A Cellulolytic Rumen Bacterium, *Micromonospora ruminantium* sp. nov.

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SUMMARY

A cellulolytic *Micromonospora* was isolated from the rumen of a sheep. The strain is related to, but not identical with, *Micromonospora propionici*, the cellulolytic actinomycete isolated by Hungate (1946) from the alimentary tract of a termite.

INTRODUCTION

In 1942 Hungate observed, in the rumen of a sheep, bacteria resembling morphologically the *Micromonospora*. In 1946 he described *Micromonospora propionici* as a new species of anaerobic cellulolytic bacterium isolated from the gut of a termite. According to Hungate (1966) and Prévot (1966), nine species of anaerobic cellulolytic rumen bacteria are known at present. They belong to the genera *Bacteroides*, *Butyrivibrio*, *Clostridium*, *Ruminococcus* and *Cillobacterium*. The taxonomic position of the new species described by Leatherwood & Sharma (1972) is uncertain.

The aim of the present work was to isolate the cellulolytic actinomycete from the rumen of a sheep.

METHODS

Isolation. The original culture of the cellulolytic strain was isolated from the rumen of a sheep fed *ad libitum* on lucerne. Samples of the rumen contents were pumped through a fistula under a CO₂ atmosphere into flasks kept at 39 °C, using a perforated stomach tube. The isolation procedure was performed with all precautions for maintaining anaerobic conditions: a CO₂ atmosphere, rubber stoppers, and sodium dithionite (0.0015 %, w/v) added to the rumen fluid. Tenfold dilutions of the rumen fluid in the solution of Bryant & Robinson (1961) were used to inoculate a liquid medium containing strips of Whatman No. 1 chromatographic paper (Mann, 1968). From the rumen fluid diluted to 1 p.p.m. we obtained micro-organisms digesting cellulose. They were purified by passing several times through the liquid and agar-solidified media containing cellulose.

Characterization. The bacteria were routinely cultured anaerobically at 39 °C in the following liquid and solid media: (i) rumen fluid, cellulose medium (Mann, 1968); (ii) rumen fluid medium (Bryant & Robinson, 1961); (iii) yeast extract and tryptose, peptone medium (van Gylswyk & Hoffman, 1970). Cultures were transferred every seven days. Grown cultures in cellulose medium were kept at 5 °C.

Morphological observations were made on bacteria grown in media (i) and (ii). Stained preparations were made from cultures which were 4 h, 12 h, 18 h, 1 day, 2 days, 3 days, 6 days and 10 days old. The following methods were used: Gram method, Hucker and Cohn

(Jacobs & Gerstein, 1965); Hiss's method for staining capsules (Jacobs & Gerstein, 1965); Schäffer and Fulton's method for staining spores (Tanner, 1944); Leifson's method for staining flagella (Harrigan & McCance, 1966; Skerman, 1967).

Observations of bacteria and bacterial sections were made with a Yeolko electron microscope, with specimens prepared as follows: For metal-shadowed specimens, 1 drop of a washed suspension was placed on a Formvar-coated copper grid (200 mesh), air-dried, and shadowed with carbon plus palladium at an angle of 24° . For negatively-stained specimens, 1 drop of a washed suspension was mixed with 1 drop of phosphotungstate (3 % w/v, pH 7.2), placed on a Formvar-coated copper grid (200 mesh), and air-dried. For ultramicrotomy, the bacteria were fixed by glutaraldehyde (4 %, w/v) in phosphate buffer, and post-fixed in osmium tetroxide (1 %, w/v). Dehydration was performed by passage through a graded series of ethanol solutions and the dehydrated bacteria were embedded in Epon; blocks were polymerized at 37°C for 12 h and then at 56°C for the next 12 h. Ultrathin sections were cut with an ultramicrotome (LKB, Sweden) and stained with uranyl acetate plus lead citrate.

Growth of bacteria was observed in aerobic and anaerobic conditions in media with different redox potentials, at $39 \pm 1^\circ\text{C}$. In anaerobic conditions the following observations were made: growth in medium without NaHCO_3 in a nitrogen atmosphere; the effect of growth on redox potential in glucose and cellobiose media; growth in media with pH values of 4.0 to 9.0 (the pH before inoculation was adjusted with NaOH or with HCl); growth in media with 2.5, 6.5, 8.5 and 9.5 % (w/v) NaCl; the production of catalase, indole (Bryant & Doetch, 1954; Skerman, 1967), acetoin (Bryant & Small, 1956), H_2S (van Gylswyk & Hoffman, 1970) and ammonium (Bryant & Burkey, 1953); the production of volatile fatty acids and of lactic acid from cellulose or from cellobiose after 24 h growth (Skulmowski, Honory, Honory & Szewczyk, 1969); gelatin liquefaction (Bryant & Burkey, 1953); casein hydrolysis in roll tubes (Blackburn & Hobson, 1962; Fulghum & Moore, 1963); starch hydrolysis after 1, 3 and 7 days incubation in a starch medium; carbohydrate fermentation in a nitrogen atmosphere after 24 h growth in media with half the usual amount of NaHCO_3 and 0.5 % (w/v) of the following carbohydrates – DL(–)arabinose, D(+)xylose, L(–)xylose, glucose, galactose, fructose, sorbose, maltose, sucrose, lactose, cellobiose, trehalose, raffinose, salicin, inulin, dulcitol, mannitol and cellulose; motility in the liquid media in capillary tubes under anaerobic conditions (observed under a phase contrast microscope); motility in semi-solid agar (Bryant & Small, 1956).

DNA isolation and purification was by the phenol method elaborated by A. Chomczyńska (1972, unpublished) at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences. Sodium citrate (0.025 M) was added to 3 l of the bacterial culture in medium (iii) at the end of exponential growth. The bacteria were then centrifuged at 0°C and washed twice with 0.1 M-EDTA plus 0.15 M-NaCl buffer, pH 8.2. The wet bacteria (1.9 g) were suspended in 17 ml of the above buffer solution, incubated 30 min at 37°C with lysozyme (0.1 %, w/v), then incubated 20 min at 56°C with Sarkosyl N.L.-30 (0.2 %, w/v). Protein digestion was performed with pronase (250 $\mu\text{g}/\text{ml}$) at 37°C for several hours. Deproteinization was repeated three times with a phenol at 4°C (pH 8.5) saturated with EDTA. DNA was precipitated with 96 % (v/v) ethanol: DNA threads on the glass rods were solubilized in 15 ml 0.015 M-NaCl + 0.0015 M-sodium citrate (DSC). Next day the DNA solution, with 1.2 ml 1.5 M-NaCl + 0.15 M-sodium citrate (CSC) added, was dialysed three times in 2 l 0.15 M-NaCl + 0.015 M-sodium citrate (SSC). When the last dialysis was finished, the solution was digested for 3 h with ribonuclease at 37°C (RNase I, conc. 200 $\mu\text{g}/\text{ml}$; RNase T_1 , conc. 20 $\mu\text{g}/\text{ml}$), and with pronase (1 mg/ml) for several hours afterwards. Then the solution was

Fig. 1. *Micromonospora ruminantium*, 24 h culture. Gram stain.

Fig. 2. *Micromonospora ruminantium*, 20 h culture. Electron micrograph, carbon-palladium shadowed, showing rods and a coccal form.

Fig. 3. *Micromonospora ruminantium*, 20 h culture. Electron micrograph, carbon-palladium shadowed, branching cell.

deproteinized three times with phenol saturated with SSC, the pH of the water phase being adjusted to 8.5, and the DNA was then precipitated with 96 % (v/v) ethanol. DNA threads on the glass rods were put into 2 ml DSC. After the DNA had dissolved, the solution was dialysed three times in 2 l SSC diluted 100 times. The concentration of DNA in the solution diluted 40 times was determined from the u.v. spectrum.

The adenine and guanine content of the DNA was determined according to Huang & Rosenberg (1966).

Redox potentials of bacterial cultures (Clark, 1960; Hankus, 1963) were determined using an LBS 66 potentiometer (Eureka, Warsaw) with platinum and calomel electrodes and an electrolytic bridge. The electrodes were immersed in cultures kept under a constant stream of carbon dioxide.

RESULTS

Pleomorphic, cellulolytic bacteria (10^6 bacteria/ml) were isolated from the rumen fluid of a sheep (Fig. 1). The bacteria were Gram-positive in very young cultures and later Gram-negative, and were non-motile and uncapsulated. They formed single spores at the end of the branched filaments. The spores absorbed malachite green less intensely than bacillus endospores. In pure culture, cocci, straight and curved rods, branched rods and filaments were observed. The width of cells varied from $0.33 \mu\text{m}$ to $0.73 \mu\text{m}$, being generally 0.4 to $0.5 \mu\text{m}$. The length of the bacteria varied considerably, the shortest (coccal forms) being $0.33 \mu\text{m}$ whereas rod forms were up to $3.2 \mu\text{m}$ and filaments were up to $100 \mu\text{m}$ in length. Figure 2 shows rods and a coccal form, and Fig. 3 a branching cell. Filaments were without cross-membranes (Fig. 4). During fission cells divided without previous cross-membrane formation (Fig. 5); this Figure also demonstrates that the wall structure is typical of that of Gram-positive bacteria.

The DNA contained $54.5 \pm 1.6\%$ GC and

$$\frac{A+T}{G+C} = 0.84.$$

Surface colonies of bacteria under a carbon dioxide atmosphere were rhizoidal (Fig. 6),

Fig. 4. *Micromonospora ruminantium*, 20 h culture. Electron micrograph, negative-stained by phosphotungstate, filament without cross membranes.

but deep agar colonies were lenticular. After 48 h growth the diameter of colonies was from 1.2 to 2.0 mm. Liquid culture became uniformly turbid and then the cells sedimented. The optimal temperature for growth was 40 °C. The bacteria grew at 45 °C, but did not grow at 50 °C. They did not grow below pH 5.5, or above pH 8.0. The maximum concentration of NaCl permitting growth was 4.5 % (w/v).

The bacteria did not produce catalase and did not grow aerobically. They grew well in media with a low redox potential. The optimum growth potential, between calomel and platinum electrodes, was -350 to -400 mV. In media without reducing agents, in which the potential before inoculation was +60 mV, the bacteria grew very slowly, the potential decreasing after 6 h incubation to -135 mV and after 8 h to -185 mV.

In 0.5 % (w/v) yeast extract and 1.5 % (w/v) tryptose media the bacteria grew without rumen fluid. They fermented several carbohydrates with the production of acids. The pH values after three days of incubation were as follows: D-glucose 4.3; fructose 4.8; galactose 4.6; maltose 4.4; raffinose 4.4; cellobiose 5.0; salicin 5.5.

Acid was not produced from: DL(-)arabinose, L(-)xylose, sorbose, sucrose, lactose, trehalose, glycerol, dulcitol, D(-)mannitol, aesculin or inulin, the pH of the medium being unchanged at 6.8.

They hydrolysed starch, carboxymethylcellulose and cellulose (Fig. 7), and disintegration of the paper in the paper strip medium occurred within 3 days of incubation.

Fig. 5. *Micromonospora ruminantium*, 20-h-old. Ultrathin section. Fixed with glutaraldehyde and osmium tetroxide and stained with uranyl acetate and lead citrate.

Fig. 6. *Micromonospora ruminantium*, 3-days-old, surface colonies (not stained) on cellobiose agar medium incubated under a constant stream of CO₂.

Fig. 7. *Micromonospora ruminantium*, 14-days-old, zones of cellulose digestion in filter-paper strips

Table 1. Acid production by *Micromonospora ruminantium* after 48 h of incubation in cellulose and in cellobiose media

Substrate	Acid (mmol/100 ml)			
	Lactic	Acetic	Propionic	Butyric
Cellulose	0.774	0.496	0.143	0.0318
Cellobiose	0.930	0.606	—	—

Valeric acid stimulated cellulolytic activity. The cellulolytic activity depended on the kind of rumen fluid used as a component of the medium. Rumen fluid from a sheep fed on lucerne gave better cellulolytic activity than rumen fluid from a sheep fed on hay. Bacteria transferred several times through yeast extract and tryptose media without rumen fluid temporarily lost cellulolytic activity, as did bacteria transferred several times through media containing only simple sugars. Cellulolytic activity was restored by 6 to 8 transfers through cellulose medium.

Mainly lactic and acetic acids with small amounts of propionic and butyric acids were produced from cellulose. With cellobiose as a substrate in the medium without rumen fluid, only acetic and lactic acids were detected (Table 1). The bacteria did not liquefy gelatin, did not decompose milk protein, produced ammonia very slowly from peptone, and did not produce H_2S , acetoin, or indole.

DISCUSSION

According to the definition of Gall & Huhtanen (1951) 'true' rumen micro-organisms are anaerobic, they produce from rumen substrates compounds normally present in the rumen, and their numbers in rumen contents are more than 10^6 /ml. Rumen microbial species should be common to ruminants living in different geographical regions.

The bacteria described in this work, identical with *Micromonospora* Orskov, 1923 as described by Prévot (1966), have the characteristics of true rumen micro-organisms. *Micromonospora* as a rumen genus was mentioned by Hungate in 1946. van Gylswyk (1970), isolating cellulolytic rumen bacteria, found as a contaminant Gram-negative rods and filaments. It seems possible that both authors observed the micro-organisms isolated by us.

The only known anaerobic cellulolytic species of *Micromonospora* (*M. propionici*) was isolated from the alimentary tract of a termite (Hungate, 1946). The species described in this work differs from *M. propionici* in source of isolation, morphology of colonies, cellulolytic activity and products of cellulose decomposition, since our strain produces mainly lactic and acetic acids. We propose for it the name *Micromonospora ruminantium*.

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The type culture of *Micromonospora ruminantium* is deposited in the American Type Culture Collection and has been assigned the ATCC accession number 27728.

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