cells per ml.

[d] Amino acids were added to the medium as casein hydrolysate (salt free, vitamin free; Nutritional Biochemical Corp., Cleveland, Ohio).

[e] NC, Not counted. The cellulose fibers in the media masked the detection of transparent spirochete colonies.

mate, acetate, and succinate as major nongaseous end products, whereas the strains in the other group did not produce succinate but instead formed ethanol, in addition to formate and acetate.

The succinate-forming strains (strains GL, GP, XA, 6M, 8G, and RUS-1) constituted a rather homogeneous group in terms of their morphological and physiological characteristics, as well as the G+C content of their DNA (36 to 38 mol%). Cells of these strains measured 0.2 to 0.3 by 5 to 8 μm. Although differences in cell coiling patterns existed among some of these strains, it seemed likely that they all were strains

TABLE 2. Characteristics of rumen spirochetes that produce formate, acetate, and ethanol from glucose

Strain	Periplasmic fibrils per cell[a]	Cell size (μm)	G+C (mol%)	Pectinolytic activity	Growth on arabinogalactan	Amylolytic activity
PA	8	0.3–0.4 by 5–8	43	−	−	+
LB	8	0.3–0.4 by 5–8	43	+	−	NA[b]
6A	8	0.3–0.4 by 5–8	43	−	−	NA
LU	12	0.4 by 10–12	41	+	+	+
CA	16–20	0.4–0.5 by 10–15	42	+	+	+
DA	≥32	0.6 by 12–15	46	+	−	−
PB	≥32	0.6–0.7 by 12–20	54	+	+	+

[a] Periplasmic fibrils have also been called axial fibrils and endoflagella. The number of periplasmic fibrils in strains DA and PB could not be determined accurately because the fibrils were present in a bundle. At least 16 fibrils were inserted at each end of the cell.

[b] NA, Not assayed.

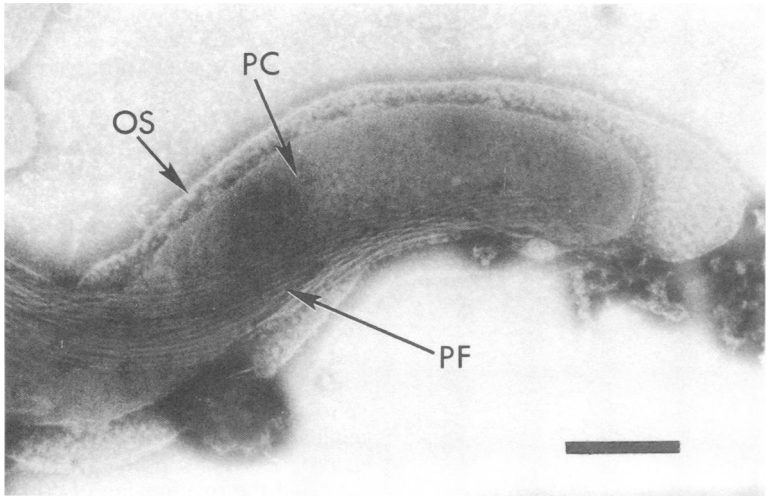

FIG. 3. Transmission electron micrograph of one end of a negatively stained cell of rumen spirochete strain PB. An outer sheath (OS), a protoplasmic cylinder (PC), and a bundle of periplasmic fibrils (PF) are visible. Bar = 0.3 μm.

of *T. bryantii* (17). They differed from the ethanol-forming strains not only in fermentation end products, but also because they were usually smaller in size, had only two periplasmic fibrils (axial fibrils, endoflagella) per cell, and had a lower G+C content of the DNA. Furthermore, they lacked the ability to utilize pectin, arabinogalactan, or starch.

In contrast to the relative uniformity of characteristics exhibited by the succinate formers, marked diversity was found among the ethanol-forming spirochetes. Depending on the strain, the ethanol formers had from 8 to more than 32 periplasmic fibrils per cell and varied considerably in cell size (Table 2; Fig. 3 and 4). Strains with larger cells tended to have a greater number of periplasmic fibrils per cell (Table 2; Fig. 3 and 4). With respect to the G+C content of their DNA, all except one of the ethanol-producing strains were clustered in the 41 to 46 mol% region (Table 2). The G+C content of the DNA of the remaining strain (strain PB) was 54 mol%. Most ethanol formers were able to ferment pectin and starch, and only some utilized arabinogalactan (Table 2). However, pectinolytic strains of spirochetes did not form ethanol when pectin was the fermentable substrate in the growth medium. The main end products of pectin fermentation were formate and acetate.

Strain 606, a rumen spirochete isolated by Ziolecki, does not ferment glucose, but ferments pectin primarily to formate and acetate (19). Cells of strain 606 measured 0.6 by 15 μm and possessed 32 or more periplasmic fibrils, as revealed by electron microscopy. The G+C content of the DNA of this spirochete was 46 mol%.

As previously mentioned, all strains of succinate formers and ethanol formers utilized glucose as a fermentable substrate. However, some strains (strains LB, 6A, LU, and CA) grew to final growth yields of approximately 5×10^7 to 8×10^7 cells per ml in RFG or MVTY broth media from which glucose had been omitted. Addition of amino acids to these media did not result in an increase in cell yields. In broth media that contained glucose (e.g., RFG or MVTY media), the growth yields of these strains were four to five times greater. The other strains required glucose in these media for growth.

DISCUSSION

Our study showed that relatively large numbers of spirochetes with diverse dissimilatory capabilities are present in rumen fluid. It is important to note that the numbers of spirochetes that we determined (Table 1) account for only a fraction of the spirochetes that were, in fact, present in the rumen of the animals we tested. This incomplete enumeration can be attributed to the following reasons. As described above, an agar medium had to be used which was transparent enough to allow accurate visualization of the spirochete colonies that developed. However, this enumeration medium did not support the growth of as many spirochetes as did more nutritionally complex, but less transparent media. In addition to being relative-

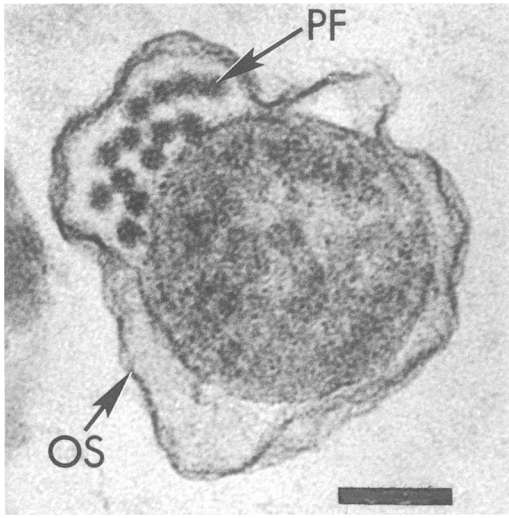

FIG. 4. Transmission electron micrograph of a thin section through a cell of rumen spirochete strain CA. Note that the periplasmic fibrils (PF) are present as a bundle and are surrounded by an outer sheath (OS). Bar = 0.2 μm.

ly transparent, the enumeration medium had the desirable feature of not supporting the development of cotton ball colonies when carbohydrate was omitted from it. This indicated that the carbohydrateless medium did not contain appreciable amounts of carbon and energy sources for the spirochetes. In contrast, richer media (e.g., RFG medium), from which the carbohydrate was omitted, supported the development of cotton ball colonies when inoculated with some of the rumen fluid samples.

Another reason for the lower enumeration values is that spirochetes that were associated with rumen epithelial surfaces (1, 13) or adhering to forage particles undergoing degradation in the rumen (6) were not counted by our procedure.

The numbers of polysaccharide-degrading spirochetes present in bovine rumen fluid are sufficiently high to justify the conclusion that these bacteria contribute to the breakdown of plant polysaccharide material ingested by the animal. This conclusion applies to plant polysaccharides, such as pectins, xylan, and arabinogalactan, but not to cellulose inasmuch as cellulolytic spirochetes were not detected in rumen fluid. Furthermore, most of the ethanol-producing isolates utilized starch as a fermentable substrate.

Rumen fluid contained relatively high population densities of spirochetes capable of utilizing as fermentable substrates the hydrolysis products of pectin, hemicelluloses, and cellulose (e.g., galacturonic acid, glucuronic acid, xylose, arabinose, galactose, cellobiose). Stanton and Canale-Parola have demonstrated that some rumen spirochetes interact with cellulolytic bacteria since they use as carbon and energy sources soluble sugars released from cellulose by the metabolic activities of the latter bacteria (17). By analogy, it is possible that interactions exist in the rumen between pectinolytic or hemicellulolytic bacteria and spirochetes that ferment breakdown products of pectin or hemicelluloses.

According to current taxonomic criteria (3), all anaerobic spirochetes that are indigenous to animal hosts are classified in the genus *Treponema*. From this genus are excluded spirochetes that are etiological agents of relapsing fever, which are placed in the genus *Borrelia*, and large spirochetes indigenous to the intestinal tract of molluscs, which are classified in the genus *Cristispira*. Borrelias probably are not anaerobic (11), and the relationship of cristispiras to O_2 is not known. There are two other recognized genera of spirochetes, *Spirochaeta* and *Leptospira* (3). None of the species of *Spirochaeta* is host-associated, and the genus *Leptospira* includes only obligately aerobic spirochetes.

In accordance with the taxonomic scheme summarized above, all of the strains of rumen spirochetes we investigated should be classified in the genus *Treponema*. However, many of these strains differ markedly from one another in morphology, physiology, and in the G+C content of their DNA, which ranges from 36 to 54 mol% in the 13 strains isolated in our laboratory and in Ziolecki's strain 606. Because of such pronounced differences it seems inappropriate to classify these 14 strains within a single genus. Thus, the information yielded by our characterization studies of rumen spirochetes emphasizes the need for developing a new taxonomic treatment of anaerobic, host-associated spirochetes.

ACKNOWLEDGMENTS

We are grateful to S. C. Holt for the use of his electron microscopy facilities, which are supported by National Science Foundation equipment grant PCM 78-05656. We thank C. D. Cox for the photographs of spirochete colonies.

This research was supported by Public Health Service grant AI-17737 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Bauchop, T., R. T. J. Clarke, and J. C. Newhook.** 1975. Scanning electron microscope study of bacteria associated with the rumen epithelium of sheep. Appl. Microbiol. **30**:668–675.
2. **Breznak, J. A., and E. Canale-Parola.** 1975. Morphology and physiology of *Spirochaeta aurantia* strains isolated from aquatic habitats. Arch. Microbiol. **105**:1–12.
3. **Buchanan, R. E., and N. E. Gibbons (ed.).** 1974. Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
4. **Canale-Parola, E.** 1978. Motility and chemotaxis of spirochetes. Annu. Rev. Microbiol. **32**:69–99.

5. **DeLey, J.** 1970. Re-examination of the association between melting point, buoyant density, and chemical base composition of deoxyribonucleic acid. J. Bacteriol. **101**:738–754.
6. **Dinsdale, D., E. J. Morris, and J. S. D. Bacon.** 1978. Electron microscopy of the microbial populations present and their modes of attack on various cellulosic substances undergoing digestion in the sheep rumen. Appl. Environ. Microbiol. **36**:160–168.
7. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore.** 1977. Anaerobic laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
8. **Holt, S. C., and E. R. Leadbetter.** 1967. Fine structure of *Sporocytophaga myxococcoides*. Arch. Mikrobiol. **57**:199–213.
9. **Holt, S. C., A. C. R. Tanner, and S. S. Socransky.** 1980. Morphology and ultrastructure of oral strains of *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. Infect. Immun. **30**:588–600.
10. **Hungate, R. E.** 1966. The rumen and its microbes. Academic Press, Inc., New York.
11. **Kelly, R. T.** 1974. *Borrelia*, p. 355–357. *In* E. H. Lennette, E. H. Spaulding, and J. P. Truant (ed.), Manual of clinical microbiology, 2nd ed. American Society for Microbiology, Washington, D.C.
12. **Lang, E., and H. Lang.** 1972. Speissifische fabreaktion zum direkten nachweis der Ameisensäure. Z. Anal. Chem. **260**:8–10.
13. **McCowan, R. P., K.-J. Cheng, C. B. M. Bailey, and J. W. Costerton.** 1978. Adhesion of bacteria to epithelial cell surfaces within the reticulorumen of cattle. Appl. Environ. Microbiol. **35**:149–155.
14. **Sabatini, D. D., K. Bensch, and R. J. Barrnett.** 1963. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. **17**:19–58.
15. **Salyers, A. A., R. Arthur, and A. Kuritza.** 1981. Digestion of larch arabinogalactan by a strain of human colonic *Bacteroides* growing in continuous culture. J. Agric. Food Chem. **29**:475–479.
16. **Stanton, T. B., and E. Canale-Parola.** 1979. Enumeration and selective isolation of rumen spirochetes. Appl. Environ. Microbiol. **38**:965–973.
17. **Stanton, T. B., and E. Canale-Parola.** 1980. *Treponema bryantii* sp. nov., a rumen spirochete that interacts with cellulolytic bacteria. Arch. Microbiol. **127**:145–156.
18. **Tomerska, H.** 1971. Decomposition of pectin *in vitro* by pure strains of rumen bacteria. Acta Microbiol. Pol. Ser. B **3**:107–116.
19. **Ziolecki, A.** 1979. Isolation and characterization of large treponemes from the bovine rumen. Appl. Environ. Microbiol. **37**:131–135.
20. **Ziolecki, A., and M. Wojciechowicz.** 1980. Small pectinolytic spirochetes from the rumen. Appl. Environ. Microbiol. **39**:919–922.