**Clostridium arbusti** sp. nov., an anaerobic bacterium isolated from pear orchard soil

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An obligately anaerobic, Gram-positive, spore-forming bacterial strain, designated SL206T, was isolated from pear orchard soils. Strain SL206T cells were straight or slightly curved rods, with motility by peritrichate flagella. Cell walls contained meso-diaminopimelic acid; wall sugars were glucose, rhamnose and mannose. The major fatty acids were C16:0, C18:1\textit{v}9\textit{c} and summed feature 10 (containing C18:1\textit{v}11\textit{c}/9\textit{t}/6\textit{t}). API 20A reactions were negative for oxidase, catalase and acid production from L-rhamnose, sucrose, trehalose, D-xylose, melezitose, salicin and D-sorbitol, and positive for acid production from D-glucose, sucrose, malrose, D-mannose and raffinose. Glucose was fermented to acetate, butyrate, CO2, H2 and ethanol in culture. The G+C content of the genomic DNA was 31.1 mol%. Based on comparative 16S rRNA gene sequence analysis, the isolate belonged to the genus *Clostridium* and formed a clade with *Clostridium pasteurianum*. The species most closely related to strain SL206T were *C. pasteurianum* (98.6 % similarity) and *Clostridium acidisoli* (97.8 % similarity). In DNA–DNA relatedness studies, the isolate had 59.5 % relatedness with *C. pasteurianum* and thus represented a unique species. On the basis of these studies, strain SL206T (\textit{KCTC 5449T} = \textit{JCM 14858T}) is proposed to represent the type strain of a novel species, *Clostridium arbusti* sp. nov.

The genus *Clostridium* is a large and phenotypically heterogeneous taxon, currently comprising more than 190 species (Wiegel et al., 2006). The DNA of these anaerobic bacteria are classified as belonging to three major groups (Wiegel et al., 2006): the low-G+C Gram-positive bacteria, the *Clostridium coccoides–Eubacterium rectale* group (cluster XIVa), and the *Clostridium leptum* group (cluster IV). The species within this genus are divided into several cluster groups based on 16S rRNA gene sequences (Collins et al., 1994). Cluster I, which includes the type species of the genus, *Clostridium butyricum*, is the largest of the clostridial groups, and members of this group exhibit similarity values above 90 % despite exhibiting a range of saccharolytic, proteolytic, psychrophilic, mesophilic and thermophilic phenotypes. Some of the strains within cluster I have been isolated and described as novel microorganisms obtained from soil or sediments (Jeong et al., 2004; Kim et al., 2006, 2007; Lee et al., 2007). In this study, we report the taxonomic description of a strictly anaerobic bacterium within cluster I, designated strain SL206T.

Strain SL206T was isolated from soil samples (30 cm depth) collected from a pear orchard in Daejeon, Republic of Korea. The samples were serially diluted in 0.85 % (w/v) saline solution, spread onto Reinforced Clostridial Medium (RCM; Difco) that was heated at 80 °C for 10 min, and incubated at 30 °C under anaerobic conditions (Forma Anaerobic System; Thermo Fisher Scientific) using a gas phase of N2/H2/CO2 (88 : 7 : 5 %, v/v). Strain SL206T was subcultured several times to obtain a pure culture on agar plates. The reference strains used in the study were *Clostridium pasteurianum* (KCTC 1674T) and *Clostridium acidisoli* (KCTC 5384T). Strains were cultured routinely on RCM in Hungate tubes (Bellco) under anaerobic conditions and stored at −80 °C as skim milk (Difco) suspensions (10 %, w/v).

To determine the phenotypic characteristics of strain SL206T, cells were grown on RCM agar at 37 °C for 48 h.
Gram staining (Gram stain kit; Difco) and the KOH test were carried out as described previously (Chang et al., 2008). Cell morphology and flagellation were examined by phase-contrast microscopy (Nikon 80i) and transmission electron microscopy (H-7600, Hitachi). Catalase activity was detected by placing drops of 3 % (v/v) H2O2 onto plate-grown cultures and observing the production of oxygen bubbles. The presence of oxidase activity was determined by using an Oxy-swab (bioMérieux). Various biochemical properties were determined by using the API 20A, 32A and ZYM strips (bioMérieux) according to the manufacturer’s instructions. The pH range for growth was determined by growing cells in 500 ml bottles (Corning) containing 250 ml RCM broth at pH 4–9 in 0.5-unit increments. Broths were buffered with Na2HPO4/NaH2PO4, succinic acid/NaOH or 2-amino-2-methyl-1,3-propanediol/HCl. The pH and NaCl (0–5 %) ranges for growth were determined after 5 days of incubation at 32 °C in RCM. The temperature range for growth (5–50 °C) was also determined. Growth was checked by monitoring optical density at 595 nm (Bio-Rad). Motility was observed in a Hungate tube containing 0.4 % semi-solid agar. The cell-wall sugar and diaminopimelic acid in cell-wall hydrolysates were analysed by using solvent systems as described by Komagata & Suzuki (1987).

Colonies of strain SL206T cultivated on RCM agar were circular (approx. 1.0–1.5 mm in diameter), entire, convex and white or ivory. Cells were Gram-positive, strictly anaerobic and straight or slightly curved rods (1.0–1.3 × 3.5–4.5 μm). Cells were motile with flagella that were primarily peritrichately arranged. Oval endospores were observed in a terminal position (Fig. 1a). Electron micrographs of thin sections of cells showed that the cytoplasmic membrane of the strain was surrounded by a peptidoglycan layer and an outer layer (Fig. 1b). Cells grew at 20–40 °C and at pH 5.5–9.0. Optimal growth was observed at 37 °C and pH 7.0. No growth occurred with greater than 3.0 % (w/v) NaCl.

The cell wall of strain SL206T contained meso-diaminopimelic acid; wall sugars were glucose, rhamnose and mannose. In contrast, C. pasteurianum KCTC 1674T contained glucose, rhamnose, mannose and galactose, and C. acidisoli KCTC 5384T contained glucose, rhamnose and galactose. Cell wall sugars of strain SL206T were significantly different from those of closely related species. Strain SL206T was similar to the most closely related species with respect to fatty acid profile, utilization of glucose, sucrose, maltose and mannose (Heyndrickx et al., 1991; Holdeman et al., 1977), and nitrogen-fixing activity in nitrogen-free media (Kasap & Chen, 2005; Kuhner et al., 2000). However, it differed from the related species in certain phenotypic characteristics such as enzyme reaction and cell wall sugar composition. Detailed phenotypic characteristics of strain SL206T are reported in Table 1 and the species description.

The nearly complete 16S rRNA gene sequence (1347 bp) for strain SL206T was determined according to Chang et al. (2002). The resulting sequence was aligned manually against sequences obtained from the GenBank database. Phylogenetic trees were inferred by using the neighbour-joining (Saitou & Nei, 1987), Fitch–Margoliash (Fitch & Margoliash, 1967) and maximum-parsimony (Fitch, 1971) methods. Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes & Cantor (1969). The PHYLIP package (Felsenstein, 1993) was used for all analyses. The tree topology was evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining methods based on 1000 resamplings.

A preliminary comparison of the 16S rRNA gene sequence for strain SL206T was performed by an initial BLAST search.

**Fig. 1.** Transmission electron micrographs of thin sections of strain SL206T cells showing (a) cell division (bar, 1 μm) and (b) Gram-positive-type cell wall (bar, 200 nm). CM, cell membrane; P, peptidoglycan layer; S, septum; SP, spore; W, outer layer.
Table 1. Differential physiological characteristics of strain SL206\textsuperscript{T} and closely related species of the genus Clostridium

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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</thead>
<tbody>
<tr>
<td>Growth temperature</td>
<td>20–40</td>
<td>25–45</td>
<td>5–37</td>
</tr>
<tr>
<td>range (optimum) °C</td>
<td>(37)</td>
<td>(32)</td>
<td>(25–30)</td>
</tr>
<tr>
<td>Growth pH range (optimum)</td>
<td>5.5–9.0</td>
<td>4.7–7.5</td>
<td>4.0–7.0</td>
</tr>
<tr>
<td>Growth in 3 % (w/v) NaCl</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Cell wall sugar*</td>
<td>R, M, Gc</td>
<td>R, M, Gc, Gl</td>
<td>R, Gc, Gl</td>
</tr>
<tr>
<td>Nitrogen-fixation</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>31.1</td>
<td>26–28</td>
<td>30.7</td>
</tr>
</tbody>
</table>

**API 20A**
- D-Mannitol: – + +
- Lactose: – + +
- Salicin: – + +
- D-Xylose: – + +
- L-Arabinose: – + +
- Aesculin: – + +
- Glycerol: – + +
- Cellobiose: – + +
- Melezitose: – + +
- D-Sorbitol: – + +
- D-Rhamnose: – + +
- Trehalose: – + –

**API 32A or ZYM**
- α-Galactosidase: + – –
- β-Galactosidase: – + +
- β-Glucosidase: – + +
- Glutamic acid decarboxylase: + – –
- Acid phosphatase: – – +
- Alkaline phosphatase: – – w

* R, Rhamnose; M, mannose; Gc, glucose; Gl, galactose.

DNA–DNA hybridization was performed, as described previously (Chang et al., 2008), with photobiotin-labelled probes at 45 °C using a Fluoroskan Ascent Fluorescent plate reader (Thermo Life Science). Three replicates of each sample were tested. DNA–DNA hybridization values between strain SL206\textsuperscript{T} and strains *C. pasteurianum* KCTC 1674\textsuperscript{T} and *C. acidisoli* KCTC 5384\textsuperscript{T} were 59.5 and 53.2 %, respectively. These values were moderate but below the suggested threshold value for species delineation (Wayne et al., 1987). These values indicate that strain SL206\textsuperscript{T} represents a novel species that is distinct from *C. pasteurianum* and *C. acidisoli*.

To determine the DNA G+C (mol%) content, DNA was prepared according to Chang et al. (2002) and analysed by reversed-phase HPLC as described by Tamaoka & Komagata (1984). The relative values were calculated by using *Escherichia coli* KCTC 2441\textsuperscript{T} DNA as the standard. The DNA G+C content of strain SL206\textsuperscript{T} was 31.1 mol%. Although this value was a little higher than those reported for *C. pasteurianum* (26–28 mol%; Holdeman et al., 1977) and *C. acidisoli* (30.7 mol%; Kuhner et al., 2000), its taxonomic position suggests that strain SL206\textsuperscript{T} represents a member of cluster I and the low-G+C Gram-positive bacteria of the order *Clostridiales* (Wieg et al., 2006).

Cellular fatty acid patterns of cells grown on PYG medium (DSMZ medium 104) at 37 °C were determined after 24 h by extracting and analysing the fatty acid methyl esters (FAMEs) according to a standard protocol (Sherlock Microbial Identification System; MIDI). The FAMEs were separated with an automated GC system (model 6890N and 7683 autosampler; Agilent) and identified by using the Microbial Identification software package (Moore library).

Cellular fatty acid patterns for the novel strain were examined and compared with the patterns of *C. pasteurianum* KCTC 1674\textsuperscript{T} and *C. acidisoli* KCTC 5384\textsuperscript{T} (Table 2). In all three strains, C\textsubscript{16:0}, C\textsubscript{18:1}ω9c and summed feature 10 (containing C\textsubscript{18:1}ω11t/9t), C\textsubscript{18:2}ω6t/ω9c and C\textsubscript{18:1}ω9c were the most common fatty acids, comprising approximately 75–86 % of total extracted lipids. The major fatty acids for strain SL206\textsuperscript{T} (C\textsubscript{16:0} 12-OH and the absence of C\textsubscript{16:0} DMA (dimethyl acetate), C\textsubscript{18:1}ω9c DMA and C\textsubscript{19:0} cyclo 9,10 DMA.

For detection of end-products from glucose metabolism, strain SL206\textsuperscript{T} was cultivated in PY basal medium (DSMZ medium 104 without glucose and Tween 80, with only 1 g yeast extract l\textsuperscript{-1}) at 37 °C for 24 h. Prior to cultivation, pre-sterilized glucose solution was added to the medium to a final concentration of 20 mM. All cultivations were carried out in 50 ml serum vials (working volume of 20 ml) under an atmosphere of N\textsubscript{2}/CO\textsubscript{2} (85 : 15, v/v). Fermentation end-products were analysed by GC (Varian 3400 GC; Varian Associates) equipped with a flame-ionization detector and CP wax 52 CB column, according to methods described by Holdeman et al. (1977) and Steer et al. (2001).
Nitrogen-fixing cultures were grown on a nitrogen-free medium, as described by Kasap & Chen (2005), consisting of: sucrose, 30 g; K2HPO4, 0.69 g; Na2SO4.10H2O, 0.19 g; mineral solution, 1 ml (KCTC medium no. 650); CaCO3, 5 g and resazurin 1 mg (per litre of distilled water). Cultures were grown at 32 °C under N2 in rubber-stoppered 160 ml serum bottles containing 50 ml of medium. Acetylene-reduction assay was used to measure nitrogenase activity, as described by Lee et al. (2002), by gas chromatography (Shimadzu 17A) with a flame-ionization detector (FID). The head space of cultures was replaced by argon gas and 10% acetylene gas was added. Tubes were incubated at 32 °C for 1 h with shaking, and then 300 μl sample was used for GC analysis.

Analysis of fermentation end-products from glucose metabolism showed that acetate, butyrate, acids, abundant CO2 and H2 were formed by strain SL206T, whereas lactate was not detected. Ethanol was produced. Pyruvate was converted principally to acetate, butyrate, CO2 and H2; lactate was not utilized (Wiegel et al., 2006).

Acetylene-reduction assay was used to measure whole-cell nitrogenase activity for strain SL206T. When grown on medium in the presence of molybdenum, the strain reduced acetylene to ethylene (approx. 0.51 mg l−1). C. pasteurianum KCTC 1674T also showed nitrogenase activity (approx. 0.64 mg ethylene l−1), but C. acidisoli KCTC 5384T did not. No nitrogenase activity was evident in the absence of Mo. Molybdenum was essential for the biosynthesis and activity of the nitrogenase involved (Wiegel et al., 2006).

On the basis of its cell wall sugar composition, nitrogenase activity, fatty acid pattern, DNA G+C content (31.1 mol%), DNA–DNA hybridization values (<59.5%), 16S rRNA gene sequence analyses (<98.6% similarity), growth temperature and pH range, strain SL206T can be differentiated from members of the genus Clostridium as a novel species, for which the name Clostridium arbusti sp. nov. is proposed.

**Description of Clostridium arbusti** sp. nov.

Clostridium arbusti (ar.‘bu.sti. L. gen. n. arbusti of an orchard, isolated from pear orchard soil).

Cells in RCM agar culture are Gram-staining-positive, straight to slightly curved rods, 1.0–1.3 × 3.5–4.5 μm, occurring singly or in pairs. Motile cells are peritrichous. Spores are oval, terminal and do not swell the cell. Cell walls contain meso-diaminopimelic acid; wall sugars are glucose, rhamnose and mannose. Surface colonies on RCM agar plates are 1–1.5 mm in diameter, circular to irregular, low convex, translucent to semioopaque, white or ivory and shiny with a smooth or erose margin and an entire internal structure. Cultures in PYG broth are turbid with a smooth surface. Colony morphology of a 30°C inoculated RCM agar plate was shiny with a smooth or erose margin and an entire internal structure. Cultures in PYG broth are turbid with a smooth surface. Colony morphology of a 30°C inoculated RCM agar plate was shiny with a smooth or erose margin and an entire internal structure.

**Table 2. Cellular fatty acid compositions of strain SL206T and closely related species of the genus Clostridium**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Strain</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
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<tbody>
<tr>
<td>C14:0</td>
<td></td>
<td>2.6</td>
<td>2.5</td>
<td>2.8</td>
</tr>
<tr>
<td>C16:1ω9c</td>
<td></td>
<td>4.6</td>
<td>5.7</td>
<td>5.5</td>
</tr>
<tr>
<td>C16:0</td>
<td></td>
<td>31.8</td>
<td>29.7</td>
<td>31.6</td>
</tr>
<tr>
<td>C16:1ω9cDMA</td>
<td></td>
<td>2.3</td>
<td>–</td>
<td>1.7</td>
</tr>
<tr>
<td>C16:0DMA</td>
<td></td>
<td>–</td>
<td>–</td>
<td>1.9</td>
</tr>
<tr>
<td>C17:0CycDMA</td>
<td></td>
<td>3.0</td>
<td>–</td>
<td>5.7</td>
</tr>
<tr>
<td>C18:1ω9c</td>
<td></td>
<td>38.0</td>
<td>44.9</td>
<td>34.2</td>
</tr>
<tr>
<td>C18:0</td>
<td></td>
<td>5.8</td>
<td>6.3</td>
<td>3.4</td>
</tr>
<tr>
<td>C18:1ω9cDMA</td>
<td></td>
<td>–</td>
<td>–</td>
<td>3.0</td>
</tr>
<tr>
<td>C18:1ω2OH</td>
<td></td>
<td>2.9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C19:0Cyc9,10DMA</td>
<td></td>
<td>–</td>
<td>–</td>
<td>1.2</td>
</tr>
<tr>
<td>Summed feature 10*</td>
<td></td>
<td>9.0</td>
<td>11.0</td>
<td>8.9</td>
</tr>
</tbody>
</table>

*Fatty acids that could not be separated by GC using the Microbial Identification System (Microbial ID) software were considered summed features. Summed feature 10 contains C18:1ω11c/9t/6t.
pH of 9.0. Growth occurs in synthetic medium. Abundant gas is detected in RCM and PYG deep agar cultures. Negative API 20A reactions for oxidase, catalase and acid production from L-rhamnose, sucrose, trehalose, D-xylose, melezitose, salicin and D-sorbitol; positive reactions for acid production from D-glucose, sucrose, maltose, D-mannose and raffinose. Positive Rapid ID 32A reactions for x-galactosidase, x-glucosidase and glutamic acid decarboxylase. Positive API 2YM reactions for naphthol-AS-BI-phosphohydrolase, x-galactosidase, x-glucosidase; negative reactions for acid phosphatase, alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, x-chymotrypsin, β-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, N-acetylmannosidase and β-fucosidase. Atmospheric N2 is fixed; molybdenum is essential for nitrogenase biosynthesis and activity of the nitrogenase involved. Cell fatty acids include mainly the straight chain C16:0, monounsaturated C18:1ω9c and summed feature 10 (containing C18:1ω11c/9t/6t). The DNA G+C content of the type strain is 31.1 mol%. Products in PYG broth cultures include butyric and acetic acids; abundant CO2 and H2 are detected. Ethanol is produced. Pyruvate is converted principally to acetate, butyrate, CO2 and H2; lactate is not utilized.

The type strain is SL206T (=KCTC 5449T =JCM 14858T), isolated from soil samples from a pear orchard in Daejeon, Republic of Korea.

Acknowledgements

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References