Clostridium aldrichii sp. nov., a Cellulolytic Mesophile Inhabiting a Wood-Fermenting Anaerobic Digestor†

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An anaerobic, mesophilic, spore-forming, cellulolytic bacterium was repeatedly isolated from a wood-fermenting anaerobic digestor. Cells of this organism were gram-positive rods, motile with a bundle of polar flagella, and formed subterminal oblong spores. The colonies in agar had an irregular shape with many platelike structures and were greyish white. Cellulose, xylan, and cellobiose served as substrates for growth. Acetate, propionate, butyrate, isobutyrate, isovalerate, lactate, succinate, H₂, and CO₂ were products of cellobiose fermentation. The optimal temperature and pH for growth were 35°C and 7, respectively. The DNA composition was 40 mol% G+C. The name Clostridium aldrichii sp. nov. is proposed. The type strain is P-1 (= OGI 112, = ATCC 49358).

MATERIALS AND METHODS

Samples and treatments. Strict anaerobic methods (7) were used for collection, sample preparation, inoculation, and identification. Samples were collected from the effluent of a continuously stirred tank, bench-scale, mesophilic (35°C), poplar-fed digester. The effluent was blended for less than 1 min in a homogenizer under oxygen-free N₂-CO₂ (80:20). The treated sample was diluted in basal medium up to 10⁻¹¹ in order to exceed the highest dilution of the samples over a period of 2 years and is described below. The name Clostridium aldrichii is proposed for this organism.

Culture media. The basal medium contained (per liter): NH₄Cl, 2 g; KCl, 0.5 g; KH₂PO₄, 1.65 g; MgSO₄·7H₂O, 0.5 g; Trypticase (BBL Microbiology Systems), 0.5 g; yeast extract (BBL), 0.5 g; clarified rumen fluid, 200 ml; clarified woody-biomass digester sludge, 100 ml; mineral solution [NaCl (6 g), (NH₄)₂SO₄ (6 g), CaCl₂ (0.6 g), H₂O (1,000 ml), 150 ml; trace mineral solution (1), 10 ml; vitamin solution (1), 10 ml; resazurin (0.5 mg/ml), 1 ml; L-cysteine hydrochloride, 0.5 g; Na₂S·9H₂O, 0.2 g. The agar concentration was 1.5% (wt/vol). The pH was adjusted to 7.0 with NaHCO₃. The medium was prepared and dispensed with continuous flushing of the gas mixture mentioned above. Medium was dispensed into serum tubes (1) (18 by 150 mm; Bellco Glass, Inc., catalog no. 2048-00150) or serum bottles, according to need, stoppered with butyl rubber stoppers (Bellco, catalog no. 2048-11800), sealed with aluminum seals (Wheaton Industries; 20-mm tear-off; catalog no. 224193), and sterilized at 121°C for 20 min.

The cellulose slurry was prepared as follows: 30 g of Whatman no. 1 filter paper was ground with 1,000 ml of distilled water for 72 h in a 5-liter alumina grinding jar with pebbles (20 to 30 mm in diameter). The cellulose slurry was added (50 ml/liter) to the basic medium to make the cellulose medium or cellulose agar medium (CAM). The amount of the cellulose slurry added was varied according to the purpose of the culture. The cellulose concentration in the medium was typically in the range of 0.2 to 0.4% (wt/vol). A Whatman no. 1 filter paper strip (50 by 5 mm) was submerged in the 4.5-ml basal medium tube as filter paper strip medium (CSM) to determine the highest dilution of the mixed population exhibiting cellulolytic activity.

Other substrates, including cellulose, pectin, xylan, and glucose, were added to the basal medium at a final concentration of 1% (wt/vol). The media and methods of Holdeman et al. (6) were used for biochemical tests and fermentation product analysis.

Isolation, purification, and enumeration. Serial dilutions of sample (0.5 ml) were inoculated in triplicate into CAM (4.5 ml per tube) for roll tubes and CSM for cellulolytic activity detection. The tubes were incubated at 35°C until colonies surrounded by clear zones appeared in CAM or until the submerged filter paper strips were digested in the CSM. Cellulolytic colonies in the highest-dilution roll tube were picked and suspended in cellulose medium, which was used to inoculate CAM roll tubes. The tubes were incubated at 35°C until cellulolytic colonies appeared again. This transfer procedure was repeated several times until a pure culture was obtained. To confirm the purity, the isolate was transferred to cellulose or glucose agar medium in roll tubes and incubated at 35°C. After ascertaining that only one morphological type of colony appeared, a single colony was picked and transferred back to CAM roll tubes to check its cellul...
FIG. 1. (A) Ultrathin section of P-1 cells, showing gram-positive-type cell wall structures (an enlarged cross section is shown at bottom right). (B) Ultrathin section showing the round particle between two cells.

lolytic activity. The colony and cell morphologies from the secondary growth of the clear-zoned colonies in CAM were compared and were identical to those of the previous isolate (8).

Numbers of cellulolytic bacteria were estimated from the reciprocal of the highest dilution of the inoculated samples in which the filter paper strip was digested in CSM and in which clear-zoned colonies appeared in CAM.

Morphological studies and microscopy. Morphological observations of the cells from 24- to 48-h growth broth cultures and single colonies were made by both light microscopy (Nikon Labophot equipped with phase optics, epifluorescence, and an automatic photomicrographic Nikon FX-35A camera with UF-XII control) and electron microscopy (Philips EM-301 transmission electron microscope). Gram stains, the KOH test (5), and the 1-alanine-4-nitroanilide (LANA) test (2) were used to complement the Gram reaction. Medium without Trypticase, yeast extract, clarified rumen fluid, and digester sludge but with the vitamin solution and cellobiose was used to induce spore formation. A fresh culture (1 to 2 days) in cellobiose medium was used for electron microscopic observation of flagella.

To prepare specimens for electron microscopy, glutaraldehyde was injected into the fresh broth culture to a final concentration of approximately 1.3%. Cells were immediately centrifuged, and the supernatant was replaced with 1% glutaraldehyde in 0.025 M sodium cacodylate buffer (pH 7.2). Cells were postfixed in buffered 1% osmium tetroxide followed by 1% aqueous uranyl acetate, dehydrated in a graded ethanol to acetone series, and embedded in Spurr low-viscosity resin. Sections were cut with a diamond knife on an LKB Ultrotome III and poststained with uranyl acetate and lead citrate. For observation of flagella, cells were gently pipetted from the fresh culture and placed on Formvar-coated 200-mesh copper grids which had been precoated with 0.1% bacitracin to improve surface wettability. Cells were allowed to settle for 3 to 5 min, and the excess medium was drawn off. The grids were washed with water to remove soluble medium components and shadowed at a 30° angle with a carbon-platinum source in an electron gun using a Balzers model MED 010 vacuum evaporator. Grids were examined by transmission electron microscopy.

Physiological and biochemical identifications. Basal medium with cellobiose was used for the measurements of optimal growth temperature and pH. Cell density was determined by measurement of optical densities of cultures with a spectrophotometer (Perkin-Elmer model 35) at 610 nm. Optimal temperature and pH measurements were determined twice, and each measurement was made in triplicate. Nutrient requirements were tested in the medium with cellobiose as the only carbon and energy source and with different combinations of yeast extract, Trypticase, clarified rumen fluid, digester sludge, and vitamin solution. Two-day-old cultures grown on cellobiose basal agar slants were washed with the salt medium to serve as inocula for these tests. The fermentation products were analyzed after incubation for 2 days in PY basal medium (6) with cellobiose as the substrate. Biochemical tests were performed in PY basal medium with carbohydrates, enzymes, and other substrates. Fermentation product analyses and biochemical tests were done in the laboratory of W. E. C. and L. V. H. Moore, Virginia Polytechnic Institute and State University, Blacksburg.

Analysis of DNA base composition. Resuspended cells from a centrifuged pellet were treated with 5% (vol/vol) Triton X-100, and DNA was extracted and purified by the method of Marmur (13). DNA base compositions were determined from the buoyant density of DNA in CsCl gradients by the method of Preston and Boone (16). DNAs from Micrococcus lysodeikticus and Escherichia coli were used as standards. The mol% G+C of the DNA was calculated by the method of Schildkraut et al. (17).

RESULTS AND DISCUSSION

The isolate was rod shaped, gram positive, motile, formed endospores, and obligately anaerobic but was unable to reduce sulfate to sulfide. These differential characters indicate that the isolate can be placed in the genus Clostridium (3). It was unlike previously described mesophilic, cellu-
lolytic clostridia in growth substrates, fermentative products, morphology, and immunospecificity. Therefore, we propose a new species, *Clostridium aldrichii*, as follows.

*Clostridium aldrichii* sp. nov. *Clostridium aldrichii* (Al. driˈchi.i., N.L. gen. n., of Aldrich, named for Henry C. Aldrich, a professor of the University of Florida, Gainesville, for his contributions to ultrastructural research on the strictly anaerobic bacteria) cells are easily decolorized in Gram stains, but KOH and LANA tests are negative. Cell wall structure is that of gram-positive bacterium (Fig. 1A). Cells are 0.5 to 1.0 μm by 3 to 5 μm, straight or slightly curved with rounded ends (Fig. 2). Pleomorphism is evident,

FIG. 2. Cell morphology of strain P-1 visualized by light microscopy. (A) Vegetative cells after 24 to 48 h of growth in basal medium with cellobiose as the substrate; a round particle (arrow) can be seen between two cells (B) Spores of strain P-1 (arrow).

FIG. 3. Carbon-platinum-shadowed whole-cell preparation; bundled flagella can be seen at both ends of the paired cells.
FIG. 4. Colonial morphology of strain P-1 in CAM with clear zones around each colony. Arrow indicates the surface colony.

with some cells up to 8 to 12 μm in length; occasionally, some cells are swollen at both attached ends of paired cells. Cells are arranged mostly in pairs but some are single, or in short chains (Fig. 2). Round particles are sometimes seen between two connected cells (Fig. 1B and 2A). Young cells of strain P-1 are motile, with a bundle of flagella at one end of the cell (Fig. 3). Oblong spores occur subterminally and are formed after 2 weeks in a medium containing salts, trace vitamins, and cellobiose as the only carbon source at 35°C. Mature spores are 0.5 to 1.0 μm by 1 to 2 μm (Fig. 2B). Spores are viable after being heated at 80°C for 10 min.

Morphologically identical colonies appear in CAM 4 to 6 days after transfer of strain P-1 from medium to medium and within 24 h in the medium with cellobiose as the substrate. The surface colonies are round, smooth, entire, soft, jelly-like, semitransparent or greyish white, and 1 to 2 mm in diameter. Clear zones caused by cellulolytic activity around colonies are usually 4 to 6 mm in diameter, and the widths of the clear zones from the edge of the colonies are 2 to 3 mm. In roll tubes, surface colonies often flow down along the agar surface to form elongated colonies; the clear zones subsequently appear around the elongated growth. Subsurface colonies are irregular in shape, with many platelike structures in different dimensions; they are greyish white and more opaque than surface colonies (Fig. 4). This distinctive colony morphology and associated clear zone facilitate recognition of colonies of strain P-1.

DNA base composition. The G+C content of strain P-1 DNA is 40 mol%.

The type strain of C. aldrichii is strain P-1 (= OGI 112, = ATCC 49358 [OGI, Collection of Methanogenic Archaeobacteria of Oregon Graduate Institute of Science and Technology]). It was isolated in 1986 from a 3-month-old, mesophilic, poplar wood-fed continuously stirred anaerobic digester. It has been considered a predominant cellulolytic bacterium, since it inhabited (at 10⁶ to 10⁷) this digester for at least 2 years.

Enumeration of strain P-1. Cellulolytic activities in CSM (digestion of the immersed part of the filter paper strip; Fig. 5) and CAM (colonies with clear zones; Fig. 4) correspondingly appear in the same or nearly the same dilution in isolations, and the highest dilutions showing positive cellulolytic activity fall in the range of 10⁻⁹ to 10⁻¹⁰.

Distinguishing characteristics. Clostridium aldrichii P-1 is considered a new species on the basis of the following evidence. Most mesophilic, cellulolytic Clostridium species which have been reported (10-12, 14, 15, 18, 19) grow and produce acids in glucose; strain P-1 does not produce acids from glucose. No immunological cross-reactions were observed between the polyclonal antibody of C. aldrichii P-1 and 14 other known bacterial species (including 11 cellu-
TABLE 1. Comparison of *C. aldrichii* and *C. polysaccharolyticum*

<table>
<thead>
<tr>
<th>Characteristic*</th>
<th><em>C. aldrichii</em></th>
<th><em>C. polysaccharolyticum</em></th>
</tr>
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<tbody>
<tr>
<td>Cell morphology</td>
<td>Rods (0.5 to 1 μm by 3 to 5 μm)</td>
<td>Rods (0.8 μm by 3 to 6 μm)</td>
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<td>Spores</td>
<td>Oblong (0.5 to 1 μm)</td>
<td>Oval/spherical (1.2 by 0.8 μm)</td>
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<td>Flagella</td>
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<td>Source of isolates</td>
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<td>Sheep rumen</td>
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*Methods used for biochemical tests and fermentative product detections were the same (6) under different laboratory conditions, except DNA base ratio measurements. Symbols: +, positive reaction; -, negative reaction; w, weak reaction.*

Clostridial (cellulolytic species) by using an enzyme-linked immunosorbent assay technique (an immunological study of *C. aldrichii* P-1 will be reported elsewhere).

Only one reported cellulolytic, mesophilic *Clostridium* species, *Clostridium polysaccharolyticum* (20, 21), does not produce acids from glucose. The important characteristics of the two species are compared in Table 1. Although the DNA base ratios are similar, the arrangement of the flagella, acid production from different carbohydrates, and the differences in fermentation products from cellobiose are the primary bases for separating them into different species.

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LITERATURE CITED


