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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An obligate anaerobic, Gram-staining-negative, mesophilic, cellulolytic bacterium, strain H1^T, 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 C_{16:0} (27.1 %), C_{14:0} (9.2 %) and iso-C_{16:0} (6.4%). Based on the 16S rRNA gene sequence analysis, strain H1^T was affiliated to the clostridial rRNA cluster XIVb and showed the highest 16S rRNA gene sequence similarity to *Clostridium lentocellum* DSM 5427^T (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 H1^T. The type strain of *Cellulosilyticum ruminicola* sp. nov. is strain H1^T (=CGMCC 1.5065^T=JCM 14822^T). *Clostridium lentocellum* was reclassified in the new genus as *Cellulosilyticum lentocellum* comb. nov. (type strain RHM5^T=ATCC 49066^T=DSM 5427^T=NCIMB 11756^T).

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 (Beguín & 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.

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 H1^T 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 H1^T 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 H1^T was examined microscopically for cell morphology and colony homogeneity. *Clostridium lentocellum* DSM 5427^T was obtained from the

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain H1^T is EF382648.

A supplementary table showing the cellular fatty acid composition of strain H1^T is available with the online version of this paper.

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 H1^T 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 H1^T 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 H1^T and *Clostridium lentocellum* DSM 5427^T 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 H1^T were formic acid, acetic acid and carbon dioxide. Trace amounts of ethanol, lactic acid and succinic acid were also produced. *Clostridium lentocellum* DSM 5427^T 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 H1^T 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

Fig. 1. Electron micrograph of an ultrathin section of a cell of strain H1^T. CM, cytoplasmic membrane; P, peptidoglycan. Bar, 0.1 µm.

Table 1. Differential characteristics of *Cellulosilyticum ruminicola* sp. nov. H1^T and *Cellulosilyticum lentocellum* DSM 5427^T

All data were from this study. Both strains were positive for utilization of cellobiose, cellulose and xylose. +, Positive; w, weak; –, negative.

Characteristic	<i>C. ruminicola</i> H1 ^T	<i>C. lentocellum</i> DSM 5427 ^T
Cell morphology	Straight to slightly curved rods	Slightly curved rods
Motility	–	+
Utilization of:		
Fructose	–	+
Galactose	–	w
Starch	–	+
Sucrose	–	+
Trehalose	–	w
Xylan	+	w
Fermentation products*	A, F, e, l, s	A, E, l, fu, s
DNA G + C content (mol%)	33.7	36

*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.

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 H1^T 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 H1^T 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 H1^T 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 H1^T used cellobiose (0.5 %), xylan (0.5 %), xylose (0.5 %) and maltose (0.5 %) as sole carbon and energy sources. The substrate profile of *Clostridium lentocellum* DSM 5427^T was tested in parallel. More details are given in Table 1 and the genus and species descriptions.

Cellular fatty acids of strain H1^T 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 C_{16:0} (27.1 %), C_{14:0} (9.2 %), iso-C_{16:0} (6.4 %), C_{16:1ω9c}

Fig. 2. Phylogenetic dendrogram of strain H1^T 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 1897^T was used as an outgroup. Bar, 2% sequence divergence.

(4.7%) and *C_{18:1ω7c}* (4.7%) (Supplementary Table S1, available in IJSEM Online).

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 H1^T, 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 H1^T had the highest 16S rRNA gene sequence similarity (96.0%) with *Clostridium lentocellum* DSM 5427^T. 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 H1^T and *Clostridium lentocellum* DSM 5427^T represent a new genus of the family '*Lachnospiraceae*'.

The cellulolytic 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 H1^T and *Clostridium lentocellum* DSM 5427^T differed from the phylogenetically related species *Clostridium lactatifermentans*, *Clostridium propionicum* and *Clostridium neopropionicum* in their capabilities to hydrolyse cellulose and xylan and inability 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 H1^T and *Clostridium lentocellum* DSM 5427^T did not. Strain H1^T and *Clostridium lentocellum* DSM 5427^T could also be distinguished from other phylogenetically related genera in the family 'Lachnospiraceae' as follows: from the genera of *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 *Robinsoniella* 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.ly'ti.cum. N.L. n. *cellulosum* 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 non-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 aesculin. 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, hippurate 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 C_{16:0}, C_{14:0}, iso-C_{16:0}, C_{16:1ω9c} and C_{18:1ω7c}. The DNA G + C content of the type strain is 33.7 ± 1.2 mol%.

The type strain, H1^T (=CGMCC 1.5065^T=JCM 14822^T), was isolated from the rumen of yak.

Description of *Cellulosilyticum lentocellum* comb. nov.

Cellulosilyticum lentocellum (len.to.cel'lum. L. adj. *lentus* slow; N.L. n. *cellulosum* cellulose; N.L. neut. adj. *lentocellum* intended to mean slowly fermenting cellulose).

Basonym: *Clostridium lentocellum* Murray *et al.* 1987.

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, xylose, 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, RHM5^T (=ATCC 49066^T=DSM 5427^T=NCIMB 11756^T), was isolated from an estuarine mud bank of a river receiving paper-mill and domestic effluent.

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