Cellulosibacter alkalithermophilus gen. nov., sp. nov., an anaerobic alkalithermophilic, cellulolytic-xylanolytic bacterium isolated from soil of a coconut garden

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An obligately anaerobic, cellulolytic-xylanolytic bacterium, designated strain A6T, was isolated from soil of a coconut garden in the Bangkuntien district of Bangkok, Thailand. The strain was Gram-stain positive, catalase-negative, endospore-forming, motile and rod-shaped with a cell size of 0.2–0.3 μm. Optimal growth of strain A6T occurred at pH 6.55, 55 °C. Strain A6T fermented various carbohydrates, and the end products from the fermentation of cellobiose were acetate, ethanol, propionate and a small amount of butyrate. The major cellular fatty acids were iso-C14 : 0 3-OH, iso-C15 : 0, iso-C16 : 0 and C16 : 0. The cell-wall peptidoglycan contained meso-diaminopimelic acid. No respiratory quinones were detected. The DNA G+C content was 30.0 mol%. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the strain represented a new phyletic sublineage within the family Clostridiaceae, with 93.0 % 16S rRNA gene sequence similarity to recognized species of this family. On the basis of phenotypic, genotypic and physiological evidence, strain A6T represents a novel species of a new genus, for which the name Cellulosibacter alkalithermophilus gen. nov., sp. nov. is proposed. The type strain of the type species is A6T (=TISTR 1915T = KCTC 5874T).

The family Clostridiaceae, as defined by the taxonomic outline of Bergey’s Manual of Systematic Bacteriology, contains as its core the genus Clostridium sensu stricto (Wiegel et al., 2006). Several species that group outside Clostridium sensu stricto but fall within the family Clostridiaceae from a 16S rRNA gene sequence phylogeny perspective have been transferred to new genera. Thus Clostridium fervidum has been transferred to the genus Caloramator as Caloramator fervidus and Clostridium pfeffnigii has been transferred to the genus Oxobacter as Oxobacter pfeffnigii (Collins et al., 1994).

The environments from which alkalithermophiles are usually isolated are alkaliphilic and thermobiotic, but some alkalithermophiles have also been isolated from mesobiotic, slightly acidic to neutrophilic habitats (Wiegel & Kevbrin, 2004). In this study, we isolated a saccharolytic, Gram-stain-positive, rod-shaped bacterium (strain A6T) with low DNA G+C content, of the family Clostridiaceae, from a coconut garden in Thailand. Soil from the coconut garden was collected from a depth of 30 cm, where the soil pH ranged...
between 6.8 and 8.0, and the temperature was 30 °C. 16S rRNA gene sequence analysis showed that the isolate fell outside of the radiation of the genus *Clostridium sensu stricto* and could be differentiated from the closely related species *Clostridium thermosuccinogenes* DSM 5807^T^ by physiological and biochemical characteristics and DNA G+C content. Therefore strain A6^T^ represents a novel species of a new genus within the family *Clostridiaceae*.

Soil samples were collected from different locations within a coconut garden in the Bangkuntien district of Bangkok, Thailand and were stored anaerobically at 4 °C until use. In order to obtain enrichment cultures, 5 g of each sample was mixed anaerobically with basal medium (BM) of the following composition (1−1): 1 g K2HPO4, 2 g urea, 7 g NaHCO3, 1 g Na2CO3, 2 g yeast extract, 0.7 g cysteine-HCl, 0.001 g reazurin and 200 µl mineral solution. Mineral solution (100 ml) consisted of 25 g MgCl2·6H2O, 3.75 g CaCl2·2H2O and 0.0312 g FeSO4·6H2O. The pH was adjusted to 9.5 with 4 M NaOH. Each soil sample was added to 15 ml of BM containing a piece of filter paper (1 cm × 10 cm) as a substrate and incubated at 55 °C. Samples that showed effective degradation of the filter paper were selected for enrichment in BM containing Sigmaticell type 20 cellulose powder (0.5 % w/v; Sigma). Samples were then isolated from repeated dilution rows of anaerobic agar shake-roll tubes technique (Hungate, 1969). Strain A6^T^ was obtained several times following the isolation of single colonies. The purity of the isolates and cell morphology were examined under a scanning electron microscope (S-2500; Hitachi). Gram staining was conducted using conventional methodology and confirmed using the KOH test (Powers, 1995). Substrate utilization was tested by growing the strain in BM containing 0.5 % (w/v) of the following substrates: lactose, D-galactose, sucrose, D-glucose, trehalose, L-arabinose, D-ribose, D-mannose, maltose, D-xyllose, D-fructose, cellulobiose, raffinose, inulin, sorbitol, mannitol, glycerol, chitin, pectin, gelatin, salicin, ascinul, sugar cane bagasse, corn hull, corn cob, rice straw, rice stalk, dextran, chitosan, gum, wheat starch, potato starch, rice starch, cellulobiose, cellulose powder, Avicel, oat spelt xylan, larchwood xylan or birchwood xylan. The utilization of the different substrates was assessed by determining the optical density at 600 nm (OD<sub>600</sub>) after incubation at 55 °C for 4 days; a positive result was classified as an OD<sub>600</sub> measurement of >0.40. The end products of cellulose, cellulobiose and corn hull fermentation were determined by gas chromatography (GC 14 A; Shimadzu) using a Carbo-pack B-DA/4 % carbowax 20 M column. Other biochemical tests were conducted according to the methods of Holdeman et al. (1977) and Summanen et al. (1993).

The G+C content (mol%) of the genomic DNA was determined by reverse-phase high-performance liquid chromatography (Tamaoka & Komagata, 1984). Cellular fatty acids were extracted, methylated and analysed using the standard Microbial Identification System (MIDI) (Sasser, 1990) by DSMZ GmbH, Germany. Peaks were identified by comparing the results with the TSBA 40 database. Diaminopimelic acid (DAP) in the cell wall and isoprenoid quinone were determined as described by Komagata & Suzuki (1987).

DNA extraction was performed using the Qiamp DNA Stool kit (Qiagen) according to the manufacturer’s protocol after incubating the cells for 2 days. The almost complete 16S rRNA gene sequence was amplified by PCR using primers 8F and 1492R (Chimtong et al., 2011). Sequences of the closest relatives were retrieved by a search of references available in the GenBank/EMBL database using the BLAST software program (Altschul et al., 1997), and were aligned using CLUSTAL_X software, version 1.83 (Thompson et al., 2003). The alignment was edited manually to remove gaps and ambiguous nucleotides prior to the construction of phylogenetic trees. The phylogenetic tree was constructed using the neighbour-joining (NJ) method (Saitou & Nei, 1987), maximum-parsimony (Kluge & Farris, 1969) and the minimum-evolution method (Felsenstein, 1997) in the MEGA software version 4.0 (Tamura et al., 2007). The confidence values for branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings. The values for sequence similarity among the closest strains were determined using the EzTaxon server (Chun et al., 2007).

Strain A6^T^ was Gram-stain-positive, with short, straight or slightly curved rods measuring 2.0–3.0 µm long and 0.2–0.3 µm wide (Fig. S1a, available in IJSEM online). Binary division occurred by constriction (Fig. S1a). A single spherical endospore was formed at the terminal site, with a diameter of 0.26–0.46 µm (Fig. S1b). The temperature range for growth at pH 9.5 was 50–65 °C, with an optimum of 55 °C (no growth at or below 50 °C, or at or above 65 °C). The pH range for growth at 55 °C was 8.0–10.0, with optimum growth at pH 9.5 (no growth at or below pH 8.0, or at or above pH 10.0). The doubling times under optimal growth conditions of 55 °C and pH 9.5 were 7.86 and 2.74 h with sole carbon sources of cellulose powder or cellulobiose, respectively. Substrate utilization was observed on lactose, D-galactose, sucrose, D-glucose, trehalose, L-arabinose, D-ribose, D-mannose, maltose, D-xylitol, melan, glycerol, chitin, pectin, gelatin, salicin, ascinul, sugar cane bagasse, corn hull, corn cob, rice straw, rice stalk, dextran, chitosan, gum, wheat starch, potato starch, rice starch, cellulobiose, cellulose powder, Avicel, oat spelt xylan, larchwood xylan or birchwood xylan. The utilization of the different substrates was assessed by determining the optical density at 600 nm (OD<sub>600</sub>) after incubation at 55 °C for 4 days; a positive result was classified as an OD<sub>600</sub> measurement of >0.40. The end products of cellulose, cellulobiose and corn hull fermentation were determined by gas chromatography (GC 14 A; Shimadzu) using a Carbo-pack B-DA/4 % carbowax 20 M column. Other biochemical tests were conducted according to the methods of Holdeman et al. (1977) and Summanen et al. (1993).

The G+C content (mol%) of the genomic DNA was determined by reverse-phase high-performance liquid chromatography (Tamaoka & Komagata, 1984). Cellular fatty acids were extracted, methylated and analysed using the standard Microbial Identification System (MIDI) (Sasser, 1990) by DSMZ GmbH, Germany. Peaks were identified by comparing the results with the TSBA 40 database. The major cellular fatty acids were C<sub>16:0</sub> (24.37 %), iso-C<sub>16:0</sub> (13.36 %), iso-C<sub>14:0</sub> 3-OH (11.29 %) and iso-C<sub>15:0</sub> (10.09 %). The less abundant cellular fatty acids were iso-C<sub>17:0</sub> (4.53 %), C<sub>14:0</sub> 2-OH (4.29 %), C<sub>16:0</sub> (3.48 %), anteiso-C<sub>15:0</sub> (2.47 %),...
The almost complete 16S rRNA gene sequence of isolate A6T (1570 nt) was compared against the 16S rRNA gene sequences of selected representatives of the family Clostridiaceae. Phylogenetic analysis of this large dataset revealed that the novel isolate belonged to a cluster within the family Clostridiaceae (Figs S2, S3 and S4). Comparison of the A6T 16S rRNA gene sequence with selected 16S rRNA gene sequences of the members of the family Clostridiaceae demonstrated that strain A6T formed a clade with members of the genus Clostridium (Fig. 1). High levels of 16S rRNA gene sequence similarity were found with Clostridium thermosuccinogenes DSM 5807^T (93.0 %), Anaerobacter polyendosporus DSM 5272^T (82.6 %), Caloranaerobacter azorensis DSM 13643^T (82.4 %), Oxobacter p fennigii DSM 3222^T (82.1 %), Natronincola histidinovorans DSM 11416^T (81.8 %), Caloramator fervidus ATCC 43204^T (81.4 %), Sarcina maxima DSM 316^T (81.3 %), Anoxynatronum sibiricum DSM 15060^T (81.2 %), Caminicella sporogenes DSM 14501^T (81.0 %), Thermohalobacter berrensis ATCC 19398^T (79.5 %) and Alkaliphilus crotonatoxidans DSM 15060^T (79.1 %). The nucleotides of isolate A6T differed from those of the closest neighbouring genera.

Strain A6T was clearly distinguished from the phylogenetically closest related species C. thermosuccinogenes DSM 5807^T, the closest related genus Clostridium, and other phylogenetically related genera of the family Clostridiaceae by physiological and biochemical characteristics and the DNA G+C content (Table 1). In addition, strain A6T was differentiated from the genera Alkaliphilus, Anoxynatronum, Caloranaerobacter, Caminicella, Natronincola, Sarcina, Thermohalobacter and Tindallia based on 16S rRNA gene sequence analyses and the following characteristics: from members of the genus Alkaliphilus by the temperature range within which growth occurs (Takei et al., 2001); from members of the genera Anoxynatronum and Caloranaerobacter in terms of spore formation (Garnova et al., 2003; Wery et al., 2001); from members of the genus Caminicella by being Gram-negative (Alain et al., 2002); from members of the genera Natronincola and Tindallia in terms of carbohydrate utilization (Kevbrin et al., 1998; Zhilina et al., 1998); from members of the genus Sarcina in terms of morphology and motility (Canale-Parole, 1986); and from members of the genus Thermohalobacter in terms of its Gram-staining reaction and spore formation (Cayol et al., 2000). Taken together, these data indicate that strain A6T represents a novel species in a new genus of the Clostridiaceae family, for which the name Cellulosibacter alkalithermophilus gen. nov., sp. nov. is proposed. The type strain of the type species is A6T (=TISTR 1915^T=KCTC 5874^T).

Description of Cellulosibacter gen. nov.

Cellulosibacter [Cel.lu.lo.si.bac’ter N.L. n. cellulosum cellulose; N.L. masc. n. bacter a rod, staff; N.L. masc. n. Cellulosibacter cellulose (degrading) rod].

Cells are Gram-stain-positive, spore-forming, motile, straight to slightly curved rods. Thermophilic and alkaliphilic, with growth at pH 8.0–10.0. Catalase-negative. Cellular fatty acids are composed mainly of 16-carbon-containing saturated, branched fatty acids (iso-C16:0, iso-C14:0 3-OH and iso-C15:0). The cell wall peptidoglycan is meso-DAP. Obligately anaerobic. The type species is Cellulosibacter alkalithermophilus.

Description of Cellulosibacter alkalithermophilus sp. nov.

Cellulosibacter alkalithermophilus [al.ka.li.ther.mo’phi.lus N.L. n. alkali (from Arabic article al the; Arabic n. qalil ashes of saltwort), alkali; Gr. n. thermé heat; N.L. adj. philus -a -um (from Gr. adj. philos -é -on), friend, loving;...
Table 1. Differential characteristics of strain A6\(^{T}\), phylogenetically related genera of the family Clostridiaceae and the phylogenetically related species Bacteroides cellulosolvens

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphology and size (μm)</strong></td>
<td>Straight to slightly curved rods, 0.2–0.3 × 2.0–3.0</td>
<td>Straight rods, 0.3–0.4 × 2.0–4.0*</td>
<td>Thick rods, 1.5–3.0 × 4.0–8.0</td>
<td>Rods, 0.65–0.75 × 2.0–2.5</td>
<td>Slightly curved rods, 0.4 × 1.6–3.5</td>
<td>Rods, 0.8–6.0</td>
<td>Straight rods, 0.5–1.7 × 2.4–7.6</td>
</tr>
<tr>
<td><strong>Motility</strong></td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Gram stain reaction</strong></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td><strong>Presence of spores</strong></td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><strong>DNA G+C content (mol%)</strong></td>
<td>30.0</td>
<td>35.9</td>
<td>29.0</td>
<td>39.0</td>
<td>38.0</td>
<td>43.0</td>
<td>27.0–28.0</td>
</tr>
<tr>
<td><strong>Optimum</strong></td>
<td>55</td>
<td>58</td>
<td>25–35</td>
<td>68</td>
<td>36–38</td>
<td>42</td>
<td>25</td>
</tr>
<tr>
<td><strong>pH for growth</strong></td>
<td>Range 8.0–10.0</td>
<td>6.0–9.0</td>
<td>5.5–8.5</td>
<td>5.5–9.0</td>
<td>6.3–8.0</td>
<td>5.7–8.0</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Optimum</strong></td>
<td>9.5</td>
<td>7.5</td>
<td>6.5–7.5</td>
<td>7.0–7.5</td>
<td>7.3</td>
<td>7.0</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Cellulose degradation</strong></td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><strong>Xylan degradation</strong></td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td><strong>End products†</strong></td>
<td>A, E, P, B(^{a})</td>
<td>F, A, L, S, H(_2)(^{b})</td>
<td>A, L, B, E, b,</td>
<td>A, E, L, i-b, i-v, n-v,</td>
<td>B, H(^{d})</td>
<td>A, E(^{a})</td>
<td>M, A, H(_2)+ (\text{CO}_2, \text{B, E}^{c})</td>
</tr>
</tbody>
</table>

*Data from Drent *et al.* (1991).
†End products from fermentation of: a, cellobiose; b, inulin; c, glucose; d, methoxybenzenoids; e, pectin.
N.L. masc. adj., alkalitherophilus, loving alkaline environment and heat.

Displays the following characteristics in addition to those described for the genus. Cells are 2.0–3.0 μm long and 0.2–0.3 μm wide. Spherical endospores (0.26–0.46 μm in diameter) are formed at a terminal position. Colonies grown on BM agar plates supplemented with cellulose powder are circular, yellowish and smooth (0.5–1.0 mm in diameter). The temperature range for growth at pH 9.5 is 50–65 °C (optimum, 55 °C), and the pH range for growth at pH 5.5–9.5. Uses lactose, D-galactose, sucrose, D-glucose, trehalose, L-arabinose, D-ribose, D-mannose, maltose, D-xylose, D-fructose, cellobiose, raffinose, sugar cane bagasse, corn hull, corn cob, rice straw, rice stalk, dextran, chitosan, gum, wheat starch, potato starch, rice starch, cellulose, cellulose powder, Avicel, oat spelt xylan, larchwood xylan and wheat starch, potato starch, rice starch, cellobiose, cellulose powder, Avicel, oat spelt xylan, larchwood xylan and birchwood xylan as carbon and energy sources, but not pectin, mannitol, glycerol, chitin, pectin, gelatin, salicin, inulin or aesculin. Does not curdle milk. H2S and indole are produced. The end products of cellobiose fermentation are ethanol, acetate, propionate and butyrate.

The type strain, A6T (=TISTR 1915T =KCTC 5874T), was isolated from a soil sample collected from a coconut garden in the Bangkuntien district of Bangkok, Thailand. The DNA G+C content of the type strain is 30.0 mol%.

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References


