Clostridium cellulolyticum sp. nov., a Cellulolytic, Mesophilic Species from Decayed Grass

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A new cellulolytic *Clostridium* species that was isolated from decayed grass is described. The colonies produced by this organism on cellulose agar are circular, translucent, and unpigmented and have undulate margins. The cells of this bacterium are gram-positive straight to slightly curved rods 3 to 6 μm long by 0.6 to 1 μm wide and are motile; they form round, terminal spores 1.5 μm in diameter. A variety of carbohydrates are fermented by this mesophilic anaerobe. The major fermentation products from cellulose are carbon dioxide, hydrogen, ethanol, acetate, lactate, and formate. The deoxyribonucleic acid base composition is 41 mol% guanine plus cytosine. The type strain of *Clostridium cellulolyticum* sp. nov. is strain H₁₀ (= ATCC 35319).

Anaerobic cellulolytic bacteria that take part in the degradation of cellulosic materials have been isolated from manure, rumen contents, and soil. In this report we describe the isolation and characterization of a new species of *Clostridium* from compost.

MATERIALS AND METHODS

Media and culture techniques. The composition of the medium which we used was the same as the composition of the cellulose-yeast extract-salts medium (CM medium) described by Weimer and Zeikus (15), except that Na₂S was omitted, the cysteine hydrochloride concentration was 1 g/liter, and the cellulose MN300 concentration was 7.5 g/liter.

For solid media CM medium was supplemented with 20 g of agar (Difco Laboratories, Detroit, Mich.) per liter. The pH was adjusted to 7.5 with NaOH. This medium was pre-reduced by the Hungate technique (5) and was dispensed under a constant flow of N₂ in 8-ml amounts (4.5-ml amounts for solid medium) into anaerobic culture tubes (16 by 125 mm; Bellco Glass, Inc., Vineland, N.J.).

For enrichment cultures, we used anaerobic tubes (25 by 142 mm; Bellco) that contained 25 ml of medium.

Carbohydrate media were prepared by replacing cellulose with filter-sterilized solutions of carbohydrates to give final concentrations of 7.5 g/liter. The cultures were incubated at 35°C without shaking.

The anaerobic culture techniques of Hungate (5), as modified by Bryant (1), were used throughout this work.

All cultures were inoculated and incubated with nitrogen as the gas.

Isolation of strain H₁₀^T. Strain H₁₀^T (T = type strain) was obtained from cultured decayed grass by enrichment and isolation.

Enrichment cultures were initiated by inoculating 1 to 2 g of grass from compost into anaerobic culture tubes (25 by 142 mm; Bellco) containing 25 ml of pre-reduced medium. After 1 week of incubation at 35°C, the cultures were heated at 80°C for 10 min, and 1 ml of each culture was then transferred to 8 ml of pre-reduced liquid medium.

After a few days of incubation, cultures that produced

discrete gas bubbles and solubilized cellulose were selected for isolation in solid medium.

Serial dilutions were incubated in cellulose agar roll tubes. After 3 to 5 days, colonies which produced cleared zones of cellulose digestion were transferred individually into the liquid medium by means of a bent Pasteur pipette (5) under a flow of N₂ gas.

Electron microscopy. A colony was picked from cellulose agar. The cells were fixed with 2% osmic acid, suspended in agar, (Difco), dehydrated, and embedded in Epon. Thin sections were cut with a diamond knife and were contrasted with lead citrate.

The sections were viewed with a Siemens Elmiskop model 102 electron microscope.

Isolation of DNA and measurement of the G+C content of the DNA. Deoxyribonucleic acid (DNA) was isolated by the method of Marmur (10).

The DNA base composition, expressed as the guanine-plus-cytosine (G+C) content, was estimated from the thermal denaturation temperature, which was determined by the method of Marmur and Doty (11).

Thermal denaturation of DNA was carried out with a Perkin-Elmer model 551 spectrophotometer equipped with a temperature program controller.

The absorbance changes at 260 nm were recorded, and the values were corrected by the method of Mandel and Marmur (9).

The G+C content was calculated from the thermal denaturation temperature by using the following equation of Marmur and Doty (11): G+C content = $2.44 \times (\text{thermal denaturation temperature} - 69.3)$.

Biochemical tests. Biochemical tests were performed by the standard procedures described in the *Anaerobe Laboratory Manual* (3). Tests were read after 3 days and again after 10 days.

Growth measurements. For determinations of growth rates on carbohydrates, the optical densities at 525 nm were measured with a Beckman model 25 spectrophotometer. For growth on cellulose, the pellet proteins were determined by the method of Gordon et al. (2), and the total protein was estimated by the method of Lowry et al. (7), using bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard.

Analytical methods. The composition of the gas in the headspace was analyzed. H₂ and CO₂ were determined by

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FIG. 1. *C. cellulolyticum*. (A) Vegetative and sporulated cells of strain H₁₀^T grown on medium containing cellulose MN300. Bar = 10 μ m. (B) Ultrathin section of vegetative and sporulated cells. Bar = 1 μ m.

using an Intersmat model IGC MB chromatograph equipped with a thermoconductivity detector (80 mA) and a column (2 m by 2 mm [inside diameter]) fitted with Carbosieve (120–140 mesh; Supelco). The column temperature was 100°C, and the carrier gas was argon (1.8 to 2 bars at the column head).

The injection temperature was 130°C and the detector temperature was 110°C. A 0.5-ml sample of the culture gas phase was injected directly into the gas chromatography apparatus by means of a 1-ml syringe.

Culture samples were centrifuged at 25,000 $\times g$ for 10 min. The resulting supernatant was retained for a determination

of alcohols and acids, and the pellet was used to determine the pellet protein.

Alcohols and volatile fatty acids were analyzed by using a Carlo-Erba Fractovap model G1 gas chromatograph. This instrument was equipped with a flame ionization detector. The column was made of stainless steel, was 2 meters long and 5 mm in diameter, and contained Porapak Q (100–120 mesh; Waters Associates, Milford Mass.).

The following conditions were selected for this analysis: column temperature, 220°C; carrier gas, nitrogen (1 bar); injector temperature, 240°C; carrier gas flow rate, 10 cm³/min; hydrogen and air flow rate, 160 cm³/min.

FIG. 2. Electron micrograph of *C. cellulolyticum*. Bar = 1 μ m.

A 1-ml culture supernatant sample was acidified with 50 μ l of 6 N HCl, and 2 μ l of the acidified supernatant was injected into the gas chromatograph.

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

Formate was determined by the colorimetric method of Lang and Lang (6).

The total reducing sugars in the supernatant were determined by using a dinitrosalicylic acid reagent with glucose as the standard and the method of Miller et al. (12).

Glucose was determined enzymatically with glucose oxidase (Boehringer-Mannheim).

RESULTS AND DISCUSSION

Our isolate from cellulose enrichment cultures of compost appears to be a new species that belongs in *Clostridium* group III, as described by Smith and Hobbs (14). A description of this species is given below.

Clostridium cellulolyticum sp. nov. *Clostridium cellulolyticum* (cel.lu.lo.ly'ti.cum. Gr. n. *cellulosum* cellulose; Gr. adj. *lyticus* dissolving; N.L. adj. *cellulolyticum* cellulose dissolving) cells (Fig. 1) are anaerobic, gram-positive, straight to slightly curved rods that are 3 to 6 μ m long by 0.6 to 1 μ m wide. Spores are present in cultures on cellulose media 3 or more days old; these spores are spherical (diameter, 1.5 μ m) and terminal, produce swollen cells (Fig. 1 A and B), and resist a temperature of 100°C for 30 min.

Spores are rarely formed on other carbohydrate media. The cells are motile by means of peritrichous flagella (Fig. 2) and have a cell wall that is typical of the cell walls of gram-positive species.

On cellulose agar medium colonies appear after 5 to 6 days of incubation at 35°C. Surface colonies are smooth, circular, approximately 0.5 mm in diameter, slightly raised, translucent, and unpigmented with undulate margins, and a butyrous texture. Clear zones showing complete solubilization of cellulose around the colonies are 1 to 4 mm in diameter.

The maximum growth temperature is 45°C, the optimum growth temperature is 32 to 35°C, and the minimum growth temperature is 25°C; growth does not occur at 20 and 50°C even after 2 months.

There is no growth with adonitol, amygdalin, dulcitol, erythritol, glycerol, glycogen, inositol, inulin, lactose, maltose, mannitol, melezitose, raffinose, rhamnose, salicin, sorbitol, sorbose, sucrose, and trehalose.

Poor growth is produced with galactose, mannose, or ribose.

Moderate growth is produced with arabinose, cellobiose, cellulose, fructose, glucose, and xylose, and the final pH is 5.5 to 5.2. The doubling times with cellobiose, cellulose, and glucose are 7, 15, and 10 h, respectively.

Esculin is hydrolyzed; starch is not hydrolyzed. Ammonia is not produced from arginine. Acetylmethylcarbinol, nitrite, and indole are not produced. Milk is unchanged, casein is not digested, and no growth occurs in chopped meat broth. Pyruvate and lactate are not utilized. Gelatin is not liquefied,

TABLE 1. Principal differences that distinguish *C. cellulolyticum*, *C. cellobioparum*, and *C. papyrosolvens*

Characteristic	<i>C. cellobioparum</i> as described by:				<i>C. papyrosolvens</i> ^e	<i>C. cellulolyticum</i>
	Hungate ^a	Skinner ^b	Smith and Hobbs ^c	Anaerobe Laboratory Manual ^d		
Carbohydrate fermentation						
Dulcitol	ND ^f	ND	+ ^g	+	—	—
Esculin	ND	ND	—	—(pH) ^h	+	—(pH) ^h
Galactose	S	w	+	+	+	w
Glucose	+	w	+	+	+	+
Glycerol	—	—	w	w	+	—
Lactose	S	w	w	w	—	—
Maltose	+	+	+	+	—	—
Mannitol	S	—	w	w	—	—
Mannose	+	+	+	+	—	w
Melibiose	+	ND	+	+	—	—
Raffinose	S	—	+	w	—	—
Ribose	ND	ND	+	+	+	S
Salicin	—	—	+	w	—	—
Sucrose	S	—	—	—	—	—
Sorbitol	ND	ND	+	w	—	—
G+C content (mol%)	ND	ND	25	28	30	41
Gram reaction	ND	—	—	—	—	+

^a See reference 4.^b See reference 13.^c See reference 14.^d See reference 3.^e See reference 8.^f ND, Not determined.^g +, Positive; —, negative; w, weakly fermented; S, occasionally slowly fermented.^h Hydrolysis occurs with no change in the pH value.

and thiosulfate is not reduced. Catalase, urease, lipase, and lecithinase are not produced.

Carbon dioxide, hydrogen, acetate, ethanol, lactate, and formate are produced from cellulose. Little glucose is detected among the reducing sugars produced.

Isolated in autumn from decayed grass compost packed for 3 to 4 months. The mean G+C content of the DNA is 41 mol%. The type strain is strain H₁₀ (= ATCC 35319).

In *Bergey's Manual of Determinative Bacteriology*, 8th ed. (14), the only member of the group III clostridia that is recognized as cellulolytic is *Clostridium cellobioparum*.

The most significant differences between *C. cellulolyticum* and *C. cellobioparum* are shown in Table 1. *C. cellulolyticum* differs from *C. cellobioparum* in not fermenting dulcitol, lactose, mannose, and sorbitol. Furthermore, from cellulose digestion *C. cellobioparum* forms only cellobiose, the strain isolated by Skinner (13) forms glucose and little or no cellobiose, and our isolate forms cellobiose and little glucose.

C. cellulolyticum also differs from *Clostridium papyrosolvens*, a new species described by Madden et al. (8), in not fermenting glycerol and particularly in being unable to grow at temperatures below 25°C. Finally, the G+C content of the DNA of *C. papyrosolvens* is 30 mol%; thus, this species can be readily distinguished from *C. cellulolyticum*, which has a G+C content of 41 mol%.

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