Clostridium populeti sp. nov., a Cellulolytic Species from a Woody-Biomass Digester

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A new anaerobic, mesophilic, sporeforming, cellulolytic bacterium is described. The cells of this organism stained gram negative, were motile rods, and formed terminal oval spores which swelled the cells. Colonies were irregular and opaque, with a yellow-pigmented center. Arabinose, xylose, fructose, galactose, glucose, cellobiose, maltose, sucrose, cellulose, xylan, and pectin served as substrates for growth. H₂, CO₂, acetate, butyrate, and lactate were produced during growth on cellulose or glucose. Optimal growth occurred at 35°C and pH 7.0. The deoxyribonucleic acid composition was 28 mol% guanine plus cytosine. The name *Clostridium populeti* sp. nov. is proposed. The type strain is strain 743A (= ATCC 35295).

The cellulolytic bacteria from bovine rumina (9, 10), human colons (2), rat ceca (23), guinea pig ceca (5), horse large intestines (4), soil (29), estuarine sediments (19), freshwater sediments (17), and decomposing vegetation (18, 26) have been extensively investigated. In contrast, very little is known about the cellulolytic bacteria in anaerobic digester systems. However, such bacteria should be present and may enter municipal waste digestors from runoff water or from sewage containing the feces of humans and animals.

Most-probable-number determinations of cellulolytic bacteria in swine manure digestors showed that the cellulolytic and hemicellulolytic bacteria comprised less than 0.1% of the total digester population (14). The cellulolytic bacteria in anaerobic digestors were presumed to be similar to those in rumina since nonsporeforming rods predominated (12, 20). However, two recent isolates show that this is not the case; thermophilic cellulolytic sporeformers were isolated from sewage sludge (24), and a cellulolytic, vibrioid, mesophilic bacterium was isolated from municipal sewage sludge (25).

We report here the isolation and characterization of an anaerobic, mesophilic, sporeforming, cellulolytic bacterium from a woody-biomass digester. Except for *Clostridium cellulovorans* (30), our isolate differs from previously described digester isolates in morphology, optimum growth temperature, substrates utilized, and fermentation products.

MATERIALS AND METHODS

Isolation. The inoculum source was a batch methanogenic fermentation of finely divided (ca. 0.8 mm in size) hybrid poplar wood (15) obtained from D. P. Chynoweth.

Culture methods and media. The anaerobic techniques of Hungate (12) were used throughout our experiments. The culture methods and media used have been described previously (30), except that fermentation products from 10 mM glucose and 10 g of Avicel per liter were determined in medium buffered with 50 mM HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid; Calbiochem-Behring, La Jolla, Calif.) (final pH, 7.0).

Morphological, biochemical, and physiological tests. Phase-contrast photomicrographs were taken as previously described (6). Gram stain reaction, lysis by KOH, and hydrolysis of L-alanine-4-nitroanilide were determined by the methods of Carlone et al. (3). Cell morphology, substrate utilization, temperature and pH optima, and growth were

determined as previously described (30). Starch hydrolysis, gelatin liquefaction, and catalase production were determined by the methods of Smibert and Krieg (31). A 0.1-ml inoculum from a 24-h HEPES-glucose-grown culture was used for fermentation balance determinations. For Avicel degradation, 0.5 ml of a 72-h HEPES-Avicel-grown inoculum was used.

Analytical techniques. Culture headspace gases were analyzed by gas chromatography (1). Volatile and non-volatile fatty acids and alcohols were measured by the methods of Sleat et al. (30). Lactate was determined enzymatically with lactate dehydrogenase (lactic acid diagnostic kit; Sigma Chemical Co., St. Louis, Mo.). Formate was determined by the procedure of Lang and Lang (16); reducing sugars were determined by using the dinitrosalicylic acid reagent (22), and glucose was determined by using the Direct Glucose Test Set (Stanbio Lab Inc., San Antonio, Tex.). Residual Avicel was measured gravimetrically by the method of Weimer et al. (33).

Analysis of DNA base composition. Whole-cell deoxyribonucleic acid (DNA) was extracted and purified by the method of Marmur (21). The buoyant density of the purified DNA was determined by preparative ultracentrifugation in a cesium chloride gradient (27). DNAs from *Escherichia coli* strain B and *Micrococcus lysodeikticus* were used as controls. The DNA base ratio (guanine-plus-cytosine content) was calculated by the method of Schildkraut et al. (28).

Reagents, chemicals, and gases. All chemicals were reagent quality, except where noted. Gas mixtures were purchased from Searle Medical Products, Cucamonga, Calif. Avicel (type PH-105; lot 5150-128) was a gift from FMC Corp., Philadelphia, Pa.

RESULTS AND DISCUSSION

Strain 743A^T (T = type strain) was strictly anaerobic, did not reduce sulfate, and formed endospores. These characteristics place it in the genus *Clostridium* (32). It differed significantly from other mesophilic, cellulolytic clostridia (7, 11, 17, 19, 26) in substrates utilized, fermentation products, morphology, and optimum growth temperature. Therefore, we propose a new species, *Clostridium populeti*, with the species description given below.

Description of *Clostridium populeti* sp. nov. *Clostridium populeti* (po.pu'le.ti. L. n. *populetum* poplar wood; L. gen. n. *populeti* of poplar wood) cells stain gram negative and are L-alanine-4-nitroanilide and KOH negative. Motile. The

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FIG. 1. Photomicrographs of a cellulose-agar-grown culture. (A) Vegetative cells and clostridium. (B) Cells with terminal, oval spores and free spores. Bar = 5 μm .

slightly curved rod-shaped cells are 1 to 1.5 μm wide and 1.7 to 3.0 μm long and often occur in pairs. Oval spores form terminally and cause a marked swelling of the cells (Fig. 1). The mature spores are 1.0 to 1.2 μm in diameter. Endospores are viable after heating to 80°C for 10 min. Vegetative cells are resistant to sodium dodecyl sulfate but are readily lysed by lysozyme and ethylenediaminetetraacetate.

Colony characteristics. In medium solidified with 2% purified agar and containing pebble-milled cellulose, zones of clearing appear within 48 h. Deep colonies are irregular, opaque, and yellow. The clear zone of cellulolysis can reach a diameter of 20 mm after prolonged incubation. The cellulose in agar roll tubes containing high numbers of cellulolytic

FIG. 2. Effect of temperature on H_2 production by a cellobiose-grown culture. Maximal H_2 production was after 17 h of incubation.

colonies is completely hydrolyzed within 2 weeks. Surface colonies are almost invisible except for a thin opaque edge. Colonies in cellobiose-agar medium after 48 h of incubation are 1 mm in diameter, irregular, opaque, and yellow. The colonies are smooth and butyrous, becoming rhizoid with age. Colonies in pectin-agar medium are yellow; those in xylan-agar medium are orangish brown.

Nutrition and growth conditions. Rumen fluid, Trypticase peptone, and added vitamins are not required for growth. Nutritional requirements can be met by the addition of 2 g of yeast extract per liter, although yeast extract cannot serve as a primary substrate. The optimum temperature for growth is 35°C, and growth occurs over a temperature range of 20 to 40°C (Fig. 2). No growth is observed at 15°C after prolonged incubation. The optimum pH for growth is 7.0, and the pH range is 6.4 to 8.1 (Fig. 3).

Metabolic characteristics. Obligate anaerobe. Catalase negative. Sulfate not reduced. Arabinose, xylose, galactose, glucose, fructose, cellobiose, maltose, sucrose, cellulose, pectin, xylan, and gum arabic are fermented. Rhamnose, glycerol, mannose, lactose, trehalose, melezitose, erythritol, arabinol, sorbitol, lactate, and pyruvate are not fermented. Gelatin is liquefied after prolonged incubation. Starch and casein are not hydrolyzed.

Growth in liquid medium containing a soluble carbohydrate is homogeneous. Motility and endospore formation are often inhibited during growth on a soluble carbohydrate in liquid medium. Cellulose is readily fermented in medium buffered (final concentration, 50 mM) with PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] or HEPES organic buffer but is not fermented in the presence of morpholine-propanesulfonic acid, *N*-2-acetamido-2-aminoethanesulfonic acid, tris(hydroxymethyl)aminomethane, or *N,N*-methylene-bisacrylamide-tris(hydroxymethyl)aminomethane-propane.

The products obtained from glucose after fermentation is complete and H_2 production ceases are acetate (16 mol/100 mol of glucose), butyrate (72 mol/100 mol of glucose), lactate (50 mol/100 mol of glucose), H_2 (98 mol/100 mol of glucose),

FIG. 3. Effect of pH on H_2 production by a cellobiose-grown culture. Maximal H_2 production was after 15 h of incubation.

FIG. 4. Fermentation of Avicel and production of H₂ and butyrate.

and CO₂ (111 mol/100 mol of glucose). These values (the means of five determinations) yield 92.5% recovery of carbon, 101% recovery of hydrogen, and give an oxidation-reduction balance of 0.92 calculated by the method of Gottschalk (8). Trace amounts of ethanol and succinate are also detected. Pyruvate and formate are not produced.

Growth on pebble-milled cellulose in liquid medium is characterized by the production of a yellow pigment which is bound to the substrate. The cellulose becomes viscous and tends to clump. The maximum rate of cellulose degradation in carbonate-buffered medium (initial pH, 7.0) is 29 mg/liter per h. H₂ and butyrate production parallel Avicel degradation during growth in HEPES-buffered medium (Fig. 4). Small quantities of reducing sugars (4 mM after 32 days) are produced when excess cellulose is included in the medium. The maximum rate of Avicel degradation is 8.7 mg/liter per h.

Guanine-plus-cytosine content. The guanine-plus-cytosine content of *C. populeti* DNA is 28 mol%.

Source. *C. populeti* was isolated from a batch methanogenic fermentation of finely divided hybrid poplar wood.

Type strain. The type strain of *C. populeti* is strain 743A (= ATCC 35295).

Distinguishing characteristics. *C. populeti* is distinguished from most mesophilic, cellulolytic clostridia by its production of butyrate as a major fermentation product during cellulose degradation. The recently isolated species *C. cellulovorans* (30) also produces butyrate, but can be distinguished from *C. populeti* by its production of formate and its endospore morphology. *C. cellulovorans* produces central or subterminal, oblong endospores, whereas *C. populeti* produces terminal, oval endospores.

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