APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1979, p. 131-135
0099-2240/79/01-0131/05$02.00/0
Vol. 37, No. 1

Isolation and Characterization of Large Treponemes from the Bovine Rumen

ALEKSANDER ZIOŁECKI

Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland

Received for publication 18 October 1978

Ten strains of strictly anaerobic spiral organisms were isolated in pure culture from a 10^{-7} dilution of bovine rumen contents. Three strains were studied in detail. These strains morphologically resembled previously isolated and described rumen treponemes except the new isolates were larger, 0.7 μm wide and 12 to 25 μm long. They rapidly fermented pectin and, less readily, L-arabinose, inulin, and sucrose. Acetic and formic acid were the main fermentation products from pectin; small amounts of succinic acid were also formed.

In the rumen contents of cattle and sheep fed on different diets we have repeatedly observed large spiral organisms. They were present in much lower concentrations than the small treponemes, and there were wide day-to-day variations in concentrations in the same animal. A similar organism was seen by Bryant (2) in the rumen contents of a cow. Attempts to isolate the large organisms in pure culture were not successful until we noticed, during studies on the fermentation of pectin by a mixed population of rumen microorganisms, that the medium containing pectin acted as an enrichment medium for the large treponemes. A number of strains was then isolated, and the present paper describes these isolates.

MATERIALS AND METHODS

Isolation. The medium used for isolation was essentially that described by Tomerska (6) modified to contain 0.3% pectin, 0.03% thioglycolate, and 0.025% cysteine. Anaerobiosis was maintained as described by Hungate (4). Samples of rumen fluid were withdrawn from a cow with a permanent rumen fistula. The cow was fed on hay and concentrates. Serial dilutions up to 10^{-8} were made in the liquid pectin medium, which was then solidified by addition to each tube of the same volume of melted medium of similar composition containing 3% agar and no pectin, and incubated at 39°C for 24 to 48 h. The agar gels were then ejected from the tubes into sterile petri dishes by blowing CO_2 through a Pasteur pipette inserted along the wall to the bottom of the tube. Colonies with the characteristic appearance of treponemes were checked microscopically. A small fragment of a treponeme-containing colony was then introduced into a test tube with the pectin medium, deposited on the wall of the tube, and macerated with a loop or a spatula. The tube was then plugged and mixed thoroughly with a Vortex type mixer; serial dilutions in the liquid pectin medium were made again, but the medium was not solidified with agar. The treponemes were isolated from the highest dilution showing growth. Isolation from the second dilution series was essential because in the first series the colonies were picked from 10^{-7} dilutions of rumen fluid and were contaminated with other organisms. The brief exposure to oxygen when picking the colonies did not seem to damage the organisms. The isolated strains were maintained in the liquid pectin medium.

Microscopy. Microscopic observations and measurements were made under a phase-contrast microscope using wet mounts. For taking cell measurements the preparations were solidified with gelatin.

Physiological tests. The medium for fermentation tests was essentially that used for isolation, with pectin replaced by similar concentrations of sugars, sugar alcohols, or glycosides. Incubation was for 7 days at 39°C. Tubes showing visible growth were checked microscopically and regarded as positive. Inoculated tubes with no carbohydrates were run in parallel as a negative control. Tests for H_2S production, gelatin liquefaction, indole production, nitrate reduction, and decomposition of casein were made according to Bryant and Doetsch (3). All tests were done in triplicate.

Growth requirements. These were studied using the same basal medium appropriately modified. Rumen fluid and yeast extract were omitted, and ammonium sulfate and cysteine were replaced by equivalent amounts of sodium sulfate and thioglycolate, respectively. Other ingredients were added as required. Growth was assessed by measuring the optical density of cultures at 570 nm.

Fermentation of pectin. The medium was similar to that used for isolation and contained 0.5% purified citrus pectin. The fermentation was in 100-ml conical flasks with rubber stoppers equipped with Bunsen valves. The medium was inoculated with 3 drops of an actively growing 18-h culture and incubated for 24 h at 39°C. The fermentation tests were repeated three times at weekly intervals, each time in triplicate.

Analyses. C_1 to C_5 volatile fatty acids were estimated by gas chromatography as previously described (9). Methyl and ethyl alcohols were also determined by gas chromatography. A 10-ml sample of deprotein-

ized medium, pH about 7.8, was pipetted into a 50-ml boiling flask, diluted to 20 ml with water, and distilled; 10 ml of the distillate was collected in a graduated test tube, and 1-μl samples were injected onto the column. Separation was at 200°C on Porapak Q in a glass column of 4-mm internal diameter and 150-cm length. A Pye-Unicam (Cambridge, England) gas chromatograph model 64 with a flame ionization detector was used. Lactic acid was determined by the colorimetric method of Barker and Summerson and succinic acid as the silver salt, both as described by Neish (5). Galacturonic acid was estimated according to Bitter and Muir (1).

RESULTS

Ten strains were isolated from 10^{-7} dilutions of rumen contents. Three of them (no. 606, 709, and 710), isolated from three samples of rumen contents collected separately at 3- to 4-week intervals, were studied in detail.

Morphology. The cells varied in length from 12 to 25 μm and were 0.6 to 0.7 μm wide. They were spiral, with tapered ends, and the prevailing number of coils was 4 to 8. The coils were not very regular; their amplitude was about 2 μm and the wavelength about 3 μm. In wet mounts the cells were motile; both lashing and corkscrew-like motion were observed, but the former predominated, as was also the case in samples of rumen contents. In older cultures, incubated over 36 h, round bodies were formed and gradually replaced the normal spiral cells (Fig. 1D). The initial stage of the formation of those bodies seemed to be a tight coiling of the cell (Fig. 1B and C). Similar round bodies are formed by the smaller rumen spirochetes (2, 7, 10). The organisms stained well with the common basic dyes and were gram negative. Deep colonies in pectin agar were regularly spherical, diffuse, and 4 to 5 mm in diameter.

Growth requirements. Growth was observed at 35 and 39°C but not at 30 or 45°C after 7 days of incubation. Rumen fluid stimulated growth. Growth, as measured by optical density of the medium at 570 nm, increased with increasing concentration of rumen fluid up to 5%; over that amount, stimulation was negligible (Table 1). The effect was partly due to the C_4 and C_5 fatty acids. Addition of iso-butyric, 2-methyl butyric, valeric, and iso-valeric acids to the medium resulted in growth of strains 709 and 710 approaching 75% of that with 5% rumen fluid. Supplementing the medium with 1% rumen fluid in addition to the fatty acids gave growth similar to that with 5% rumen fluid. The response of strain 606 to the fatty acids was considerably less; 2% rumen fluid in addition to the fatty acids was needed to obtain growth comparable to that with 5% rumen fluid. Acetic and propionic acids did not stimulate growth.

Biotin could substitute for rumen fluid for strains 709 and 710 when added to the medium supplemented with C_4 and C_5 fatty acids. Yeast extract was slightly inferior to biotin in this respect, and *p*-aminobenzoic acid substituted for rumen fluid only to a minor extent. The response of strain 606 to the vitamins or yeast extract was negligible.

Ammonium sulfate or casein hydrolysate (Casitone, Difco; 13.2% N on a dry basis) were utilized as sources of nitrogen for growth. Growth with $(NH_4)_2SO_4$ was slightly better at the equivalent N level than with Casitone; joint addition of 50:50 $(NH_4)_2SO_4$ and Casitone did not increase growth over that with $(NH_4)_2SO_4$ as the sole source of nitrogen. Although the utilization of $(NH_4)_2SO_4$ and Casitone by all strains was similar, addition of 0.1% Casitone to the growth medium stimulated the production of pectinolytic enzymes as judged by the higher pectinolytic activity of the fermented medium (unpublished data).

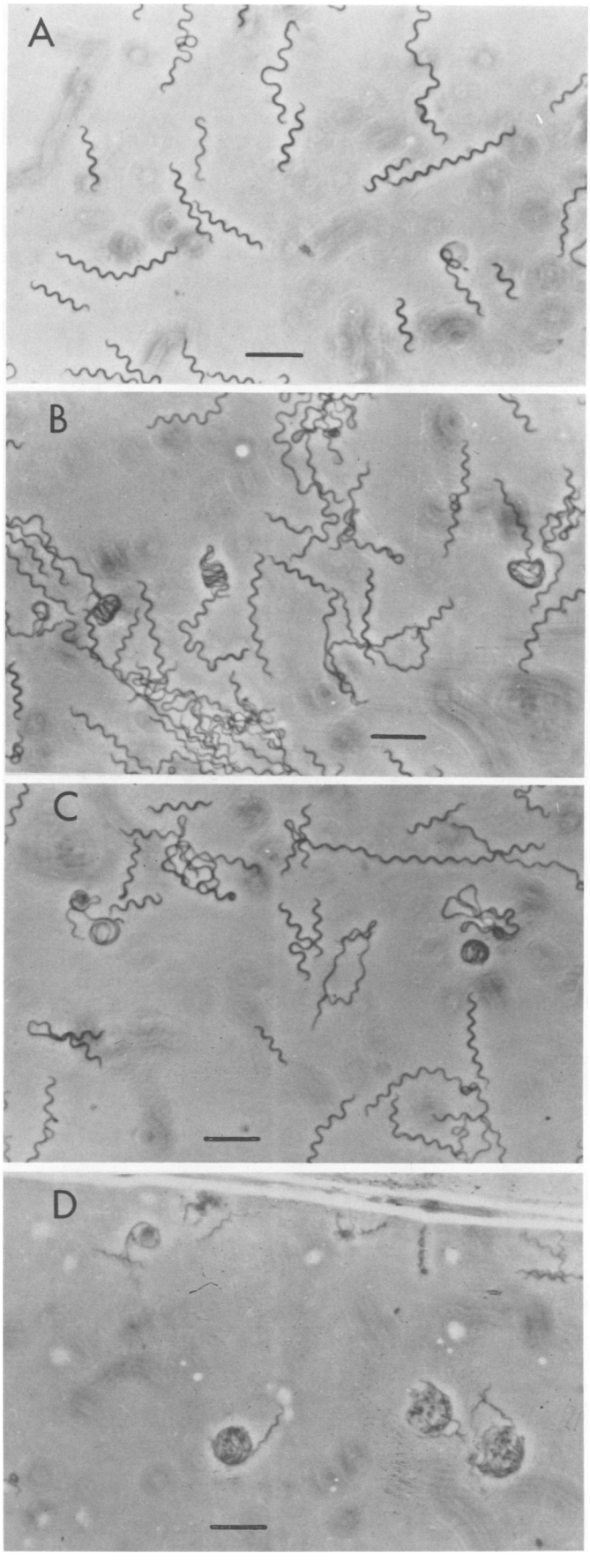
Casein was not hydrolyzed, gelatin was not liquefied, indole was not produced, and nitrates were not reduced.

All strains fermented L-arabinose, inulin, pectin, and sucrose, but only pectin was fermented vigorously and completely, whereas with 0.5% sucrose, 10 to 40% unfermented sugar remained in the medium after fermentation ceased. Growth of all strains on galacturonic acid was very poor. Strain 709 fermented also D-xylose. After about 2 years of maintenance on a pectin medium, the strains lost their ability to ferment sugars other than pectin. None of the strains fermented glucose, fructose, galactose, mannose, lactose, rhamnose, maltose, cellobiose, trehalose, raffinose, melezitose, starch, gum arabic, xylan, glycerol, mannitol, adonitol, sorbitol, salicin, esculin, and lactate.

All strains were obligately anaerobic and did not grow if the resazurin in the medium was not reduced to colorless. The maintenance of cultures was difficult. Stock cultures in pectin medium kept at 2°C did not survive for more than 5 to 6 days. No attempts were made to freeze-dry the cultures.

Fermentation products of pectin. Acetic and formic acids were the main fermentation

FIG. 1. *Large pectinolytic rumen treponemes. (A) 18-h culture in pectin medium; (B) and (C) 30-h culture showing initial stages of the formation of spherical bodies; (D) spherical bodies in a 48-h culture. Bar = 10 μm.*


A
B
C
D

products of pectin (Table 2); small amounts of succinic acids were also formed. Propionic, butyric, and lactic acids were not produced. A certain amount of methyl alcohol was always found in the fermented medium, probably derived from de-esterification of pectin rather than being a true fermentation product. The fermentation products of sucrose were similar except for the lack of methyl alcohol.

DISCUSSION

The large spirochetes share some characteristics of the small rumen spirochetes so far isolated and described. Their morphology is similar, except for the much larger size; they exhibit the same type of motility and produce similar round bodies in older cultures. The main products of carbohydrate fermentation are also similar: acetic and formic acids. The capacity of the large treponemes to utilize different carbohydrates or their derivatives is limited to the fermentation of pectin, inulin, sucrose, and L-arabinose. The latter was the only monosaccharide fermented by these organisms; glucose and fructose were not utilized, and growth on galacturonic acid was very poor. There were some differences between the three strains studied. The growth and fermentation rates were greatest with strain 709, least with 606, and intermediate with 710. Strain 606 seemed also to have more complex growth requirements, as its response to certain growth stimulants was less than that of the other two strains. All strains required a combination of C_4 and C_5 branched-chain fatty acids and valeric acid for good growth. The requirement for those acids seems to be a common feature of the rumen treponemes, having been observed also in other organisms (7, 10).

The most outstanding physiological characteristic of the large treponemes is their pectinolytic activity; the fermentation of the other sugars was less rapid and often incomplete. There is the possibility, of course, that this might have resulted from the fact that the strains described here had been isolated on a pectin medium, and other strains may be more active towards other substrates. The pectinolytic activity of the treponemes was comparable to that of the predominant pectinolytic rumen bacteria, *Lachnospira multiparus* and *Bacteroides ruminicola*. Growth in pectin medium was very rapid; in a tube with 10 ml of medium, inoculated with a loopful of stock culture, within 8 h there was a visible turbidity, which reached a maximum within about 12 h. The doubling time of strain 709 during the exponential phase of growth was about 60 min (unpublished data). The treponemes produced and released into the external environment a complex of pectinolytic enzymes, predominantly pectin lyase (8). The enzymes seemed to be constitutive rather than

TABLE 1. *Effect of rumen fluid, fatty acids, and other growth stimulants on growth of rumen treponemes*

Supplement[a]	Optical density at 570 nm of strain:		
	606	709	710
None	0.000	0.000	0.000
Rumen fluid			
1%	0.041	0.032	0.036
2%	0.097	0.097	0.108
5%	0.222	0.244	0.310
10%	0.237	0.260	0.377
20%	0.252	0.276	0.398
Fatty acid mixture[b]	0.022	0.208	0.194
+1% rumen fluid	0.125	0.268	0.276
+2% rumen fluid	0.194	0.276	0.268
+biotin[c]	0.022	0.229	0.319
+0.1% yeast extract	0.018	0.208	0.260

[a] With $(NH_4)_2SO_4$ as the sole nitrogen source.
[b] Isobutyric, valeric, isovaleric, and 2-methyl butyric acids, each at 25 mg/100 ml.
[c] 10 μg/100 ml.

TABLE 2. *Fermentation products of pectin by pectinolytic rumen treponemes*

Strain no.	Product (mmol/100 ml of fermented medium)[a]					Cell dry matter		Carbon recovery[b]
	Formic acid	Acetic acid	Succinic acid	Methyl alcohol	CO_2[c]	mg	mmol of C[d]	
606	1.435	3.793	0.033	0.820	2.358	22.0	0.917	90.98
709	1.616	3.616	0.026	0.874	2.000	24.1	1.006	87.54
710	1.440	3.946	0.016	1.082	2.506	24.8	1.033	94.69

[a] Fermentation medium contained 0.55% pectin, equivalent to 442 mg of pectin galacturonic acid per 100 ml; H_2 production not measured.
[b] Calculated relative to galacturonic acid C, hence not including methyl alcohol derived probably from de-esterification of pectin.
[c] Calculated as equimolar to acetic acid less formic acid.
[d] Assuming 50% C in cell dry matter.

induced, because the pectinolytic activity of cultures grown on sucrose was only slightly less than of those grown on pectin (unpublished data).

The large treponemes do not seem to be of major importance in rumen metabolism. They may be effective in decomposing pectin to no lesser degree than are the other pectinolytic species. As to the other carbohydrates, L-arabinose and sucrose are fermented by several other species, each of them, except perhaps *Streptococcus bovis,* more numerous in the rumen microbial population than the large treponemes. Inulin is rarely a component of the ruminant diet, but one is tempted to speculate that the ability to ferment inulin may be indicative of the ability to utilize other fructosans, especially those produced and stored by many species of grasses.

Apart from their relative importance for the rumen processes, the large treponemes have been observed by different workers in various geographically distant places. They produce either normal end products or intermediates in the rumen from substrates present in ruminant diets, are strictly anaerobic, and can be isolated from dilutions exceeding 10^{-6} ml. All this suggests that they may be regarded as a true rumen organism. In view of their general resemblance to the smaller rumen treponemes, they may be classified as belonging to the same genus.

LITERATURE CITED

1. **Bitter, T., and H. N. Muir.** 1962. A modified uronic acid carbazole reaction. Anal. Biochem. **4**:330–334.
2. **Bryant, M. P.** 1952. The isolation and characteristics of a spirochete from the bovine rumen. J. Bacteriol. **64**:325–335.
3. **Bryant, M. P., and R. N. Doetsch.** 1954. A study of actively cellulolytic rodshaped bacteria of the bovine rumen. J. Dairy Sci. **37**:1176–1183.
4. **Hungate, R. E.** 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. **14**:1–49.
5. **Neish, A. C.** 1952. Analytical methods for bacterial fermentations. Prairie Regional Laboratory, National Research Council of Canada, Saskatoon.
6. **Tomerska, H.** 1971. Decomposition of pectin in vitro by pure strains of rumen bacteria. Acta Microbiol. Pol. Ser. B **3**:107–115.
7. **Wegner, G. H., and E. M. Foster.** 1960. Fatty acid requirements of certain rumen bacteria. J. Dairy Sci. **43**:566–568.
8. **Wojciechowicz, M., and A. Ziołecki.** 1979. Pectinolytic enzymes of large rumen treponemes. Appl. Environ. Microbiol. **37**:136–142.
9. **Ziołecki, A., and E. Kwiatkowska.** 1973. Gas chromatography of C_1 to C_5 fatty acids in rumen fluid and fermentation media. J. Chromatogr. **80**:250–254.
10. **Ziołecki, A., H. Tomerska, and M. Wojciechowicz.** 1975. Characteristics of pure cultures of Borrelia sp. isolated from the bovine rumen. Acta Microbiol. Pol. Ser. B **7**:45–50.