Emendation of the Genus *Acetivibrio* and Description of *Acetivibrio cellulosolvens* sp. nov., a Nonmotile Cellulolytic Mesophile†

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The genus *Acetivibrio* is emended to include nonmotile, gram-negative, obligately anaerobic rods that produce acetic acid and ethanol from fermentation of carbohydrates. A new species, *Acetivibrio cellulosolvens*, which was isolated from a cellulose-enriched culture of sludge, is described. The colonies on agar are entire, raised, and cream colored. The cells are straight rods, gram-negative, nonsporeforming, and nonmotile. The isolate which we studied ferments cellobiose, cellulose, esculin, and salicin and does not require yeast extract or rumen fluid for growth. The major end products produced from cellobiose or cellulose are acetic acid, ethanol, CO₂, and H₂. Lactic acid and glucose also are produced in small amounts. The type strain of *A. cellulosolvens* is strains BAS (= NRC 2936).

A cellulolytic anaerobe that was isolated from a heat-treated, cellulose-enriched culture (8) started from sewage sludge is described. This nonsporeforming, mesophilic, rod-shaped anaerobe has the ability to survive heating at 80°C for 10 min in mixed cultures, but not in pure culture. The new isolate is described as *Acetivibrio cellulosolvens* sp. nov.

MATERIALS AND METHODS

Media. The basal medium used for isolation and culture maintenance was the same medium that was used for the development of cellulose-enriched cultures (7). This basal medium contained the following components (in milligrams per liter): NaHCO₃, 2.060; 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; H₃BO₄, 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. This medium was supplemented with cellulose (type CF-11; Whatman) or tissue paper (Kleenex; Kimberly-Clark of Canada, Ltd.) and with 2% (wt/vol) agar (Difco Laboratories, Detroit, Mich.) for culture isolation. The pH of the medium was adjusted to 7.0 ± 0.2, and the medium was reduced by using a cysteine-sodium sulfide solution and the Hungate technique (5). A gas mixture containing 80% N₂ and 20% CO₂ was used as a headspace gas. Usually, 10-ml portions of the prereduced medium were dispensed into 60-ml serum vials. All other media and reagents required for characterization tests were prepared as described by Holdeman et al. (3).

Isolation and stock culture maintenance. The inoculum used for isolation was obtained from a fermentor that was started by using a cellulose-enriched culture and was heated at 80°C for 10 min (8). The cellulolytic anaerobes present in this heat-treated culture survived repeated heating at 80°C for 10 min. The inoculum was serially diluted in the basal medium. Each dilution (0.1 ml) was disseminated onto a

filter paper placed over agar slants. For this purpose, the agar slants were prepared inside 160-ml serum vials; any excess moisture present inside these vials was removed, and presterilized filter papers were placed over the slants. All of these procedures were carried out under an N₂-CO₂ atmosphere and aseptic conditions. This method was preferred over the conventional petri dish technique, as growth of these anaerobes depends on direct contact between them and the cellulose. After incubation for 4 to 7 days, the colonies were picked and transferred into broth basal medium containing tissue papers (2 g/liters). After complete dissolution of the tissue paper, the broth was serially diluted and plated onto an agar slant. This procedure was repeated three times. A stock culture of the isolate was maintained on basal medium containing tissue paper by transferring at 3- to 4-day intervals. Incubation was carried out at 35 to 37°C with shaking.

Biochemical tests and electron microscopy. Biochemical tests and substrate utilization tests were carried out in basal medium containing the desired substrate (0.5 or 1.0%, wt/vol) by using the procedures described by Holdeman et al. (3). Inoculated vials were incubated at 35°C and tested at 48-h and 7-day intervals. All negative tests were held for 2 weeks. Analyses were made to determine gas volume and composition (H₂ and CO₂), as well as volatile and nonvolatile acids and alcohols (3, 6). To determine the optimum pH for growth, the initial pH of the broth was adjusted in about 0.5-pH unit increments between pH 5 and 9. Cells harvested from a 72-h-old cellobiose broth culture were used for extraction of deoxyribonucleic acid, and the guanine-plus-cytosine content was determined as described by Marmur (11), Markov and Ivanov (10), and Ulitzur (17). For electron microscopy, cultures were grown in basal medium containing cellobiose as the sole carbon source and were incubated without shaking. Electron microscopy was carried out on 2- and 4-day-old cultures as described previously (2). Gel electrophoresis of extracellular proteins was carried out by using the method of Wood (18).

RESULTS AND DISCUSSION

A new strain was isolated by serial dilution of heat-treated cellulose-enriched domestic sewage sludge streaked onto cellulose layered agar. On the basis of Gram staining,

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FIG. 1. Cell morphology of a culture grown in cellobiose broth.

metabolic properties, and guanine-plus-cytosine content of the deoxyribonucleic acid, this obligately anaerobic rod-shaped organism was assigned to the genus *Acetivibrio* (4, 12). The two species currently in this genus are motile by single or multiple flagella. Therefore, we emend the description of the genus *Acetivibrio* (14) to include nonmotile aflagellate species with cellular and metabolic properties similar to those of *Acetivibrio cellulolyticus* (15) and *Acetivibrio ethanolgignens* (16).

A description of *A. cellulosolvans* sp. nov. is given below.

***Acetivibrio cellulosolvans* sp. nov.** *Acetivibrio cellulosolvans* (cel. lu. lo. sol. vens. L. verb *solvere* to dissolve; N.L. adj. *cellulosolvans* cellulose dissolving, indicating the ability of this organism to ferment cellulosic substrates) cells are obligately anaerobic, gram negative, rod shaped, and straight and occur singly, in pairs, or in chains of three or more cells (Fig. 1). Single cells are about 0.3 to 0.7 by 2 to 3 μ m. No spores have been detected, and 72-h- and 2-week-old cultures grown on cellobiose or cellulose medium fail to survive heating at 80°C for 10 min or exposure to 50% ethanol (9). The cells are nonmotile with no flagella visible in electron micrographs of young cells grown without shaking in broth containing cellobiose (Fig. 2). Electron micrographs show that one end of each cell is round and uniform and the other end is rough and uneven. This characteristic is observed in both young and old cells. The cells have an outer membrane (Fig. 3).

Colonies on cellulose agar are visible after 4 to 7 days of incubation. They are about 2 to 3 mm in diameter, entire, raised, and cream colored, showing no clear zones surrounding the colonies. Growth in cellulose-containing broth under an N_2 - CO_2 gas phase has little or no color.

The optimum growth temperature is between 35 and 37°C, and there is no growth at 20 or 45°C. The optimum pH is between 6.5 and 7.5, and there is no growth at pH 6.0 or 8.0.

Only cellulose, cellobiose, esculin, and salicin support growth. Yeast extract or rumen fluid is not required for growth on these carbon sources. Bile (2% Oxgall; Difco) inhibits growth.

No growth is produced in medium supplemented with adonitol, amygdalin, arabinose, Casamino Acids, casein, chopped meat broth, dulcitol, erythritol, fructose, galactose, glucose, glycerol, glycogen, inositol, inulin, lactate, lactose, maltose, mannitol, mannose, melezitose, melibiose, milk, peptone, pyruvate, raffinose, rhamnose, ribose, sorbitol,

FIG. 2. Electron micrograph of a strain BAS^T cell from a 2-day-old culture grown in cellobiose medium under stationary culture conditions.

FIG. 3. Ultrastructure of strain BAS^T, showing the outer membrane. Fixation was carried out by using the glutaraldehyde-osmium tetrachloride technique.

FIG. 4. Polyacrylamide gel electrophoretic analysis of extracellular cellulase proteins obtained from *A. cellulosolvens* (a) and *B. cellulosolvens* (b).

sorbose, starch, sucrose, trehalose, urea, xylose, or yeast extract.

Esculin is hydrolyzed to give a positive reaction with ferric ammonium citrate. Sulfate is not reduced to sulfite, gelatin is not hydrolyzed, and catalase is not produced. Indole and nitrate reduction tests are negative.

The metabolic products of cultures in basal medium containing cellobiose incubated for 4 to 8 days under 100% N₂ or 80% N₂-20% CO₂ are acetic acid (18 mM), ethanol (3 mM), CO₂, and H₂. Trace amounts of lactic acid also are produced. In cellulose-containing medium, glucose (8 mM) also is produced.

The deoxyribonucleic acid base composition of the type strain is 41 ± 1.4 mol% guanine plus cytosine ($n = 4$).

The type strain of *A. cellulosolvens* is strain BAS, which has been deposited in the National Research Council of Canada culture collection as strain NRC 2936.

A. cellulosolvens differs from *A. cellulolyticus* (15) and *A. ethanoligignens* (16) in that it is nonmotile, hydrolyzes esculin, and gives a positive reaction with ferric ammonium citrate. *A. cellulosolvens* differs from *Bacteroides succinogenes* (1), another cellulolytic anaerobe in the family *Bacteroidaceae*, in its ability to hydrolyze esculin, in producing H₂, in not using glucose, and in not producing succinic acid. It differs from *Bacteroides cellulosolvens* (13) in not producing a bright yellow pigment in cellulose or cellobiose broth,

in hydrolyzing esculin to give a positive reaction with ferric ammonium citrate, and in showing distinct differences in gel electrophoretic analyses of extracellular cellulase proteins (Fig. 4).

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