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

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A new anaerobic mesophilic cellulolytic clostridium isolated from a methanogenic cellulose-enriched culture is described. Optimal growth occurs at 35°C and pH 7.0. Fructose, maltose, rhamnose, lactose, glucose, xylose, ribose, mannose, raffinose, cellobiose, arabinose, galactose, mannitol, adonitol, esculin, salicin, and trehalose serve as substrates for growth. The major fermentation products from cellulose or cellobiose are ethanol, acetate, formate, butyrate, isobutyrate, isovalerate, caproate, lactate, succinate, CO₂, and H₂. The deoxyribonucleic acid base composition is 38 mol% guanine plus cytosine. The type strain is 18A (= CECT 954).

The potential for converting cellulosic wastes into industrial substrates has stimulated current interest in cellulose fermentation. Among the methods proposed for this purpose, anaerobic digestion offers economic and pollution control advantages.

Many cellulolytic microorganisms have been isolated from the human colon (1), soil (18), estuarine sediments (13), freshwater sediments (9), decomposing vegetation (10, 17), and the bovine rumen (4, 5). In contrast, little attention has been paid to cellulolytic bacteria involved in artificially confined methanogenic systems. This work refers to the isolation and characterization of a distinct cellulolytic bacterium from a methanogenic digester.

MATERIALS AND METHODS

Media. The basal medium was CM3 described by Weimer and Zeikus (24), except that Na₂S was omitted and the final cysteine hydrochloride concentration was 0.55 g/liter. The pH was adjusted to 7.2 with 1 M NaOH. Basal medium, supplemented with 0.5% (wt/vol) Whatman CF-11 cellulose powder and 1.0% agar, was used for strain isolation. For detecting cellulolytic activities, the carbon source was a strip (6 by 1 cm) of Whatman no. 1 filter paper. For culture maintenance, basal medium was supplemented with 0.5% (wt/vol) Whatman CF-11 cellulose powder under oxygen-free nitrogen. Soluble carbohydrates, used as the carbon source for biochemical tests, were autoclaved separately (30 min, 110°C) and added to the sterile basal medium just before inoculation.

Anaerobic culture methods. Throughout this study, the anaerobic technique of Hungate (7) modified by Bryant (2) was followed. Standard test tubes (16 × 125 mm; Bellco Glass, Inc.) sealed with butyl rubber stoppers and screw caps (Bellco Glass) were used as culture tubes. All cultures were incubated at 37°C without agitation.

Isolation procedures. A methanogenic culture, started with a cow manure inoculum, was the source of the new microorganism. Isolation was effected from a semicontinuous enrichment (hydraulic retention time, 105 days) obtained by weekly feeding for 1 year a suspension of Whatman CF-11 cellulose powder in the defined medium of Khan et al. (8). Serial dilutions in prerduced physiological saline were used to inoculate cellulose agar plates in an anaerobic chamber. The inoculated plates were incubated in GasPak anaerobic

jars under 80% N₂-20% CO₂ at 37°C. Colonies with clearing zones of cellulose digestion were picked and transferred into tubes containing CM3 medium supplemented with a strip of filter paper. The tubes were incubated until the filter paper showed signs of decay, and samples were replated on cellulose agar. The procedure was repeated several times to assure the purity of the culture by colony appearance and microscopic examination. To confirm the purity of the culture, brucella blood agar, prepared according to the Wadsworth manual (23), was used. The isolate used in this study was maintained by weekly transfers in liquid medium.

Morphology. Living and stained cells were examined by light microscopy. Flagella were examined by electron microscopy. For this last purpose, a colony grown on brucella blood agar plates was suspended in physiological saline; a drop of the suspension was placed on a Formvar-coated copper grid and stained with 1% (wt/vol) phosphotungstic acid solution. Observations were made with a Zeiss model EM-10C electron microscope.

Biochemical reactions. Biochemical tests were performed by the standard procedures described in the *Anaerobe Laboratory Manual* (6). Tests were read as soon as microorganisms showed good growth (optical density at 625 nm = 0.8, equivalent to 0.88 mg of dried cells per ml) or one day after they achieved constant turbidity.

DNA base composition. The deoxyribonucleic acid (DNA) was isolated and purified by the method of Marmur (11) from cells grown for 24 h in basal medium with cellobiose (6 g/liter) as the carbon source. Moles percent guanine plus cytosine (G+C) was estimated from the thermal denaturation temperature by the method of Marmur and Doty (12). G+C content was calculated from the measured thermal denaturation temperature by the Owen and Hill (15) equation, $\%(G+C) = 50.9 + 2.08 (T_m - T_m \text{ ref})$, with DNA from *Escherichia coli* as a reference.

Electrophoretic study of soluble proteins. Cells from 24-h-old cultures in 5 ml of brain heart infusion broth were harvested by centrifugation at 8,000 × g for 10 min, washed twice with 50 mM phosphate buffer solution (pH 7.0), sedimented in conical tubes by centrifugation, and then suspended in 0.1 ml of 0.062 M tris(hydroxymethyl)amino-methane (Tris) chloride buffer (pH 6.8). After 0.15 g of 250- to 300-μm diameter glass beads was added, tubes were placed in an ice bath and vortexed for two 2-min periods (3, 25). The cellular debris was removed by centrifugation at 15,000 × g for 10 min. After protein denaturation by heating (100°C, 10 min) in the presence of excess sodium dodecyl

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TABLE 1. Differentiation of strain 18A from other mesophilic cellulolytic clostridia

Characteristic	Reaction ^a					Strain 18A
	<i>C. cellobioparum</i>	<i>C. papyrosolvans</i>	<i>C. cellulolyticum</i>	<i>C. cellulovorans</i>	<i>C. populeti</i>	
Growth on:						
Adonitol	ND	—	—	ND	ND	+
Dulcitol	+	—	—	ND	ND	—
Gelatin	ND	—	—	—	+	+
Glycerol	W	+	—	—	—	—
Maltose	+	—	—	+	+	+
Mannitol	—	—	—	ND	ND	+
Mannose	+	—	W	+	—	+
Raffinose	—	—	—	ND	ND	+
Rhamnose	—	—	—	—	—	+
Salicin	W	—	—	ND	ND	+
Trehalose	—	—	—	—	—	+
Indole production	—	—	—	ND	ND	+
Milk test	—	—	—	ND	ND	+
% (G+C)	25–28	30	41	25–27	28	38

^a +, Positive; —, negative; W, weak; ND, not determined.

sulfate and mercaptoethanol, samples were analyzed by electrophoresis in polyacrylamide slab gels by using 4% (stacking gel) and 12% (separating gel) acrylamide (21). Electrophoresis was run at 10°C at an increasing voltage from 80 to 130 V and at about 25 mA initial current.

Temperature and pH studies. The temperature and pH effects on the growth rate of the organism were determined in cellobiose broth. Three tubes were inoculated and incubated for each temperature and pH. Growth was measured as optical density at 625 nm on a Bausch & Lomb Spectronic 20 spectrophotometer, and the average value was calculated.

Fermentation end-product analysis. Volatile fatty acids, alcohols, and nonvolatile acids were determined in fermentation broth samples (2.5 ml) after deproteinizing with 0.1 ml of ZnCl₂ (10% wt/vol) and centrifuging at 12,000 × g for 15 min. The preparation of ether extracts and the chromatographic analysis of the extracted components were carried out by the methods of Holdeman et al. (6).

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cellular proteins of some cellulolytic clostridia. Lanes: 1, *C. papyrosolvans*; 2, *C. cellulolyticum*; 3, strain 18A; 4, *C. cellulovorans*. The numbers on the right refer to the molecular weights of the standards in track S.

Headspace gases were analyzed by gas chromatography at 50°C on Chromosorb 102 (80 to 100 mesh) in a 2-m-long by 2-mm-inside-diameter column. The effluent from the column was fed into a molecular sieve 0.5-nm column (2-m long by 2-mm inside-diameter) at room temperature. Argon was used as the carrier gas with a flow rate of 27 ml/min. Effluent gases were detected by thermal conductivity.

RESULTS AND DISCUSSION

The enrichment and isolation procedures yielded four anaerobic cellulolytic isolates from which strain 18A was selected for identification on the basis of its cellulolytic capacity for filter paper degradation.

Preliminary tests showed that the isolate was a rod-shaped, gram-positive, obligately anaerobic sporeforming bacterium. The DNA base composition was 38 mol% G+C.

On the basis of the identification scheme of the last edition of *Bergey's Manual of Systematic Bacteriology* (22), the isolate was identified as a member of the genus *Clostridium*. According to this manual, the only mesophilic members of the genus *Clostridium* recognized as cellulolytic are *C. cellobioparum* and *C. papyrosolvans*.

Isolate 18A differs from *C. cellobioparum* and *C. papyro-*

FIG. 2. Forty-eight-hour-old cells grown on brucella blood agar.

FIG. 3. Electron micrograph of *C. celerecrescens*.

solvans (Table 1) by fermenting mannitol, raffinose, rhamnose, and trehalose, and in that it is positive for indole production and for milk tests (digestion with clot formation). The G+C contents of *C. cellobioparum* and *C. papyrosolvans* are 28 and 30 mol%, respectively.

Other mesophilic cellulolytic clostridia include *C. cellulolyticum* (17), *C. cellulovorans* (19), and *C. populeti* (20). The biochemical characteristics of these species (Table 1) also differ from those of our isolate. The protein electrophoretic pattern of isolate 18A can clearly be distinguished from those of *C. papyrosolvans*, *C. cellulolyticum*, and *C. cellulovorans* (Fig. 1). In addition, isolate 18A differs from *C. cellobioparum*, *C. papyrosolvans*, and *C. cellulolyticum* by producing butyrate as a major metabolic product.

Another distinguishing characteristic of isolate 18A is its high specific growth rate, 0.564 h^{-1} , attained at a cellobiose concentration of 6 g/liter. Table 2 compares this value with those published for other cellulolytic microorganisms.

We therefore propose the establishment of a new species, *Clostridium celerecrescens* sp. nov. (ce'le. re. cres. cens. L.

adv. *celere*, fast; *crescens*, L. pres. part. of verb *crescere*, to grow; M.L. adj. *celerecrescens*, intended to reflect the fast growth of the organism).

Cells are straight to slightly curved rods, 2- to 4- μm long by 0.5- to 0.8- μm wide. Vegetative cells stain gram positive, although they decolorize readily in older cultures. Endospores are spherical and terminal and produce swollen cells (Fig. 2). Cells are motile by means of peritrichous flagella (Fig. 3).

On cellulose agar medium, colonies appear after 10 to 14 days of incubation at 37°C and exhibit halos of cellulolysis. Deep colonies are smooth, circular, approximately 0.5 mm in diameter, translucent, and unpigmented.

C. celerecrescens ferments cellulose, cellobiose, glucose, fructose, galactose, mannitol, maltose, adonitol, rhamnose,

FIG. 4. Effect of temperature on growth of *C. celerecrescens*.

TABLE 2. Specific growth rate of some cellulolytic microorganisms at optimal conditions of culture

Microorganism	Temp (°C)	pH	Concn of cellobiose (g/liter)	μ (h^{-1})	Reference
<i>Acetivibrio cellulolyticus</i>	35	7.0	10.0	0.170	16
<i>Clostridium cellulolyticum</i>	35	7.0	10.0	0.099	17
<i>Clostridium papyrosolvans</i>	37	7.0	6.0	0.266	— ^a
<i>Clostridium thermocellum</i> H1	60	7.0	6.8	0.330	14
Strain 18A	35	7.0	6.0	0.564	

^a Unpublished results obtained in this laboratory.

FIG. 5. Effect of pH on growth of *C. celerecrescens*.

xylose, ribose, mannose, raffinose, trehalose, arabinose, and esculin. It weakly ferments lactose, melezitose, melibiose, sorbose, and sucrose. It does not ferment dulcitol, erythritol, glycerol, inulin, sorbitol, and starch.

Fermentation products in cellobiose and cellulose broth are ethanol, acetate, formate, butyrate, isobutyrate, isovalerate, caproate, lactate, succinate, H₂, and CO₂.

Gelatin is liquefied and indole is produced. Catalase, lipase, lecithinase, and urease are negative. Nitrate reduction and casein hydrolysis are negative. Acetyl methyl carbinol is not produced. Milk digestion and curd formation are positive.

Optimum growth temperature is between 30 to 37°C. Growth rate decreases substantially at 23°C (Fig. 4).

Optimum pH is near 7.0. Growth rate decreases substantially under pH 7 and over pH 8 (Fig. 5).

The DNA base composition is 38 mol% G+C.

The source was a methanogenic culture started with a cow manure inoculum.

The type strain is 18A (= CECT 954) (Colección Española de Cultivos Tipo).

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