Bacteroides cellulosolvens sp. nov., a Cellulolytic Species from Sewage Sludge†

WILLIAM D. MURRAY,* L. C. SOWDEN, AND J. ROSS COLVIN

Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada K1A 0R6

A new mesophilic, cellulolytic species of *Bacteroides* was isolated from a methanogenic cellulose enrichment culture of municipal sewage sludge and is described. This species ferments only cellulose and cellobiose. The fermentation products are acetic acid, CO₂, H₂, ethanol, and small amounts of lactic acid. The deoxyribonucleic acid base composition is 43 mol% guanine plus cytosine. The name *Bacteroides cellulosolvens* is proposed. Type strain WM2 is deposited in the National Research Council of Canada culture collection as strain NRCC 2944.

A mesophilic coculture consisting of a cellulolytic anaerobe and a saccharolytic anaerobe was isolated from a cellulose enrichment culture started from municipal sewage sludge. These two anaerobes grew on cellobiose agar as a single, slimy colony (6). The saccharolytic member of this coculture, *Clostridium saccharolyticum*, has been isolated and described previously (13). In this paper, we describe the isolation and characteristics of the cellulolytic microbe and propose for it the name *Bacteroides cellulosolvens*.

MATERIALS AND METHODS

Media. The basal medium used for isolation and maintenance of the strain which we studied had the following composition (in milligrams per liter): NaHCO₃, 2,000; NH₄Cl, 680; K₂HPO₄, 296; KH₂PO₄, 180; (NH₄)₂SO₄, 150; MgSO₄ · 7H₂O, 120; CaCl₂ · 2H₂O, 61; FeSO₄ · 7H₂O, 21; N(CH₂COOH)₃, 15; NaCl, 10; MnSO₄ · H₂O, 5; CoCl₂ · 6H₂O, 1; ZnSO₄ · 7H₂O, 1; CuSO₄ · 5H₂O, 0.1; AlK(SO₄)₂ · 12H₂O, 0.1; H₃BO₃, 0.1; Na₂MoO₄ · 2H₂O, 0.1; pyridoxine hydrochloride, 0.1; thiamine hydrochloride, 0.05; riboflavin, 0.05; nicotinic acid, 0.05; *p*-aminobenzoic acid, 0.05; lipoic acid, 0.05; biotin, 0.02; folic acid, 0.02; vitamin B₁₂, 0.005; and resazurin, 1 (7). This medium was prepared by the procedure of Holdeman et al. (3) and was pre-reduced by the Hungate technique (5), using 250 mg of cysteine hydrochloride per liter and 250 mg of Na₂S · 9H₂O per liter as the reducing agents. The reduced medium was dispensed under 80% N₂-20% CO₂ in 10-ml amounts into 60-ml serum vials (11) containing preweighed amounts of insoluble substrates, such as cellulose. The vials of media were then autoclaved at 104 kPa for 15 min. The soluble substrates were filter sterilized and were injected by means of hypodermic syringes into the basal medium after the vials had been autoclaved and cooled. The final pH of all media was 7.0 ± 0.2. The isolated strain was maintained at 35°C in basal medium containing approximately 0.5% (wt/vol) cellulose in the form of small squares of 4-ply facial tissue.

Isolation. All isolation procedures were conducted anaerobically in serum vials or in an anaerobic chamber.

Biochemical tests. The procedures of Holdeman et al. (3) were used for biochemical characterization. Selected substrates were added to the basal medium at concentrations of 1% (wt/vol). When a test required the addition of a fermentable substrate (e.g., sulfate reduction medium), 0.5% cellobiose was used. The test media were inoculated with 0.05 ml of

a 72-h-old culture grown in cellulose broth and were incubated at 35°C. Media containing cellulose were incubated on a rotary shaker. Test results were determined after 72 h and 7 days. Negative tests were held for 4 weeks. Metabolic products were quantified by gas chromatography. Gas volume was measured with a gas manometer, and the gas composition (N₂, CO₂, and H₂) was determined by gas chromatography, using the method of van Huyssteen (16). Alcohols and volatile acids were assayed by the method of Ackman (1), and nonvolatile acids were methylated by the procedure of Holdeman et al. (3) before determination by gas chromatography.

Temperature and pH studies. Temperature and pH ranges and optima were determined in cellobiose broth. Growth was measured spectrophotometrically at 650 nm in cuvettes with a 1-cm light path.

Electron micrographs. For electron microscopy, cells were diluted in distilled water, placed on Formvar films, and dialyzed overnight. The films were then placed on carbon-coated grids, shadowed with Pt-Pd, and observed with a Siemens model 101 electron microscope.

DNA base composition. The deoxyribonucleic acid (DNA) was isolated and purified by the Marmur method (9) from cells grown for 72 h. The DNA base composition was determined by ultraviolet spectroscopy, using the method of Ulitzer (15). *Escherichia coli*, *Bacillus subtilis*, and salmon sperm DNAs, which had guanine-plus-cytosine contents of 51, 42.5, and 44.4 mol%, respectively, were used as standards.

RESULTS AND DISCUSSION

Cellulolytic strain WM2^T (T = type strain) was obtained in pure culture by penicillin counterselection. The failure of conventional techniques to separate the cellulolytic anaerobe from *C. saccharolyticum* was presumed to be due to the slimy nature of the symbiotic coculture (6). However, the cellulolytic microbe did not grow in glucose-yeast extract medium and, unlike *C. saccharolyticum*, was not affected by the action of penicillin. Basal medium containing 1% glucose and 1% (wt/vol) yeast extract was inoculated with a coculture and incubated at 35°C for 2 h before penicillin G was added at a concentration of 0.5 mg/ml. This initial 2-h incubation period allowed the cellulolytic species time to exhaust its endogenous energy reserves and cease growth. After 24 h of incubation the cells were washed free of penicillin. Dilutions were spread onto basal agar medium containing 1% cellobiose, and the cultures were incubated at 35°C until colonies were visible. Colonies were picked and

* Corresponding author.

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replica-plated on cellobiose agar and glucose-yeast extract agar. Colonies which grew on cellobiose agar but not on glucose-yeast extract agar were found to be free of the contaminating *C. saccharolyticum*.

Strain WM2^T is an anaerobic, gram-negative, nonspore-forming, nonmotile, rod shaped organism which produces acetic acid as its major metabolic product and appears to belong in the genus *Bacteroides*. The morphological and biochemical characteristics of strain WM2^T do not match the characteristics of any of the species listed previously in the genus *Bacteroides* (3, 4). Therefore, we propose the establishment of a new species, *Bacteroides cellulosolvans* (cell. u.lo.sol'vens. L. v. *solvere* to dissolve; N.L. adj. *cellulosolvans* cellulose dissolving), so named because of its ability to ferment cellulosic substrates.

The cells are straight, rod-shaped, approximately 0.8 by 6 μ m, and occur singly. They are nonmotile, and no flagella are detected by staining (10) or electron microscopy. Extensions of the cell wall give this microbe a distinctive rough amorphous appearance when it is grown on insoluble cellulosic substrates (Fig. 1). An ultrastructure study has shown that these projections are extensions of the cell wall rather than capsular material. The projections do not occur when the organism is grown in cellobiose broth. Thin cross-sections show the typical tripartite cell wall structure of gram-negative aerobes.

Spores are not demonstrated by malachite green staining of 1-week-old cultures or by treatment of cultures with 50% ethanol for 1 h (8). Cultures grown in facial tissue broth that are 2 days old are still viable after being held for 10 min at temperatures up to 75°C; however, heat tolerance decreases with culture age.

Bright yellow, circular, convex colonies with rough margins are produced on cellobiose agar. The colonies reach a maximum diameter of 1.0 to 1.25 mm after 5 days of growth at 35°C. On Solka Floc agar, the colonies reach their maximum size after 10 days of growth and show zones of

cellulose clearing around the periphery of the colonies (Fig. 2) and in the agar below.

The temperature range for growth is 20 to 45.5°C, with optimum growth at 42°C. Facial tissue squares in basal broth are hydrolyzed within 21 days at 20°C. A bright yellow sediment is produced in cellulose broth and cellobiose broth at growth temperatures between 20 and 40°C, but not at temperatures above 40°C. *B. cellulosolvans* grows within a pH range of 5.7 to 8.0, with optimum growth at pH 7.0.

Anaerobic medium is required for growth. Of the substrates tested, only cellulose and cellobiose support growth. *B. cellulosolvans* ferments facial tissue, ball-milled pulp (Solka Floc), microcrystalline cellulose (Avicel), filter paper, absorbent cotton batting, cheesecloth, and steam-exploded aspen wood chips. No growth occurs in basal medium supplemented with adonitol, amygdalin, arabinose, dulcitol, esculin, erythritol, fructose, galactose, glucose, glycerol, glycogen, inositol, inulin, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sorbose, starch, sucrose, trehalose, xylan, xylose, Casamino Acids, casein, chopped meat, milk, peptone, yeast extract, hippurate, lactate, pyruvate, or urea.

The following fermentation products from cellobiose broth are detected after 3 days of incubation (in millimoles per liter): H₂, 36.7; CO₂, 30.0; acetic acid, 17.1; ethanol, 8.6; and lactic acid, 1.2. The same products and yields are formed after 5 days from fermentation of cellulose. Acetylmethyl-

FIG. 1. Electron micrographs of *Bacteroides cellulosolvans* grown in cellobiose broth (a) and in cellulose (facial tissue) broth (b). Bars = 1 μ m.

FIG. 2. *Bacteroides cellulosolvans* colony growth on Solka-Floc agar. Bar = 1 mm.

carbinol is produced; ammonia, indole, catalase, urease, and gelatinase are not. Sulfate and nitrate are not reduced.

The addition of bile (2% oxgall) to cellobiose broth inhibits growth, whereas Tween 80, hemin, and vitamins B₁₂ and K₁ have no effect. Rumen fluid increases metabolite formation and growth. Unlike other species of *Bacteroides* (2, 17), the addition of complex substances to the basal medium does not influence the formation of fermentation products by *B. cellulosolvens*. Rumen fluid increases product yield but does not shift product ratios.

The DNA base composition is 43 mol% guanine plus cytosine.

B. cellulosolvens type strain WM2 is deposited in the National Research Council of Canada culture collection as strain NRCC 2944.

Cellulolytic *Bacteroides succinogenes* differs from *B. cellulosolvens* by fermenting glucose, lactose, maltose, sorbose, starch, and trehalose, by consuming CO₂ during cellulose fermentation, and by producing succinic acid (4).

Metabolically, *B. cellulosolvens* resembles *Acetivibrio cellulolyticus*. Both of these organisms were isolated from a methanogenic cellulose enrichment culture started from municipal sewage sludge. They use cellulose and cellobiose as substrates, and their major metabolic products are acetic acid, CO₂, and H₂ (14). However, *B. cellulosolvens* is morphologically distinct, and unlike *A. cellulolyticus*, it is nonmotile and unable to metabolize salicin.

Miyoshi (12) reported the isolation of 11 strains of anaerobic, cellulolytic, marine bacteria, all tentatively belonging to the genus *Bacteroides*. Six of these strains produced a yellow pigment, used cellulose and cellobiose as their sole carbon sources, and produced acetic acid as their major product. However, these strains differ from *B. cellulosolvens* in producing isobutyric acid, liquefying gelatin, and having an optimum growth temperature of 30°C.

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