Cellulosilyticum ruminicola gen. nov., sp. nov., isolated from the rumen of yak, and reclassification of Clostridium lentocellum as Cellulosilyticum lentocellum comb. nov.

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Lignocellulose is the main component of biomass generated by photosynthesis (Pérez et al., 2002) and it is degraded and utilized by various micro-organisms in nature (Beguin & Aubert, 1994). It is well known that ruminant rumens harbour abundant fibrolytic anaerobes that enable them to live on lignocellulose-based plant straws. Therefore, rumen microbiology has been studied for the primary fibrolytic potential. The yak (Bos grunniens) is a ruminant living mainly on Qinghai–Tibetan Plateau, China, more than 3000 m above sea level, and grazes exclusively on grasses and straws. Hence, it is reasonable to speculate that fibrolytic anaerobes could be active in yak rumen. In this study, we describe the isolation and characterization of a fibrolytic strain isolated from the rumen of a domesticated yak.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain H1T is EF382648.

A supplementary table showing the cellular fatty acid composition of strain H1T is available with the online version of this paper.

An obligate anaerobic, Gram-staining-negative, mesophilic, cellulolytic bacterium, strain H1T, was isolated from the rumen content of yak. Cells were straight to slightly curved rods, 0.8–1.0×3.0–4.0 μm in size, non-motile and encapsulated with mucous materials. Elliptical and terminal spores that swelled the cells were produced occasionally. The strain grew at 25–45 °C (optimum, 38 °C) and pH 6.0–7.8 (optimum, pH 6.7). Cellulose, cellobiose, xylan, xylose and maltose were used as carbon and energy sources, but not glucose. Products from cellulose and cellobiose fermentation were formic acid, acetic acid, carbon dioxide and trace amounts of ethanol, lactic acid and succinic acid. The genomic DNA G+C content was 33.7 ± 1.2 mol%. The predominant fatty acids were C16:0 (27.1%), C14:0 (9.2%) and iso-C16:0 (6.4%). Based on the 16S rRNA gene sequence analysis, strain H1T was affiliated to the clostridial rRNA cluster XIVb and showed the highest 16S rRNA gene sequence similarity to Clostridium lentocellum DSM 5427T (96.0%). These two strains formed a distinct lineage of the family ‘Lachnospiraceae’. Based on data from this polyphasic taxonomic study, a new genus, Cellulosilyticum gen. nov., is proposed. Cellulosilyticum ruminicola sp. nov. is proposed for strain H1T. The type strain of Cellulosilyticum ruminicola sp. nov. is strain H1T (=CGMCC 1.5065T=JCM 14822T). Clostridium lentocellum was reclassified in the new genus as Cellulosilyticum lentocellum comb. nov. (type strain RHM5T=ATCC 49066T=DSM 5427T=NCIMB 11756T).

RC medium, a modified RGCA medium (Bryant & Burkey, 1953), containing (per 100 ml): 40 ml yak rumen fluid, 30 ml distilled water, 2.0 g Whatman No. 1 filter paper, 0.05 g cysteine hydrochloride, 20 μl 0.2% resazurin, 45 mg K2HPO4, 45 mg KH2PO4, 90 mg (NH4)2SO4, 90 mg NaCl, 9 mg MgSO4, 9 mg CaCl2 and 1% sodium bicarbonate (w/v), was prepared with 100% CO2 as the gas phase. After sterilization, the pH of the medium was pH 6.7. RC-CB medium was prepared by adding 0.2 g cellobiose per 100 ml instead of filter paper. These media were used for the isolation of strain H1T at 38 °C in anaerobic tubes sealed with butyl rubber stoppers. RC medium was inoculated with rumen content and, after subculturing the enrichment several times, was used to inoculate RC-CB medium. A pure culture of strain H1T was obtained by using serial dilution and the Hungate roll-tube technique (Hungate, 1969) and finally picking single colonies to inoculate RC-CB medium, which was incubated at 38 °C for 2 days. The purity of strain H1T was examined microscopically for cell morphology and colony homogeneity. Clostridium lentocellum DSM 5427T was obtained from the
DSMZ, Germany. Both strains were cultured routinely in RC-CB medium (pH 6.7) at 38 °C unless otherwise indicated.

Cell morphology was examined both under a light microscope (Olympus BH-2) and an electron microscope (Hitachi H-600A). For electron microscope examination of ultrathin cell-wall sections, cells were grown in RC-CB medium for 1 day and prepared by using the method described previously (Chen & Dong, 2005). Cells of strain H1T were Gram-negative-staining throughout the growth phase; however, a typical Gram-positive structure was revealed by electron microscopy (Fig. 1). Cells were straight to slightly curved rods, 0.8–1.0×3.0–4.0 μm in size and encapsulated by abundant slime. Flagella were never observed. Strain H1T grew exclusively in the pre-reduced media and growth was completely inhibited by air. Elliptical spores were occasionally formed, which swelled the cells at one terminal. Colonies on RC-CB agar medium were yellow-green, circular and slightly convex, 1.0–2.0 mm in diameter after 1 day of incubation.

The fermentation products of strain H1T and Clostridium lentocellum DSM 5427T were determined using GC (GC-14B; Shimadzu) and gaseous products were determined by GC-14C (Shimadzu), according to Chen & Dong (2004), after culture in RC medium for 7 days and in RC-CB medium for 3 days. The major fermentation products of strain H1T were formic acid, acetic acid and carbon dioxide. Trace amounts of ethanol, lactic acid and succinic acid were also produced. Clostridium lentocellum DSM 5427T produced ethanol, acetic acid, hydrogen, carbon dioxide and trace amounts of lactic, succinic and fumaric acids (Table 1).

The temperature range for growth of strain H1T was determined in RC-CB medium in a water bath at 22–50 °C with intervals of 1 °C. To determine the pH range for growth, 30 % CO2 and 70 % N2 was used as the gas phase for RC-CB medium (pH 7.0) and the medium was adjusted to pH 5.0–10.0 by using 1 M HCl or 1 M NaOH. The pH range for growth was determined at 38 °C. Growth was determined by measuring OD600 of cultures at 3 and 7 days in duplicate. Strain H1T grew at 25–45 °C, with optimum growth at 38 °C, and at pH 6.0–7.8, with optimum growth at pH 6.7. The generation time of strain H1T was determined as 2.85 h by monitoring OD600 in RC-CB medium at 0.5 h intervals for 24 h.

Biochemical tests were performed according to the procedures described by Holdeman et al. (1977). Strain H1T did not produce indole, catalase, gelatinase, urease or acetyl-methyl-carbinol, did not reduce sulfate or nitrate and did not hydrolyse aesculin.

Substrate utilization tests were performed according to Murray et al. (1986) in RC medium by addition of various tested substances instead of filter paper. Utilization was defined as the increase of OD600 by 0.1. In addition to cellulose, strain H1T used cellobiose (0.5 %), xylan (0.5 %), xyllose (0.5 %) and maltose (0.5 %) as sole carbon and energy sources. The substrate profile of Clostridium lentocellum DSM 5427T was tested in parallel. More details are given in Table 1 and the genus and species descriptions. Cellular fatty acids of strain H1T were extracted from exponential-phase cells from 1-day cultures in RC-CB medium. The extracts were methylated and analysed using the standard Microbial Identification system (Miller, 1982; Sasser, 1990). The major cellular fatty acids were C16:0 (27.1 %), C14:0 (9.2 %), iso-C16:0 (6.4 %), C16:1ω9c

### Table 1. Differential characteristics of *Cellulosilyticum rumininicola* sp. nov. H1T and *Cellulosilyticum lentocellum* DSM 5427T

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>C. rumininicola H1T</th>
<th>C. lentocellum DSM 5427T</th>
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</thead>
<tbody>
<tr>
<td><strong>Cell morphology</strong></td>
<td>Straight to slightly curved rods</td>
<td>Slightly curved rods</td>
</tr>
<tr>
<td><strong>Motility</strong></td>
<td>–</td>
<td>+</td>
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<tr>
<td><strong>Utilization of:</strong></td>
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<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Trehalose</td>
<td>–</td>
<td>w</td>
</tr>
<tr>
<td>Xylan</td>
<td>+</td>
<td>w</td>
</tr>
<tr>
<td><strong>Fermentation products</strong></td>
<td>A, F, e, l, s</td>
<td>A, E, l, fu, s</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>33.7</td>
<td>36</td>
</tr>
</tbody>
</table>

*Upper-case letters indicate major end products and lower-case letters indicate minor end products. a, acetate; e, ethanol; f, formate; fu, fumaric acid; l, lactic acid; s, succinic acid.*

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**Fig. 1.** Electron micrograph of an ultrathin section of a cell of strain H1T. CM, cytoplasmic membrane; P, peptidoglycan. Bar, 0.1 μm.
The genomic DNA was extracted and purified using the method of Jaufeerally-Fakim & Dookun (2000). The G+C content of the genomic DNA was determined to be 33.7 ± 1.2 mol% by using the thermal denaturation method (Marmur & Doty, 1962) with a DU800 spectrophotometer (Beckman) and *Escherichia coli* K-12 as the reference strain. To determine the phylogenetic position of strain H1T, the 16S rRNA gene sequence was amplified by PCR and sequenced as described previously (Chen & Dong, 2004). The 16S rRNA gene sequence (1547 bp) was submitted to GenBank to search for similar sequences using the BLAST algorithm. The best-matching sequences were retrieved from the database and aligned, and a similarity analysis was performed using CLUSTAL_X (Thompson et al., 1997). A phylogenetic tree (Fig. 2) based on 1268 bp 16S rRNA gene sequence was constructed using neighbour-joining methods implemented with MEGA2 software (Kumar et al., 2001). The robustness of the tree was evaluated by bootstrap analysis (Felsenstein, 1985) with 1000 resamplings.

The phylogenetic analysis showed that strain H1T had the highest 16S rRNA gene sequence similarity (96.0 %) with *Clostridium lentocellum* DSM 5427T. These two strains formed a distinct lineage within the clostridial rRNA cluster XIVb with the unculturable polysporogenic strain ‘*Metabacterium polyspora*’ clone M 1.6 and ‘*Epulopiscium*’ sp. morphotype B. The lineage was supported by a bootstrap value of 99 % and exhibited 16S rRNA gene sequence similarities of less than 87.7 % with other species in cluster XIVb. Collins et al. (1994) proposed 19 rRNA clusters for *Clostridium* members because this genus is extremely heterogeneous and species in clusters II–XIX all fell outside the genus *Clostridium sensu stricto*. Recently, species in cluster XIVb were reassigned to the family ‘*Lachnospiraceae*’ (Wiegel et al., 2006). On the basis of 16S rRNA gene sequence divergence, this study concluded that strain H1T and *Clostridium lentocellum* DSM 5427T represent a new genus of the family ‘*Lachnospiraceae*’.

![Fig. 2. Phylogenetic dendrogram of strain H1T and related strains based on 16S rRNA gene sequences, constructed using the neighbour-joining method. Bootstrap values (>50 %) based on 1000 resamplings are shown at branch nodes. *Propionibacterium acnes* DSM 1897T was used as an outgroup. Bar, 2 % sequence divergence.](http://ijs.sgmjournals.org)
The cellulytic property is found in many phylogenetically diverse clostridial species (Rainey & Stackebrandt, 1993) and many of them fall into the rRNA clusters III and XIVa. Phenotypically, the strain H1T and Clostridium lentocellum DSM 5427T differed from the phylogenetically related species Clostridium lactisfermentans, Clostridium propionicum and Clostridium neopropionicum in their capabilities to hydrolyse cellulose and xylan and inabilities to ferment lactate and pyruvate (van der Wielen et al., 2002; Cardon & Barker, 1946; Tholozan et al., 1992). Furthermore, all of these three non-fibrolytic species produce propionate as the major fermentation product whereas strain H1T and Clostridium lentocellum DSM 5427T did not. Strain H1T and Clostridium lentocellum DSM 5427T could also be distinguished from other phylogenetically related genera in the family ‘Lachnospiraceae’ as follows: from the genera Roseburia, Hespellia and Anaerostipes by the ability to sporulate and inability to produce butyric or propionic acids (Stanton & Savage, 1983; Whitehead et al., 2004; Schwiertz et al., 2002); from the genus Robisoniella by the absence of succinate as a major end product (Cotta et al., 2009); from Lachnospira by cell and colony morphology (filamentous and woolly, respectively, for Lachnospira; Cornick et al., 1994); and from the genus Parasporobacterium by the ability to grow without sulfide (Lomans et al., 2001).

On the basis of the collective data presented above, a novel genus Cellulosilyticum gen. nov. is proposed, consisting of the newly isolated Cellulosilyticum ruminicola sp. nov. and Cellulosilyticum lentocellum comb. nov.

Description of Cellulosilyticum gen. nov.

Cellulosilyticum [Cel.lu.lo.si.li’ty.i.cum. N.L. n. celluloseum cellulose; N.L. neut. adj. lyticum (from Gr. neut. adj. lutikon) able to loosen, able to dissolve; N.L. neut. n. Cellulosilyticum a bacterium able to dissolve cellulose].

Straight to slightly curved rod-shaped cells (0.3–1.0 × 2.5–4.0 µm). Cells form terminal spores. Anaerobic. Mesophilic, optimum growth temperature is approximately 40 °C. Produce acetate as one of the major end products. Butyric acid is not formed. Hydrolyse cellulose and xylan and ferment cellobiose. The DNA G + C content is 32.9–36 mol%. The type species is Cellulosilyticum ruminicola.

Description of Cellulosilyticum ruminicola sp. nov.

Cellulosilyticum ruminicola [ru.mi.ni’co.la. L. n. rumen -inis the rumen; L. suff. -cola (from L. n. incola) inhabitant, dweller; N.L. n. ruminicola rumen dweller].

Exhibits the following properties in addition to those given in the genus description. Cells are Gram-negative-staining but have a Gram-positive cell-wall structure and are 0.8–1.0 × 3.0–4.0 µm in size, occurring singly. Cells are motile and encapsulated with slime. Elliptical and terminal spores are produced occasionally. Colonies are yellow-green, circular and slightly convex, 1.0–2.0 mm in diameter. Obligate anaerobic growth. Growth occurs at 25–45 °C (optimum, 38 °C) and at pH 6.0–7.8 (optimum, pH 6.7). Indole, catalase, gelatinase, urease and acetyl-methyl-carbinol are not produced. Sulfate and nitrate are not reduced. Does not hydrolyse esculin. Ferments xylose and maltose and weakly ferments lactose, pectin and sorbitol. Does not utilize arabinose, fructose, galactose, glucose, glycogen, pyruvate, sucrose, trehalose, starch, amygdalin, erythritol, glycerol, inositol, lactate, mannitol, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, hippurrate or mannose. Fermentation products from cellulose and cellobiose are formic acid, acetic acid, carbon dioxide and trace amounts of ethanol, succinic acid and lactic acid. Major cellular fatty acids are C16:0, C14:0, iso-C16:0, C16:1ω9c and C18:1ω7c. The DNA G + C content of the type strain is 33.7 ± 1.2 mol%.

The type strain, H1T (=CGMCC 1.5065T = JCM 14822T), was isolated from the rumen of yak.

Description of Cellulosilyticum lentocellum comb. nov.

Cellulosilyticum lentocellum (len.to.cell’um. L. adj. lentus slow; N.L. n. celluloseum cellulose; N.L. neut. adj. lentocellum intended to mean slowly fermenting cellulose).


Characteristics are those as given by Murray et al. (1986) and from this study. Cells are Gram-negative-staining, motile, slightly curved rods, 0.3–0.5 × 2.5–4.0 µm in size, occurring singly. Cells form round terminal spores. Colonies are flat, colourless and transparent with undulate margins, 7–10 mm in diameter. Obligate anaerobic growth. Growth occurs at 15–46.5 °C (optimum, 40 °C) and pH 5.7–9.13 (optimum, pH 7.5–7.7). Indole, catalase, gelatinase are not produced. Sulfate and nitrate are not reduced. Acetyl-methyl-carbinol is produced and urease is weakly produced. Utilizes arabinose, cellobiose, cellulose, aesculin, fructose, galactose, glucose, glycogen, lactose, maltose, pyruvate, salicin, sucrose, trehalose, xylene, xylan, milk, starch, bile and chopped meat. Does not utilize adonitol, amygdalin, arginine, dulcitol, erythritol, glycerol, hippurate, inositol, inulin, lactate, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, sorbitol, sorbose or threonine. Major fermentation products from cellulose are ethanol, acetic acid, hydrogen and carbon dioxide. The DNA G + C content of the type strain is 36 mol%.

The type strain, RHM5T (=ATCC 49066T = DSM 5427T = NCIMB 11756T), was isolated from an estuarine mud bank of a river receiving paper-mill and domestic effluent.

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References


