***Fusobacterium polysaccharolyticum* sp. nov.,
a Gram-negative Rod from the Rumen that Produces Butyrate
and Ferments Cellulose and Starch**

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A new Gram-negative, non-sporulating, rod-shaped, anaerobic bacterium capable of fermenting cellulose and starch was isolated from the rumens of sheep fed supplemented maize stover diets. The organism fermented few carbohydrates, showing a preference for polysaccharides. The main acid products of carbohydrate fermentation were butyrate and formate. Acetate was utilized.

INTRODUCTION

The Gram-negative, non-sporulating, cellulolytic, rod-shaped bacteria that have been isolated most frequently from the rumen are *Bacteroides succinogenes* and *Butyrivibrio fibrisolvens*. Others that have been found on only one occasion include one that produces mainly acetate (Margherita & Hungate, 1963) and one, also unnamed, that produces butyric acid (Leatherwood & Sharma, 1972).

This paper describes some of the characteristics of three isolates of a novel Gram-negative, rod-shaped bacterium isolated from sheep rumens.

METHODS

Source of isolates. Isolates were obtained from roll-bottles containing cellulose agar inoculated with high dilutions of rumen ingesta from sheep (Mackie *et al.*, 1978). The new bacterium was isolated from sheep A72 after it had been fed on a diet containing 13.7 % maize grain and 67.5 % maize stover for 7 d, and again when the animal had been fed for 7 d on a diet containing 50.2 % maize grain and 28.3 % stover. Isolates were also obtained from sheep A18 on the first and seventh days of feeding the latter diet. The dilutions of rumen ingesta from which the bacterium was isolated were 10^{-6} and, in one case, 10^{-7} . Three isolates, designated B, E and G, were obtained in pure culture after repeated culturing on cellulose agar and picking of colonies that produced zones of clearing.

Culture techniques. These, including procedures to obtain and maintain strictly anaerobic culture conditions, were as described by van Gylswyk & Hoffman (1970).

Media. Except where otherwise stated, media contained (g l^{-1}): K_2HPO_4 , 0.225; KH_2PO_4 , 0.225; NaCl, 0.45; $(\text{NH}_4)_2\text{SO}_4$, 0.45; CaCl_2 (anhydrous), 0.045; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.09; NaHCO_3 , 6.37; cysteine.HCl.H₂O, 0.25; $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 0.25; cellobiose, 5; indigo carmine, 0.005; together with rumen fluid from sheep fed lucerne hay (partially clarified by centrifuging at 1500 g for 30 min), 400 ml; the gas phase was 98 % CO_2 and 2 % H_2 (gases were purchased from Afrox Ltd, Industria West, Germiston, South Africa).

The cysteine and sulphide were prepared as a combined, concentrated solution under nitrogen, made alkaline before addition to the medium (medium 1). For the preparation of maintenance slopes, the concentration of the mineral nutrients was doubled and agar (15 g l^{-1}) and Difco Casitone (5 g l^{-1}) were added. Cultures were grown in this medium when tested for the ability to produce gas and catalase. The cellobiose concentration of medium 1 was reduced to 1 g l^{-1} for culturing bacteria that were examined for the presence of flagella. For fermentation tests, the concentration of NaHCO_3 in medium 1 was reduced to one-tenth and the gas phase was 88 % N_2 , 10 % CO_2 and 2 % H_2 (medium 2). Soluble sugars and other substances were added

in filter-sterilized solution to heat-sterilized medium. Insoluble substrates were heat-sterilized together with the basal medium. Temperature tolerance tests were done in medium 2 containing cellobiose as energy source.

The reaction of the bacteria to oxygen was tested in medium 1 containing filter-sterilized rumen fluid and with resazurin (1 mg) replacing indigo carmine. One batch of medium was used without modification. From three further batches the cysteine and sulphide were omitted. To two of these batches were added, under pressure, either 5 or 30 ml sterile air per 28 ml McCartney bottle containing 5 ml medium. Test media for indole and acetylmethylcarbinol production contained Difco Casitone (20 g l⁻¹) and Difco yeast extract (10 g l⁻¹) instead of rumen fluid and indigo carmine was omitted. A similar medium was used in the test for nitrate reduction but cysteine and sulphide were omitted and KNO₃ (1 g l⁻¹; in filter-sterilized concentrated solution) was added.

For H₂S production medium 1 was supplemented with BBL Trypticase (15 g l⁻¹) and ferric ammonium citrate (0.2 g l⁻¹). The gelatin liquefaction test was conducted with medium 1 containing 4 % (w/v) gelatin. Media for studying the types and quantities of fermentation end-products contained either cellobiose or ball-milled Whatman no. 1 filter paper cellulose (14.3 g l⁻¹ in each case). For determining the G + C content, bacteria were grown in medium containing 300 ml rumen fluid (clarified by centrifuging at 12000 g for 1 h) and the cysteine and sulphide were each reduced to 0.0625 g l⁻¹. The ability of the bacteria to grow in the absence of rumen fluid was tested in medium in which peptone (Parke, Davis & Co.) and yeast extract (each 10 g l⁻¹) replaced rumen fluid.

A chemically defined medium containing mineral salts, trace elements, vitamins, purines, pyrimidines [components of groups of nutrients and their concentrations were essentially similar to those employed by Roché *et al.*, (1973)], one-tenth of the usual concentration of NaHCO₃ and a gas phase of 88 % N₂, 10 % CO₂ and 2 % H₂ was tested for its ability to support growth.

All media had a pH of about 6.8 and were incubated at 38 ± 1 °C.

Identification tests. Growth in liquid media was usually assessed turbidimetrically. In the tests for temperature tolerance, ability to ferment different substrates and ability to grow in the chemically defined medium, a change in pH was taken as indicative of growth. For the gelatin liquefaction test, cultures were incubated for 24 h and 1 week and then kept at about 4 °C until the medium in uninoculated, but similarly incubated, control bottles had gelled. Production of catalase, acetylmethylcarbinol and indole was assessed as described by Cato *et al.* (1973). To determine whether nitrite was produced from nitrate, the test described by Conn (1951) using α -naphthylamine was employed. Staining for spores was done according to Schaeffer & Fulton (1933) and heat tests (80 °C, 10 min) for spores were as described by Cato *et al.* (1973). Hydrogen sulphide production was indicated by blackening of the medium. Gas production was assessed by making stabs in 28 ml McCartney bottles containing 15 ml medium (similar to that used for maintenance slopes) and checking for the splitting of agar after 24 and 48 h.

Fermentation end-products were determined using 1 week-old cultures. Ethanol was determined enzymically (Bonnischen, 1962). Volatile and non-volatile acids were determined by gas-liquid chromatography according to Cato *et al.* (1973). Concentrations of the acids were estimated using external standards. For cellulose-containing cultures, residual cellulose was determined as follows. Cultures were centrifuged and the bacteria were dissolved in 1 M-NaOH for 15 min. The cellulose was washed with 5 M-acetic acid and three times with deionized water, followed each time by centrifugation. The material was dried *in vacuo* at 65 °C and weighed.

For determining G + C mol %, cells were lysed with sodium dodecyl sulphate according to the method of Marmur (1961). Highly polymerized DNA was isolated and purified by the method of Meyer & Schleifer (1975) and base composition was estimated from the A₂₄₅/A₂₇₀ ratio by the method of Ulitzur (1972). DNA standards used for calibration were from *Micrococcus lysodeikticus*, calf thymus and salmon sperm (all from Sigma).

All identification tests were done at least twice.

RESULTS

Colony morphology and growth in solid and liquid media

After 3 d incubation, surface colonies on 0.5 % cellobiose/40 % rumen fluid agar in roll-bottles were about 1 mm in diameter, whitish and translucent, and round with smooth edges and raised centres. Cultures grown for 4 d in roll-bottles with similar medium containing 1.2 % filter paper cellulose instead of cellobiose produced well-defined zones of clearing, 2 to 3 mm in diameter, showing complete cellulolysis. Small, discreet, round colonies were present in the centres of the zones. The zones enlarged on longer incubation.

For Fig. 1, the bar marker represents 20 μm . For Figs 2 to 7, the bar marker (in Fig. 7) represents 1 μm .

Fig. 1. Gram-stained cells of *Fusobacterium polysaccharolyticum*.

Figs 2, 3 and 4. Transmission electron micrographs of cells of *F. polysaccharolyticum* isolate B after negative staining with phosphotungstic acid (3 %, pH 6).

Figs 5, 6 and 7. Transmission electron micrographs of cells of *Butyrivibrio fibrisolvens* isolate 12, stained as above.

Table 1. *Acid production from different carbohydrates by three isolates of a novel cellulolytic rumen bacterium*

Carbohydrate	Final pH* in poorly buffered medium					
	Isolate B		Isolate E		Isolate G	
	After 48 h	After 1 week	After 48 h	After 1 week	After 48 h	After 1 week
L-Arabinose	6.8	5.8	6.8	5.7	6.9	5.8
Xylose	6.8	5.9	6.8	5.8	6.8	6.1
Fructose	6.9	5.9	6.8	6.8	6.9	6.8
Cellobiose	5.8	5.5	5.8	5.7	5.8	5.7
Maltose	6.6	5.7	6.5	5.7	6.7	5.9
Dextrin	6.0	5.9	6.1	6.0	6.1	6.1
Pectin	6.6	6.0	6.6	6.5	6.7	6.5
Starch	5.7	5.6	5.7	5.6	5.5	5.7
Xylan	5.6	5.7	5.7	5.7	5.7	5.7

* Initial pH varied from 6.8 to 6.9.

When an inoculum was placed on the surface of agar slopes containing rumen fluid and 1 % cellulose, the cellulose was almost completely solubilized after 5 d incubation. Cellulolysis still occurred after many transfers of the organisms on cellobiose-containing medium, showing that the ability to solubilize cellulose is not easily lost. Growth on the surface of agar slopes appeared as a transparent mucous mass.

In liquid media, after initial active growth, a mucous sediment was formed, particularly in the case of isolate G which tended to produce a large amount of extracellular slime. This made the medium viscous and it was difficult to resuspend the sediment by shaking. On no occasion in any medium was pigment production observed.

Morphology and Gram reaction

There was some doubt as to whether the organisms were Gram-negative as they stained considerably darker than cells of *Escherichia coli* and *Proteus vulgaris*. Accordingly the three isolates, together with *Streptococcus bovis*, a Gram-positive organism, were stained in the usual way except that the counter stain (carbol fuchsin) was not applied. On microscopic examination it appeared that the cells of *S. bovis* became heavily stained whereas the cells of the new organisms remained unstained. It was concluded that the new isolates were Gram-negative but that they stained unusually intensely with the counter stain.

Bacteria grown for 16 to 24 h on 0.1 or 0.5 % cellobiose/rumen fluid agar slopes and stained by Gram's method were seen as straight rods with rounded ends (Fig. 1), usually occurring singly, but chains and long cells were often observed in young cultures. Their width varied from 0.6 to 1.1 μm and length from 2 to more than 15 μm (typically, 0.8 μm wide and 3 to 6 μm long). In cellobiose/rumen fluid medium adjusted to pH 7.2, aseptate filaments, 50 μm and more in length, were observed. Cells in older cultures lysed very readily, often swelling before disintegrating. The bacteria possessed many peritrichous flagella (Figs 2 to 4). Numerous tests for the presence of spores in different media all proved negative.

Effects of oxygen, temperature and pH on growth

The isolates were anaerobes in that they failed to grow in media under air. They grew well in media free of added oxygen irrespective of whether cysteine and sulphide were present. Medium free of these compounds appeared orange due to partially reduced resazurin. During growth of the bacteria this colour did not change and remained the same as that in uninoculated bottles indicating that the redox potential was not lowered significantly during growth.

Table 2. *Acids produced or utilized by three isolates of a novel cellulolytic rumen bacterium in media containing cellobiose or cellulose*

The uninoculated medium contained [mmol (100 ml medium)⁻¹]: acetate, 4.5; propionate, 0.9; butyrate, 0.4.

Acid	Production (+) or utilization (–) of acid [mmol (100 ml medium) ⁻¹]					
	Isolate B		Isolate E		Isolate G	
	Cellobiose	Cellulose	Cellobiose	Cellulose	Cellobiose	Cellulose
Formate	+3.1	+1.3	+4.0	+1.8	+3.6	+1.7
Acetate	–0.7	–2.3	–0.2	–2.5	+0.1	–2.5
Propionate	–0.1	–0.1	–0.1	–0.1	–0.2	–0.1
Butyrate	+2.2	+3.0	+2.3	+3.2	+1.7	+3.1

None of the isolates grew at 22 °C (pH unchanged). After 4 d at 30 °C the final pH was between 5.7 and 5.8, the same as at 38 °C. At 45 °C the final pH was 6.4 and 6.1 for isolates B and E, respectively, while isolate G did not grow.

The optimum pH for growth was about 6.8 (A. Kistner, personal communication).

Fermentation

Substrates fermented by the three isolates are given in Table 1. Media containing L-arabinose or xylose did not show a fall in pH after 48 h but an appreciable decrease was noted after 1 week. Fructose was fermented only by isolate B after 1 week. Only a slight fall in pH (0.4 pH units or less) after 48 h and a significant decrease (1 pH unit or more) after 1 week was observed with medium containing maltose. Pectin was poorly fermented, only isolate B showing a decrease of about 0.9 pH units after 1 week. The greatest consistent decrease in pH (more than 1 pH unit) was found for cellobiose, starch and xylan. It appeared that the bacteria were utilizers of highly polymerized substrates common in ruminant feeds. Cellulose was also always vigorously attacked. No growth or fall in pH was noted in media containing galactose, glucose, glycerol, inositol, inulin, lactose, mannitol, mannose, raffinose, rhamnose, salicin, sodium DL-lactate, sucrose or trehalose.

The isolates produced gas, as shown by the splitting of agar, which was very pronounced after 48 h. Only traces of ethanol were produced and a weakly positive reaction was obtained in the test for acetylmethylcarbinol for all three isolates after 5 d incubation. They did not produce lactate or succinate. The extent to which the isolates produced or utilized formic, acetic, propionic and butyric acids in media containing cellobiose or cellulose is shown in Table 2. Formate and butyrate were the major products of fermentation. The difference in the amount of acetate utilized between media containing cellobiose and cellulose corresponded with the difference in butyrate production. The less butyrate was produced in cellobiose medium the more formate was produced in compensation. This was supported by results of calculations on the stoichiometry of the reactions $\text{CO}_2 \rightarrow \text{formate}$, and $\text{acetate} \rightarrow \text{butyrate}$ in terms of hydrogen equivalents required. Quantitative differences in end-products found with the two substrates were probably due to differences in growth and fermentation rates, suggesting that cellulolysis may be growth rate-limiting.

In cellulose medium, isolates B and E solubilized 75 % of the substrate, equivalent to 6.6 mmol glucose (isolate G was not examined). Of this, less than 30 % appeared in butyrate (after subtracting butyrate derived from acetate in the medium). It is possible that a large proportion of the solubilized cellulose was not metabolized.

Nutritional and other characteristics

The isolates grew in peptone/yeast extract medium free of rumen fluid only if a fermentable carbohydrate was added. Growth was stimulated by acetate. Moderate growth occurred

in the chemically defined medium (final pH 6.1 for isolates B and E and 6.4 for isolate G). No growth occurred on this medium when CO₂ and bicarbonate were omitted (gas phase was 98 % N₂ and 2 % H₂). Growth improved when peptone and yeast extract were added (final pH 5.8 to 5.9). Addition of 30 % rumen fluid had an even greater stimulatory effect as shown by a much greater cell density (final pH, 5.5 to 5.7).

Catalase, indole and H₂S were not produced; gelatin was not liquefied and nitrite was not detected 1 and 5 d after inoculation. Semi-quantitative tests for nitrate indicated that it had not disappeared during growth.

The G + C mol % for isolate B was 41.6, compared with 41.4 for *Butyrivibrio fibrisolvens* 12 (described by van Gylswyk & Roché, 1970) and 50.9 for *Eubacterium cellulosolvens* 6 (described by van Gylswyk & Hoffman, 1970). Leatherwood & Sharma (1972) obtained a value of 41.2 for *B. fibrisolvens* A38.

DISCUSSION

The new isolates resemble *B. fibrisolvens* and the bacterium described by Leatherwood & Sharma (1972) in that they are all Gram-negative, anaerobic, non-sporulating rods from the rumen that produce butyric acid and therefore appear to be physiologically similar. They resemble *Butyrivibrio* in G + C content and differ from it in morphology and type of flagellation (compare Figs 2 to 4 with Figs 5 to 7). They differ morphologically from the bacterium isolated by Leatherwood & Sharma (1972) in that the latter possessed no flagella, occurred in long twisted chains and had a G + C content of 36.3 mol %.

According to *Bergey's Manual of Determinative Bacteriology* (Buchanan & Gibbons, 1974), the new isolates would fall in the genus *Fusobacterium* (Knorr) in that they are motile with peritrichous flagella, Gram-negative anaerobes which produce butyric acid from carbohydrate and have a pH optimum close to 7. The G + C content of the DNA in most species of fusobacteria examined ranges from 26 to 34 mol % but one species, *F. symbiosum*, was found to have a G + C mol % of 48 (Buchanan & Gibbons, 1974). The G + C mol % of the new bacterium would not exclude it from the genus *Fusobacterium*; however, the novel isolates differ from all the species described under this genus. An appropriate specific epithet for the new bacterium would be *polysaccharolyticum* in view of its assumed function in the rumen, i.e. that of degrading polymerized carbohydrates such as cellulose, xylan (hemicellulose) and starch. Isolate B is the type strain and has been deposited in the American Type Culture Collection (accession number ATCC 33142).

The occurrence of *F. polysaccharolyticum* in the rumens of sheep fed supplemented maize stover diets was not merely sporadic as bacteria of this type were found in the rumens of two sheep, when fed different levels of grain on different occasions. Isolates were obtained from random roll-bottles used when making counts of cellulolytic bacteria and it is possible that a search for this organism may have revealed a more general occurrence in the rumen ingesta of different sheep. The dilutions of ingesta in medium from which the organisms were isolated indicate that they occurred at levels of at least 10⁶ per g ingesta.

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