**Clostridium xylanolyticum** sp. nov., an Anaerobic Xylanolytic Bacterium from Decayed *Pinus patula* Wood Chips

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An anaerobic, mesophilic, sporeforming, xylanolytic bacterium was isolated from decayed *Pinus patula* wood chips. The cells of this organism are gram-negative rods, had peritrichous flagella, and formed terminal round endospores which distended the cell wall. Xylan, cellulose, fructose, glucose, maltose, mannose, melezitose, raffinose, rhamnose, salicin, sucrose, and xylose were utilized. The optimum temperature and optimum pH for growth were 35°C and 7.2, respectively. The DNA composition was 40 mol% guanine plus cytosine. The name *Clostridium xylanolyticum* sp. nov. is proposed for this bacterium. The type strain is strain ATCC 49623.

The potential use of biological hydrolysis of the hemicellulose components of wood has attracted much interest in the forestry and pulp and paper industries (9). As a result of biopulping research in our laboratories, an obligately anaerobic bacterium was isolated from wood chips. The cells of this organism were gram-negative rods, had peritrichous flagella, and formed terminal round endospores which distended the cell wall. Xylan, cellulose, fructose, glucose, maltose, mannose, melezitose, raffinose, rhamnose, salicin, sucrose, and xylose were utilized. The optimum temperature and optimum pH for growth were 35°C and 7.2, respectively. The DNA composition was 40 mol% guanine plus cytosine. The name *Clostridium xylanolyticum* sp. nov. is proposed for this bacterium. The type strain is strain ATCC 49623.

**MATERIALS AND METHODS**

**Media.** The basal medium (BM) used in this study contained 1.2% (wt/vol) Bacto-Agar (Difco), 230 ml of clarified rumen fluid, 12.5 ml of mineral solution I (2), 12.5 ml of mineral solution II (2), 0.25 g of yeast extract (Merck), 1.0 g of peptone (Merck), 2.5 ml of a vitamin solution (3), 5.0 ml of a volatile fatty acid mixture (4), 5.0 ml of hemin (0.01%, wt/vol), 0.5 ml of Pfennig trace element solution (12), 230 ml of distilled water. Sodium bicarbonate was used as a buffering agent, indigocarmine (0.05%, wt/vol) was used as a redox indicator, and 1.0 ml of cysteine monohydrochloride (12.5%, wt/vol) and 1.0 ml of Na2S (12.5%, wt/vol) were used as redox poisoning agents. The pH was adjusted to pH 6.8 with 1 N HCl or 1 N NaOH as required. BM supplemented with 3% oat spelt xylan (Fluka Chemicals) was also used, and for culture maintenance BM was supplemented with 0.3% cellobiose (Merck).

**Anaerobic culture methods.** In this study an anaerobic cabinet (Forma Scientific, Marietta, Ohio) was used to culture organisms under strict anaerobic conditions. Cultures were maintained in 3-oz (ca. 90-ml) McCartney bottles that had been purged with an O2-free gas mixture containing 5.5% H2, 31.8% CO2, and 62.7% N2 prior to use and sealed with butyl rubber stoppers and screw caps. All cultures were incubated at 35°C without agitation.

**Isolation procedure.** Samples of decayed *P. patula* wood chips were obtained from a chipping plant in Richards Bay, South Africa. The chips were collected in gas-tight containers and transported to our laboratory. The chips were cut into small (1.0-cm3) sections and immersed in liquid nitrogen before being milled in a liquid nitrogen mill (model A-70; F. Morat KG, Eissenbach, Federal Republic of Germany) which had been swabbed with alcohol and flamed to ensure sterility; 10-fold dilutions of each sample were prepared by using anaerobic diluent (2). The dilutions were plated onto BM containing 3% oat spelt xylan in the anaerobic cabinet and incubated at 35°C for 48 h. Colonies which produced clearing zones of xylan degradation were removed and restreaked to obtain pure cultures. The isolate used in this study was maintained by weekly transfers on slopes of BM containing 0.3% cellobiose.

**Morphology.** Living and stained cells of the purified isolate were examined by light microscopy. Flagella were examined by electron microscopy. To do this, a colony grown on BM containing 3% oat spelt xylan was suspended in physiological saline, and 1 drop of the resulting suspension was placed onto a Formvar-coated copper grid and stained with 1.0% (wt/vol) phosphotungstic acid (Sigma Chemical Co., St. Louis, Mo.). Observations were made by using a JEOL model 100C transmission electron microscope. Cell wall structure was also examined by using transmission electron microscopy. For this procedure, cells were prepared as described previously (6).

**Biochemical reactions.** Biochemical tests were performed by using the standard procedures described in the *Anaerobe Laboratory Manual* (8). Test results were read as soon as the microorganisms exhibited good growth (optical density at 625 nm, 0.8) or 1 day after they achieved constant turbidity.

**Temperature and pH studies.** The temperature and pH optima for growth were determined in BM broth. Three tubes were inoculated and incubated for each temperature and pH tested. Growth was measured by using a Bausch & Lomb Spectronic 1001 spectrophotometer at 625 nm, and the average values were calculated.

**Fermentation and product analysis.** Volatile fatty acids and organic acids were determined in cultures grown in peptone-yeast extract-glucose broth (8). Ether extracts were prepared and chromatographic analyses were performed as described in Holdeman et al. (8).

**DNA base composition.** The guanine-plus-cytosine (G+C) content was determined by using the melting point method of Marmur and Doty (11). Salmon sperm DNA (Boehringer

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Mannheim) and DNA from Clostridium clostridiiforme DSM 933T (T = type strain) were used as the standards. The DNAs were isolated by using the methods of Marmur (10) and Meyer and Schleifer (13). The melting temperature ($T_m$) was determined by measuring the relative change in $A_{260}$ with a Varian Cary model 2200 spectrophotometer. The G+C content was calculated by using the following equation (11): $T_m = 69.3 + 0.41(G+C)$.

**DNA-DNA hybridization.** In the DNA-DNA hybridization study DNA from our isolate was hybridized with DNA from *C. clostridiiforme*. Genomic DNA was isolated by the method of Marmur (10). The DNA was purified on a cesium chloride gradient as described below. The DNA was suspended in 4.0 ml of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5). The DNA concentration was adjusted to give a concentration of 50 to 100 μg/ml. A 4.3-g portion of CsCl was added to 4.0 ml of TE buffer, 200 μl of ethidium bromide (Sigma) was added, and the mixture was centrifuged at 450,000 × g for 4 h at 15°C. The gradient was visualized with UV light. The DNA band was removed by using a 15-gauge needle and a 3.0-ml syringe, and the ethidium bromide was extracted with CsCl-saturated isopropanol. The DNA was dialyzed overnight against 2.0 liters of TE buffer. The DNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 0.6 volume of isopropanol. DNAs from the isolate and *C. clostridiiforme* were denatured by heating them at 95°C for 5 min and then transferred to a nitrocellulose filter (Amersham, Buckinghamshire, United Kingdom) by using a Bio-Rad dot blot apparatus connected to a vacuum source. The blots were washed with 100 μl of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and fixed at 80°C for 2 h in a vacuum oven. DNA from the isolate was labeled with $^{32}$P by using a nick translation kit (Amersham). The
blots were hybridized overnight submerged in a water bath at 42°C in 30 ml of a solution containing 10 μl of the probe, 500 μl of salmon sperm DNA, 50 μl of 10 M NaOH, 300 μl of 2 M Tris (pH 7.4), and 475 μl of 1 M HCl. After hybridization the filter was rinsed in 0.2× SSC-0.02% sodium dodecyl sulfate for 5 min and then washed three times (20 min each) at room temperature. Then the filter was washed twice (20 min each) in 0.1× SSC-0.1% sodium dodecyl sulfate under stringent conditions at 60°C. The nitrocellulose filter was air dried and autoradiographed overnight at −70°C on X-ray film (Fuji Photo Film Co., Tokyo, Japan). The densities of the autoradiographs were measured by using an LKB model 2202 Ultrascan laser densitometer.

RESULTS AND DISCUSSION

The isolation procedures which we used yielded one anaerobic xylanolytic isolate which was selected for identification on the basis of hydrolysis of xylan. This isolate is an obligately anaerobic, sporeforming, rod-shaped bacterium which does not reduce sulfate to hydrogen sulfide and therefore is a member of the genus *Clostridium* Prazmowski (5).

*Clostridium xylanolyticum* sp. nov. *Clostridium xylanolyticum* (x.y.lan.o. ly’ ti. cum. Gr. n. xylanosum, xylan; Gr. adj. lyticus, dissolving; N. L. adj. xylanolyticum, xylan dissolving). Cells are gram-negative, obligately anaerobic, and rod shaped (length, 1.8 to 3.0 μm; width, 0.5 to 0.8 μm). A single, terminal, round endospore is formed by each cell; the spore distends the cell wall (Fig. 1). The cells are motile by means of peritrichous flagella (Fig. 2). Thin sections reveal a five-layer cell wall structure (Fig. 3).

Xylan is a good substrate for growth. Amygdalin, arabinose, glycerin, inositol, lactose, mannitol, ribose, sorbitol, and trehalose are not fermented. Gelatin is not liquefied; urease, indole, lecithinases, lipase, and catalase are not produced; and nitrate is not reduced. The fermentation end products from PYG (5) are formic, lactic, and acetic acids. The optimum temperature for growth is 35°C (Fig. 4), and the optimum pH for growth is 7.2 (Fig. 5).

The DNA G+C content is 40 mol%. DNA-DNA hybridization results show that under stringent conditions (60°C) the DNA of *C. clostridiiforme* exhibits only 14% homology with the DNA of our isolate. This suggests that although there is a low level of homology between the two DNAs, these organisms are not the same genetically.

Hydrolysis of starch and nonreduction of nitrate distinguish our isolate from *C. clostridiiforme*. When grown on blood agar, our isolate is beta-hemolytic, whereas *C. clostridiiforme* is nonhemolytic. *C. clostridiiforme* is susceptible to clindamycin, whereas our isolate is resistant. The G+C content of *C. clostridiiforme* is 48 mol%, whereas the G+C content of our isolate is 40 mol%. The position of the endospores of *C. clostridiiforme* is central to subterminal, whereas the position of the endospores of our isolate is terminal. Our isolate also differs from *Clostridium aerotolerans* ATCC 43524T, which ferments lactose, ribose, and trehalose. *C. aerotolerans* also utilizes xylan. The DNA G+C content of *C. aerotolerans* is 40 mol%, which is the same as the G+C content of our isolate. Another *Clostridium* strain which utilizes xylan as a growth source is *Clostridium cellulovorans* DSM 3052T. However, this organism differs from our isolate in that it ferments lactose and does not ferment melezitose, rhamnose, and xylose. The DNA G+C content of *C. cellulovorans* is 26 mol%, which is markedly different from the DNA base composition of our isolate.

The type strain is strain ATCC 49623. This strain was isolated from a P. patula chip pile which had been exposed to environmental conditions for 10 months.

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REFERENCES


