INTERNATIONAL JOURNAL OF SYSTEMATIC BACTERIOLOGY, Jan. 1982, p. 87-91 Vol. 32, No. 1
0020-7713/82/010087-05$02.00/0

Isolation and Characterization of an Anaerobic, Cellulolytic Bacterium, *Clostridium papyrosolvens* sp. nov.

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Clostridium papyrosolvens, a new species of cellulolytic, sporeforming, anaerobic bacteria, is described. The colonies produced by these bacteria in cellulose agar roll-tubes were spherical, translucent, unpigmented, and of granular appearance. Single cells of the bacterium were straight rods, 0.5 to 0.8 μm by 2 to 5 μm, peritrichous, and motile. Spherical terminal spores 1 to 1.2 μm in diameter were formed. Fermentation products from cellulose included hydrogen, carbon dioxide, ethanol, acetate, and lactate. The deoxyribonucleic acid base composition of the type strain of *C. papyrosolvens*, NCIB 11394, is 30 mol% guanine plus cytosine. The specific epithet *papyrosolvens* reflects the ability of the organism to ferment filter paper.

The Microbiology Department of Aberdeen University has conducted studies on the microbial ecology of the estuarine sediments of the River Don, Aberdeenshire, Scotland, for several years (9, 11). Part of these studies concentrated on the population of cellulolytic bacteria which was maintained by the input of paper-mill effluent.

During studies on the organisms comprising the community present in the anoxic sediments which mineralized the cellulose, a pure culture of a strictly anaerobic, cellulolytic bacterium was isolated. This report describes the isolation and characterization of this organism.

MATERIALS AND METHODS

Media. The solid medium used in this study consisted of: NH_4Cl, 2.0 g; yeast extract, 1.2 g; agar no. 3 (Oxoid), 20.0 g; K_2HPO_4, 1.65 g; cysteine hydrochloride, 0.5 g; resazurin solution (0.1%, wt/vol), 1 ml; filtered seawater, 200 ml; mineral solution, 150 ml; cellulose suspension, 200 ml; and distilled water to 1 liter. The pH was adjusted to 7.2 with 5 M NaOH.

The cellulose suspension used was 4% (wt/vol) Whatman CF11 cellulose powder, ball-milled for 72 h.

The mineral solution consisted of: $(NH_4)_2 SO_4$, 6.0 g; NaCl, 6.0 g; $MgSO_4$, 0.6 g; $CaCl_2$, 0.6 g; and distilled water to 1 liter.

The liquid medium used was similar to the solid medium except that it lacked agar and the cellulose suspension and had lower concentrations of NH_4Cl and yeast extract (1.0 and 0.6 g/liter, respectively). Cellulose was provided as a strip (9.0 by 1.0 cm) of Whatman no. 1 filter paper in each tube.

The solid medium was dispensed in tubes in 4.5-ml volumes, and the liquid medium was dispensed in 10-ml volumes.

When carbon sources other than cellulose were used, the material was dissolved in the liquid medium (3%, wt/vol) which was then boiled to remove oxygen. The medium was cooled under nitrogen, and 2-ml volumes were added to previously autoclaved (121°C for 15 min) tubes of liquid medium (10 ml) by filter sterilization.

Blanks for serial dilutions were prepared from 2% (wt/vol) NaCl containing 0.5 g of cysteine hydrochloride and 1 mg of resazurin per liter. The diluent was adjusted to pH 7.0 with 0.5 M NaOH prior to being dispensed in 9-ml volumes into McCartney bottles.

Anaerobic culture methods. The anaerobic techniques of Hungate (7) were used throughout this study. Standard test tubes (19 by 150 mm, Corning Glass Works) sealed with Suba Seal no. 29 closures (William Freeman Ltd., Barnsley, Great Britain) were used as culture tubes. Hungate roll tubes of solid media were used for the identification and selection of cellulolytic bacteria. Routine incubations were performed at 25°C. Oxygen-free nitrogen was used as the atmosphere.

Isolation procedures. Anaerobic mud was obtained from intertidal mud banks of the River Don, Aberdeenshire, Scotland, by means of the sampling system described by Parkes et al. (11). Sediment from a depth of 5 cm was diluted 100-fold, and 0.2-ml samples were inoculated into triplicate tubes of liquid medium. After 10 days, 0.2-ml samples were subcultured into fresh liquid medium; this procedure was repeated twice. The final series of tubes was incubated for 2 months.

Medium from the final cultures was then serially diluted into roll tubes which were heat shocked at 70°C for 15 min, prior to rolling, to induce spore germination. After 3 weeks of incubation, cellulolytic colonies were transferred to cellobiose medium by means of a bent Pasteur pipette (7). After 5 days, subcultures (0.2-ml inocula) were made in fresh cellobiose medium. The subcultures were again incubated for 5 days and were then serially diluted into roll tubes. Subcultures

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(0.2-ml inocula) in liquid medium were also made.

Culture purity was then assessed by microscopic examination and by examination of roll tubes for noncellulolytic colonies.

Morphology. Living and stained cells were examined by light microscopy. Flagella were examined with a Seimens Elmiskop 102 transmission electron microscope. A 2-day-old culture grown on cellobiose was harvested by centrifugation for 20 min at 1,500 × *g* (average) and suspended in distilled water and centrifuged as before. This washing procedure was repeated twice. A drop of the suspension of washed cells was placed on a Formvar-coated copper grid and was shadowed with gold-palladium (40:60) at an angle of 20°C.

Biochemical reactions. Biochemical characteristics were studied by the methods described by Holdeman et al. (4).

DNA base composition. Deoxyribonucleic acid (DNA) was isolated by the method of Ng et al. (10). The guanine-plus-cystosine content of the DNA was determined by thermal denaturation (3). *Escherichia coli* K-12 DNA (Sigma Chemical Co.) was used as a standard.

Fermentation end-product analyses. To detect volatile fatty acids and alcohols as end products of fermentation, samples of media (2.5 ml) were deproteinized by the addition of 0.1 ml of $ZnCl_2$ (10%, wt/vol) and were centrifuged at 12,000 × *g* (average) for 15 min.

Five microliters of supernatant was injected into a gas chromatograph fitted with a Chromosorb 101 (80/100 mesh) column, 1.5 m by 3.18 mm (inside diameter). The chromatograph was fitted with a flame ionization detector, and the following conditions were selected for the analysis: column temperature, 110°C on injection increasing by 15 C degrees/min to 180°C, where it was held until all of the volatile fatty acids had eluted; carrier gas, 1 bar of nitrogen at the column head; injector temperature, 180°C; detector temperature, 240°C; hydrogen flow rate, 25 cm^3/min; and air flow rate, 400 cm^3/min.

Gases were analyzed by removing a sample (0.5 ml) from the headspace of a culture tube by means of a 1-ml syringe and injecting the sample into a gas chromatography apparatus fitted with a silica gel (80/100 mesh) column, 1.5 m by 3.18 mm (inside diameter). The effluent from the silica gel column was fed into a molecular sieve 5A (40/60 mesh) column, 1.5 m by 3.18 mm (inside diameter). The column temperature was 80°C, and the carrier gas was argon. The injection temperature was 150°C, and the detector temperature was 160°C. Effluent gases were detected by thermal conducter with a filament current of 100 mA.

Growth rate and temperature for optimal growth. For growth-rate determinations, 100 ml of liquid medium plus 10 strips (4.5 by 1 cm) of Whatman no. 1 filter paper were placed in a 250-ml spherical flask and sealed with a butyl rubber bung. The flask was inoculated with 10 ml of medium from a 5-day-old culture grown on filter paper. The flask was incubated at 28°C and sampled at 12-h intervals. Samples (0.5 ml) were obtained by piercing the bung with a syringe and needle. The pH's, at 25°C, of the samples were noted before the quantities of acetate and ethanol were determined.

The temperature for optimal growth was determined by inoculating a batch of 18 tubes of liquid medium with 0.2 ml of a 5-day-old culture grown in a similar medium. Triplicate tubes were then incubated for 14 days at each temperature studied. The pH of the medium was then measured at 25°C, and the quantity of cell protein produced was determined.

Cell protein was estimated by centrifuging the entire contents of a tube at 10,000 × *g* (average) for 15 min. The supernatant was discarded, and the pelleted cells were suspended in 10 ml of 2% (wt/vol) NaCl. The washed cells were again harvested by centrifugation at 10,000 × *g* (average) for 15 min. The supernatant was discarded, and the cells in the pellet were lysed by suspending them in 0.2 M NaOH (2 ml). The protein in the suspension was determined by the method of Lowry et al. (8). Before measurement of the optical density, the medium was clarified by centrifugation at 5,000 × *g* (average) for 5 min to pellet residual cellulose. Bovine serum albumin (Sigma Chemical Co.) was used as a standard.

Lactate determination. Lactate was determined enzymatically by means of a lactate dehydrogenase-based kit (Boehringer Mannheim GmbH, Biochemica, Mannheim, West Germany).

RESULTS

The enrichment and selection procedures used resulted in the isolation of a pure culture of an obligately anaerobic, cellulolytic organism. When heat-shocked spore inocula were used, visible colonies were produced in roll tubes in 7 to 10 days, and after 3 weeks, the colonies were 1 to 2 mm in diameter, unpigmented, and granular in appearance. Further incubation resulted in a darkening of the colonies to a pale-tan color. Clear zones, showing cellulose hydrolysis, were generally 1 to 2 cm in diameter in high-dilution tubes after an incubation of 3 weeks, but they continued to extend, and zones larger than 3 cm in diameter were not uncommon after prolonged incubation (1 to 2 months).

During this study it was noted that spores inoculated into roll tubes did not germinate unless a thermal shock was applied. One batch of four roll tubes was incubated for 1 year, and no cellulolytic colonies were visible. However, after a heat shock, cellulolytic colonies appeared 10 to 14 days later.

The isolate consisted of straight rods, 0.5 to 0.8 μm by 2.0 to 5.0 μm, with terminal spores 1 to 1.2 μm in diameter. It was motile and peritrichous (Fig. 1).

Growth in nutrient broth without fermentable carbohydrate was slight; with fermentable carbohydrate, growth was moderate. Milk was unchanged.

Table 1 lists the biochemical characteristics of the isolate.

The fermentation of cellulose resulted in the production of acetate, ethanol, lactate, carbon dioxide, and hydrogen. The mean generation time of a cellulose-grown culture incubated at 28°C was estimated as 20 h, based on the total

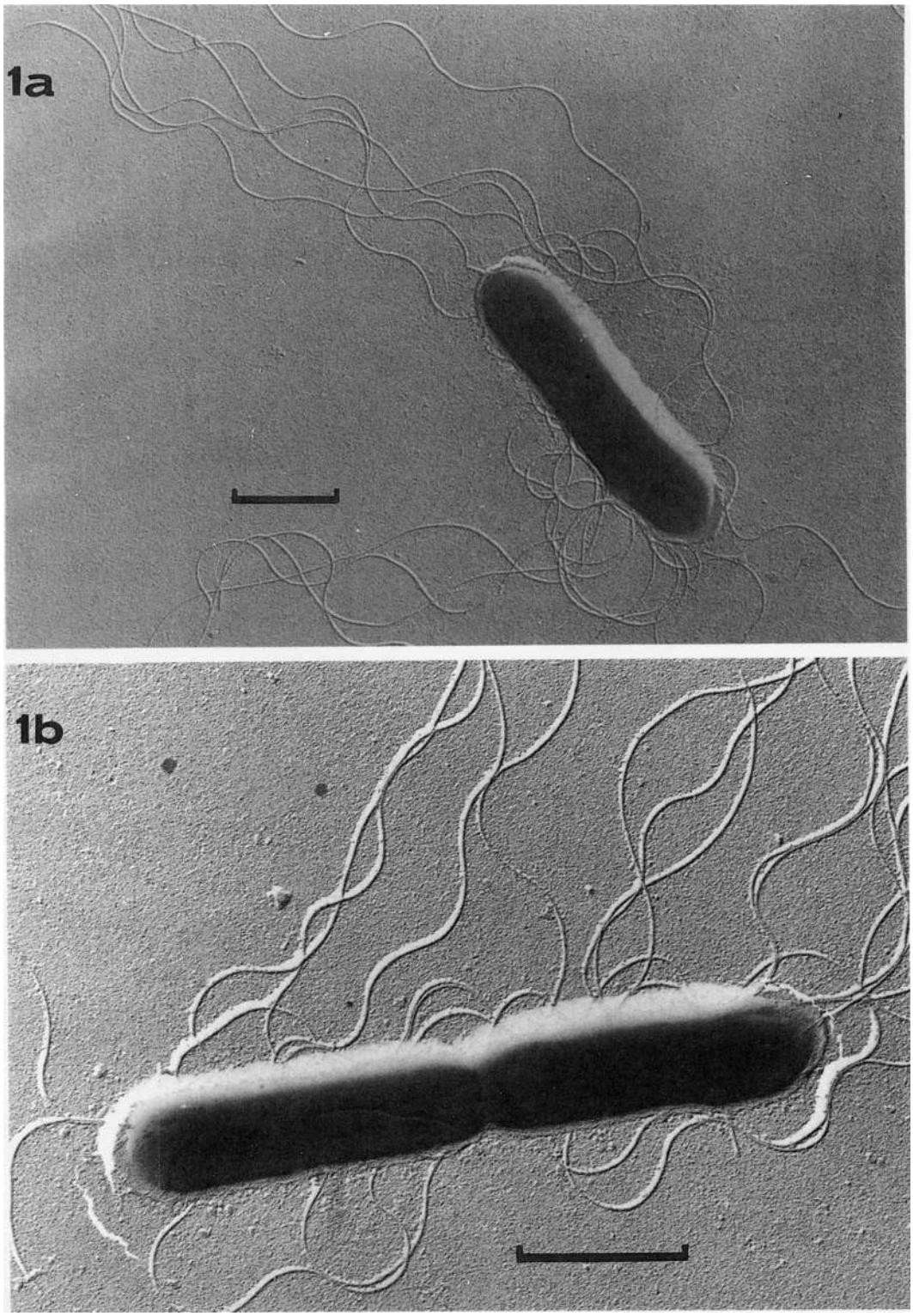

FIG. 1. Electron micrographs depicting cell morphology and flagella. (A) Single peritrichous cell. The bar represents 1 μm (×15,000). (B) Dividing cell. The bar represents 1 μm (×24,000).

TABLE 1. Biochemical characteristics of the new isolate

Assays giving negative results:				
Acetylmethylcarbinol production		Blood hemolysis		Casein hydrolysis
Catalase production		Gelatin liquefaction		H_2S production
Lecithinase production		Lipase production		Nitrate reduction
Urease production				
Carbohydrates not fermented:				
Adonitol	Amygdalin	Chitin	Dulcitol	Erythritol
Glycogen	Inositol	Inulin	Lactose	Mannitol
Maltose	Mannose	Melezitose	Melibiose	Raffinose
Rhamnose	Salicin	Sorbitol	Sorbose	Sucrose
Trehalose				
Carbohydrates fermented:				
Arabinose	Cellobiose	Cellulose	Esculin	Fructose
Galactose	Glucose	Glycerol	Ribose	Xylose

concentration of ethanol and acetate produced (9).

The temperature range for optimal growth on cellulose was 25 to 30°C. Growth occurred at 15°C. Slight growth was noted at 45°C.

During investigations to determine the temperature for optimal growth, it was discovered that spore inocula did not result in viable cultures at 37°C or above, whereas vegetative cells did grow at these temperatures. Subsequently, in all experiments involving growth vegetative cells were used as inocula.

The guanine-plus-cytosine content of the DNA of the isolate was 30 mol%.

Vegetative cells were readily decolorized and hence were gram negative. However, transmission electron microscopy of sections revealed a cell wall structure typical of gram-positive organisms.

DISCUSSION

On the basis of the identification scheme in the 8th edition of *Bergey's Manual* (1), the isolate was identified as a member of group III of the genus *Clostridium*. The 1980 Approved Lists of Bacterial Names (12) were used to exclude comparison of the isolate with species whose names had no standing in nomenclature. Subsequent comparison of the isolate with published descriptions of all group III species (4, 13) revealed that the isolate was most similar to *Clostridium cellobioparum*, the only member of this group currently recognized as cellulolytic.

Comparison of the new isolate with the original description of *C. cellobioparum* (5) showed significant differences in colony morphology. Young colonies of *C. cellobioparum* were disk shaped and compact in glucose shakes but were irregular in cellulose agar (5). The new isolate produced regular spherical colonies in similar media. Cell morphology was similar to that of *C. cellobioparum*, as originally described. However, a later description noted that *C. cellobioparum* can produce oval spores (13), and these were never seen in cultures of the new isolate. Descriptions of the biochemical characteristics of *C. cellobioparum* differ slightly, but on the basis of these, *C. cellobioparum* can be readily distinguished from the new isolate. The principal differences are shown in Table 2. The growth of the new isolate at 15°C contrasts with the inhibition of growth of *C. cellobioparum* at 25°C (13), although it should be noted that a temperature of 18°C was originally recorded for the latter (5). It is also significant that *C. cellobioparum* was isolated by means of enrichment and selection procedures performed at 38°C (5). Such procedures favor the isolation of sporeforming organisms (6), but since the spores of the new isolate do not produce viable cultures at this temperature, the organism would be actively selected against. Finally, *C. cellobioparum* has been reported to produce butyrate when fermenting glucose (2), but butyrate was never detected in cultures of the new isolate during fermentation of glucose, cellobiose, cellulose, or esculin.

In summary, a cellulolytic organism was isolated, and its initial characterization showed it to be a member of group III of the genus *Clostridium*. A comparison of the isolate with the most similar organisms of this group showed significant differences, and the isolate is therefore proposed as a new species, *Clostridium papyrosolvens* (L. noun *papyrus* paper; L. verb *solvere* to dissolve; M.L. adj. *papyrosolvens* paper-dissolving; intended to reflect the organism's ready fermentation of filter paper).

Clostridium papyrosolvens sp. nov. Straight rods, 0.5 to 0.8 μm by 2.0 to 5.0 μm. Motile and peritrichous. Endospores are spherical and terminal. Gram negative.

Deep colonies in cellulose agar are 1 to 2 mm in diameter, granular, and unpigmented.

TABLE 2. Principal differences between the new isolate and *C. cellobioparum* as described by Hungate (5) and in *Bergey's Manual* (13) and the *Anaerobe Laboratory Manual* (4)[a]

Determination	*C. cellobioparum*			New isolate
	Hungate	*Bergey's Manual*	*Anaerobe Laboratory Manual*	
Carbohydrate fermentation				
Dulcitol	NR	+	+	−
Glycerol	−	W	W	+
Lactose	S	W	W	−
Maltose	+	+	+	−
Mannitol	NR	W	W	−
Mannose	+	+	+	−
Melibiose	+	+	+	−
Raffinose	S	+	W	−
Sorbitol	NR	+	W	−
Mol% guanine plus cytosine	NR	25	28	30

[a] NR, not determined; W, weakly fermented; S, occasionally and slowly fermented.

Slight growth occurs in nutrient broth without fermentable carbohydrate; moderate growth occurs in the presence of a fermentable carbohydrate.

Ferments cellulose. Fermentation products include ethanol, acetate, lactate, hydrogen, and carbon dioxide.

Acetylmethylcarbinol is not formed.

Milk is unchanged.

Found in an estuarine anaerobic sediment.

The guanine-plus-cytosine content of the DNA is 30 mol%.

Type strain: National Collection of Industrial Bacteria strain NCIB 11394. Since the species is based on a single strain, the type strain, the description of the type strain is the same as that given above for the species.

ACKNOWLEDGMENTS

We thank A. MacKenzie and G. Hobbs for advice and assistance during this work. S. J. Joles provided essential technical assistance in obtaining sediment samples, and W. Hodgkiss produced the electron micrographs.

REPRINT REQUESTS

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