**Clostridium josui** sp. nov., a Cellulolytic, Moderate Thermophilic Species from Thai Compost

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A new strictly anaerobic, moderate thermophilic (optimum temperature, 45°C), cellulolytic, spore-forming bacterium was isolated from Thai compost. The cells of this organism stained gram positive but became gram negative as cultures reached stationary phase. They were nonmotile rods and formed terminal oval spores which swelled the cells. The deep colonies of this organism were spindle shaped and yellowish white. A variety of carbohydrates, such as cellobiose, esculin, and xylose, served as substrates for growth. Ethanol, acetate, butyrate, hydrogen, and carbon dioxide were produced during growth on cellulose or cellobiose. This organism hydrolyzed crystalline cellulose, rice straw, and other cellulosic materials without any chemical pretreatment. Optimal growth occurred at 45°C and pH 7.0. The deoxyribonucleic acid base composition was 40 mol% guanine plus cytosine. The name *Clostridium josui* sp. nov. is proposed for this new isolate, and the type strain has been deposited in the Fermentation Research Institute, Tsukuba, Japan, as strain FERM P-9684.

**MATERIALS AND METHODS**

**Cellulosic materials.** Ball-milled cellulose (BMC) was prepared by ball milling a 3% water suspension of pure cellulose (KCI flocks W-300; Sanyo Kokusaku Pulp Co., Ltd., Tokyo, Japan) for 3 days. The microcrystalline cellulose used was Avicel (E. Merck AG, Darmstadt, Federal Republic of Germany). Agricultural cellulosic wastes, such as rice straw, rice husks, corn cobs, sawdust, and bagasse, were obtained from sources in Thailand and were used without any chemical pretreatment. After drying, these cellulosic materials were ground for 1 min in a vibrating sample mill (model Ti-200; Heiko Seisakusho, Ltd., Tokyo, Japan), sieved through a 100-mesh sieve (149 μm), and used as main carbon sources in media. Other chemicals used were commercial products of the highest purity available.

**Media and culture techniques.** (i) Rumen fluid medium. A rumen fluid medium was prepared by modifying the medium described by Taya et al. (20). The procedure used was as follows. A mixture of 7.5 ml of mineral I (K₂HPO₄, 7.5 ml of mineral II (K₂HPO₄, NaCl, (NH₄)₂SO₄, MgSO₄·7H₂O, CaCl₂), 30 ml of rumen fluid, 0.02 g of cellobiose, and 0.1 ml of a 1% resazurin solution were stirred together well. The medium was adjusted to 100 ml by adding distilled water, and the pH was adjusted to 7.0 with 0.1 N HCl. After Na₂CO₃ (0.45 g) was added, the mixture was maintained at about 50°C. Then CO₂ gas was bubbled through the medium for at least 30 min, so that the medium turned pink. Cysteine (0.0025 g) was then added. These procedures lowered the medium pH to 6.8. Then, Na₂S (0.0025 g) was added. CO₂ gas bubbling was continued until the pink color of the medium disappeared. The medium was then dispensed into test tubes, which were plugged with butyl rubber stoppers and held in a press rack during autoclaving at 110°C for 10 to 15 min.

(ii) Cellulose-rumen fluid medium. In order to enhance growth of anaerobes, a cellulose-rumen fluid medium was used. This medium was prepared by adding 1% yeast extract or 0.59% Gifu Anaerobic Medium (Nissui Co., Tokyo, Japan) and 1% cellulose to the rumen fluid medium described above.

(iii) BMC-, Avicel-, and agricultural cellulose-enriched rumen fluid media. BMC-, Avicel-, and agricultural cellulose-enriched rumen fluid media were prepared by adding cellulose substances at concentrations of 1% to the rumen fluid medium described above.

Solid media for use in the roll tube method described by Hungate (9) were prepared by adding 2% agar to BMC-rumen fluid medium or Avicel-rumen fluid medium. Media were prepared and microorganisms were cultivated under anaerobic conditions by using O₂-free CO₂ gas.

**Isolation of cellulolytic anaerobic bacteria.** Bacteria were isolated from samples collected from compost heaps in the Bangkok area and some other provinces of Thailand. One loopful of each sample was inoculated into cellulose-rumen fluid medium and incubated at 37 and 45°C. After 48 h of incubation the cultures producing discrete gas bubbles and solubilized cellulose, as judged by a decrease in the bulk of cellulose precipitate, were selected for isolation on solid media. Anaerobes with high cellulolytic activity were isolated by the roll tube method. Colonies with large clear zones were transferred individually into liquid medium by using a sterile bent Pasteur pipette. The isolation procedure...
FIG. 1. Light micrograph (A) and scanning electron micrograph (B) of C. josui grown in 0.9% BMC–rumen fluid medium for 4 days at 45°C. (A) Bar = 10 μm. (B) Bar = 1 μm.

was repeated more than three times until the bacterial cells appeared to be homogeneous microscopically. By using this procedure a pure potent cellulolytic strain was isolated. The isolate was stored at −80°C as a deep agar culture in rumen fluid agar medium containing 0.1% Avicel. It was transferred every 2 to 3 months into fresh medium.

Cultivation in cellulose-rumen fluid medium. The culture of the organism kept at −80°C was activated before cultivation by inoculating it into 5 ml of cellobiose-rumen fluid medium and incubating the preparation for 24 h at 45°C. Then, 0.2 ml of this activated culture was inoculated, in duplicate, into 5-ml portions of rumen fluid medium containing 0.05 g of BMC, Avicel, or ground natural cellulosic material, such as rice straw, rice husks, corncobs, sawdust, or bagasse. Inoculated culture tubes were placed in a press test tube rack and incubated at 45°C for 7 days. The contents of the remaining cellulose and the products, such as ethanol and volatile fatty acids, released into the supernatant broth were then determined.

Identification. The most potent cellulolytic strain, strain III (T = type strain), was examined and classified by using the methods described in the Anaerobe Laboratory Manual (7) and by Holdeman and Moore (8). Biochemical tests were performed by using prereduced anaerobically sterilized medium (Scott Laboratories, Inc., Fiskeville, R.I.) supplemented with 10% rumen fluid. Clostridium innocuum ATCC 14051 was used as a control. Tests were conducted at day 3 and again at day 7.

Electron microscopy. Cells grown on BMC-rumen fluid medium were negatively stained with 1% phosphotungstic acid (pH 6.2) for detecting the presence of flagella with an electron microscope. Scanning electron micrographs were taken with a JEOL model JSM 25S electron microscope.

Isolation of DNA and measurement of its G+C content. Deoxyribonucleic acid (DNA) was isolated by a modification of the Marmur method (13). The DNA base composition, expressed as guanine-plus-cytosine (G+C) content, was determined directly by using the method of Katoh et al. (10). In this method, DNA extracted from bacterial cells and highly purified was completely degraded to deoxyribonucleotides by a Penicillium enzyme, nuclease P₁. After that, the nucleotide composition was determined by high-performance liquid chromatography, using a Licrosorb RP-18 (Merck) column.

Growth measurements. To determine the growth rates of the strain on cellobiose-rumen fluid medium, the optical densities of the culture broth were measured at 570 nm with a Hitachi model 100-20 spectrophotometer.

Analyses. The cell mass, residual cellulose, carboxymethyl cellulose-hydrolyzing activity, ethanol, acetic acid, and other volatile products in the culture broth were determined by using previously described methods (17–19).

RESULTS AND DISCUSSION

The isolate was strictly anaerobic, rod shaped, and non-motile and formed endospores, but was unable to reduce sulfate. These characteristics place it in the genus Clostridium (1). It differed significantly from other mesophilic cellulolytic species of Clostridium in motility, gram staining, optimum growth temperature, and ability to produce products. Therefore, we propose a new species, Clostridium josui; a species description is given below.

Description of Clostridium Josui sp. nov. Clostridium josui (jo.su'i) (the first four letters were obtained by combining the initial letters of the names of the authors, and the last letter was added according to the requirements of the International Code of Nomenclature of Bacteria [11]). The rod-shaped cells are gram positive, becoming gram negative as cultures reached stationary phase, and are nonmotile. The slightly curved rods are 0.2 to 0.3 μm wide and 3 to 5 μm long and are found as individuals and short or long chains.
TABLE 1. Comparison of characteristics of Clostridium species with cellulosolytic activity

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>C. josui sp. nov.</th>
<th>C. cellulolyticum</th>
<th>C. papyro-solvens</th>
<th>C. stercorarium</th>
<th>C. cellulosaparum</th>
<th>C. populetii</th>
<th>C. thermocellum</th>
<th>C. cellulosovorans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Products from PYG*</td>
<td>A,2,p,b</td>
<td>A,1,f,2</td>
<td>A,1,2</td>
<td>A,2,l</td>
<td>A,1,f,2</td>
<td>B,1,a</td>
<td>A,2,1</td>
<td>B,f,a</td>
</tr>
<tr>
<td>Motility</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Acid produced from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>−</td>
<td>+</td>
<td>±</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Galactose</td>
<td>−</td>
<td>w</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Lactose</td>
<td>w</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>w</td>
<td>−</td>
<td>NA</td>
<td>+</td>
</tr>
<tr>
<td>Maltole</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Mannose</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NA</td>
<td>+</td>
</tr>
<tr>
<td>Melibiose</td>
<td>w</td>
<td>−</td>
<td>−</td>
<td>NA</td>
<td>w</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Salicin</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Sucrose</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>NA</td>
<td>+</td>
<td>NA</td>
<td>+</td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>40d</td>
<td>41c</td>
<td>30c</td>
<td>39c</td>
<td>28c</td>
<td>28c</td>
<td>39c</td>
<td>26</td>
</tr>
<tr>
<td>Optimum temp (°C)</td>
<td>45</td>
<td>31–35</td>
<td>25–30</td>
<td>65</td>
<td>30–37</td>
<td>35</td>
<td>60</td>
<td>37</td>
</tr>
<tr>
<td>Min/max tempf</td>
<td>25/60</td>
<td>25/45</td>
<td>10/50</td>
<td>25/45</td>
<td>20/40</td>
<td>37/70</td>
<td>20/40</td>
<td></td>
</tr>
<tr>
<td>Gram stain</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* Products listed in order of amount usually detected: in peptone-yeast extract-glucose medium (PYG): a and A, acetic acid; b and B, butyric acid; f, formic acid; l and L, lactic acid; p, propionic acid; 2, ethanol. Capital letters indicate at least 1 mg/100 ml of culture; small letters indicate less than 1 mg/100 ml.

+ Reaction positive for 90–100% of the strains (pH of sugars below 5.5); −, reaction negative for 90–100% of the strains; ±, 50 to 89% of the strains positive; w, weak reaction (pH of sugars between 5.5 and 5.9).

NA, Not available.

Determined by high-performance liquid chromatography.

Maximum temperature for growth/minimum temperature for growth.

The organisms form terminal oval spores which cause a marked swelling of the cells (Fig. 1). The mature spores are 0.4 to 0.5 μm in diameter. Endospores are still viable after heating at 80°C for 20 min.

In agar medium containing BMC, cultures show a clear zone around each colony within 3 days. Deep colonies are spindle shaped and a dull yellowish color. The clear zone of cellulolysis reaches a diameter of 10 mm after prolonged incubation. Cellulose (1%) in agar roll tubes containing large cellulose materials, such as BMC, Avicel, rice straw, and sawdust, whereas propionate and butyrate are the only carbon sources.

The organisms do not utilize these materials. All of these compounds are negligibly produced when rice husks, sawdust, and bagasse are produced in large quantities when cultures are grown on cellulosic materials, such as BMC, Avicel, rice straw, and corn cobs, whereas propionate and butyrate are detected only in trace amounts (Table 2). All of these compounds are not produced. Milk is unchanged, casein is not digested, and gelatin is not liquefied.

TABLE 2. Degradation of cellulosic materials and generation of products by C. josui sp. nov. after 7 days of cultivation at 45°C

<table>
<thead>
<tr>
<th>Cellulosic material (10 g/liter)</th>
<th>Extent of cellulose degradation (g/liter)*</th>
<th>Ethanol (g/liter)</th>
<th>Acetic acid (g/liter)</th>
<th>CMCase activity (U/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMC</td>
<td>8</td>
<td>0.97</td>
<td>0.99</td>
<td>57</td>
</tr>
<tr>
<td>Avicel</td>
<td>6</td>
<td>1.16</td>
<td>0.79</td>
<td>70</td>
</tr>
<tr>
<td>Rice straw</td>
<td>4</td>
<td>0.04</td>
<td>0.14</td>
<td>53</td>
</tr>
<tr>
<td>Corn cobs</td>
<td>2</td>
<td>0.005</td>
<td>0.01</td>
<td>29</td>
</tr>
</tbody>
</table>

* The extent of degradation was estimated from the bulk change.

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LITERATURE CITED


