Proteiniclasticum ruminis gen. nov., sp. nov., a strictly anaerobic proteolytic bacterium isolated from yak rumen

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Two strictly anaerobic, proteolytic bacterial strains, designated strain D3RC-2^T and D3RC-3r, were isolated from a cellulose-degrading mixed culture enriched from yak rumen content. The strains were Gram-stain negative and non-spore-forming with cell sizes of 0.5–0.8×0.6–2.0 µm. The temperature range for growth was 24–46 °C (optimum 38–39 °C) and the pH range was between 5.6 and 8.7 (optimum 7.0–7.3). Both strains used soya peptone, tryptone, L-phenylalanine, L-leucine, L-methionine, L-serine, L-valine, L-threonine and L-histidine as carbon and nitrogen sources, but did not use any of the saccharides tested. The major fermentation products from PY medium were acetate, propionate and iso-butyrate. The DNA G + C contents of strains D3RC-2^T and D3RC-3r were 41.0 ± 0.1 mol% and 41.3 ± 0.1 mol% (HPLC), respectively. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the two strains represented a new phyletic sublineage within the family *Clostridiaceae*, with <93.8 % 16S rRNA gene sequence similarity to recognized species. On the basis of the phenotypic, genotypic and physiological evidence, strains D3RC-2^T and D3RC-3r are proposed as representing a novel species of a new genus, for which the name *Proteiniclasticum ruminis* gen. nov., sp. nov. is proposed. The type strain of the type species is D3RC-2^T (=AS 1.5057^T=JCM 14817^T).

Yak (*Bos grunniens*) are ruminants that feed exclusively on grasses and live mainly on the Qinghai–Tibetan Plateau, China, at a height of 3000 m above sea level. Previous studies based on an uncultured approach (An *et al.*, 2005) in our laboratory have indicated that more than half of the microbes in yak rumen belong to as yet uncultured groups. The strains all showed <90 % 16S rRNA gene sequence similarity levels with recognized species and 10.8 % of the sequences retrieved were related to known rumen fibrolytic bacteria. By using filter paper as the sole carbon source, a cellulose-degrading mixed culture enriched from yak rumen content was obtained. A few fibrolytic and non-fibrolytic bacterial strains were isolated from the mixed culture. In this paper, two proteolytic bacterial strains isolated from yak rumen are described.

Strains D3RC-2^T and D3RC-3r were isolated from a fibrolytic mixed culture enriched from yak rumen. The yak rumen content was inoculated into a modified basal

The GenBank accession numbers for the 16S rRNA sequences of *Proteiniclasticum ruminis* strains D3RC-2^T and D3RC-3r are DQ852338 and FJ527031, respectively.

An extended phylogenetic tree and a table detailing the cellular fatty acid content of strain D3RC-2^T are available with the online version of this paper.

medium (Bryant & Burkey, 1953; Hungate, 1966) with filter paper as the sole carbon source and grown under a gas phase of N₂/CO₂ (80:20). A fibrolytic mixed culture was obtained by subculturing the enrichment in the same medium. By means of serial dilution in peptone-yeast extract-glucose medium (PYG, Holdeman *et al.*, 1977) and the Hungate roll-tube technique (Hungate, 1969), single colonies were picked and transferred to the same medium. The roll-tube procedure was repeated several times before strains D3RC-2^T and D3RC-3r were obtained. The purity of the isolates was examined by light microscopy. All inoculations and transfers were performed with syringes and needles and the cultures were incubated at 39 °C unless otherwise indicated.

Cell morphology was examined under a light microscope (BH-2; Olympus) and an electron microscope (H-600A; Hitachi). Gram staining was carried out according to Johnson *et al.* (1995). For electron microscopy studies, bacterial cells were negatively stained with uranyl acetate. Spore formation was examined by phase-contrast microscopy under different growth conditions and at different growth phases as well as by heat treatment in a water bath at 85 °C for 10 min in growth medium.

Cells were Gram-stain negative, rod-shaped (with sizes of 0.5–0.8 × 0.6–2.0 µm), occurred singly or in pairs and were

non-motile (Fig. 1a). The Gram-negative cell-wall structure was also confirmed by the KOH-lysis test (Smibert & Krieg, 1994) and by means of an electron micrograph of an ultrathin section (Fig. 1b). Spores were never observed under various growth conditions or at different growth phases. The novel strains were unable to survive after incubation at 85 °C for 10 min.

Growth was determined by monitoring OD₆₀₀ of the cultures in PY medium at 39 °C. The strains were strictly anaerobic, no growth occurred when the cultures were exposed to air. A generation time of 1.8 h was determined for strain D3RC-2^T by monitoring the culture OD₆₀₀ at 30 min intervals up to 24 h. The temperature profile of strain D3RC-2^T was determined in a water bath from 20 to 50 °C at 1 °C intervals. Growth was observed between 24 °C and 46 °C, with optimal growth at 38–39 °C (pH 7.0). The pH range for growth was pH 5.6–8.7 with optimal growth around pH 7.0–7.3 as determined in PY

broth at various pH values adjusted by the addition of Na₂HPO₄ and NaH₂PO₄.

Substrate utilization was tested by growing the two novel strains in basal medium containing each of the substrates as follows: 1 % (w/v) cellobiose, glucose, xylose, arabinose, maltose, mannose, trehalose, salicin, lactose, melezitose, raffinose, rhamnose, mannitol and glycerol, 0.5 % (w/v) soya peptone and tryptone, and 0.2 % (w/v) L-alanine, L-cysteine, L-aspartic acid, L-glutamic acid, L-phenylalanine, L-glycine, L-histidine, L-isoleucine, L-lysine, L-leucine, L-methionine, L-asparagine, L-proline, L-arginine, serine, L-threonine, valine and tryptophan. Substrate utilization was also detected using the API 50CH system (bioMérieux) according to the manufacturer's instructions. Other physiological characteristics were determined according to Holdeman *et al.* (1977). The short-chain fatty acids were analysed by GC (GC-14B, Shimadzu) according to Chen & Dong (2004).

The two novel strains exhibited almost identical physiological and biochemical profiles using both conventional methods as well as the API 50CH system. The results indicated that the two isolates represented a single species. Strain D3RC-2^T utilized the following compounds: soya peptone, tryptone, L-phenylalanine, L-leucine, L-methionine, L-serine, L-valine, L-threonine and L-histidine. None of the carbohydrates or alcohols tested were used. The major fermentation products from PY medium were acetate, propionate and iso-butyrate. Propionate, iso-butyrate and acetate were produced from L-methionine and L-leucine, L-threonine and L-serine, and L-histidine, respectively. Other physiological characteristics are listed in the species description below.

Cellular fatty acids were extracted, methylated and analysed using the standard MIDI (Microbial Identification) system (Miller, 1982; Sasser, 1990). The cellular fatty acids of strain D3RC-2^T comprised mainly iso-branched fatty acids, predominantly iso-C_{14:0} (19.4 %), iso-C_{13:0} (19.2 %), iso-C_{15:0} (15.2 %), C_{14:0} (15.1 %), anteiso-C_{13:0} (10.0 %) and anteiso-C_{15:0} (9.2 %) (see Supplementary Table S1 in IJSEM Online).

The genomic DNA was extracted and purified according to Marmur (1961). The G+C contents of the genomic DNA of strains D3RC-2^T and D3RC-3r were determined to be 41.0±0.1 mol% and 41.3±0.1 mol%, respectively, by using HPLC (Mesbah *et al.*, 1989) with the modification of Lee *et al.* (2005). Both strains were subjected to DNA–DNA hybridization analysis and the DNA relatedness value of 96 % confirmed that the two strains represented a single species.

The 16S rRNA genes were amplified using the bacterial universal primers 27F and 1541R (Weisburg *et al.*, 1991). The purified PCR products of approximately 1.5 kb in length were cloned to *Escherichia coli* DH5α and sequenced by the Sangon Biological Engineering Technology Service, Shanghai, China. The 16S rRNA gene sequences were

Fig. 1. Electron micrographs of cells of strain D3RC-2^T. (a) Negatively stained cells, Bar, 1.0 μm. (b) Ultrathin section of a cell. Bar, 0.2 μm.

Fig. 2. Unrooted neighbour-joining phylogenetic tree showing strains D3RC-2^T, D3RC-3r and related genera of the family *Clostridiaceae* based on 16S rRNA gene sequence comparisons. Numbers at nodes represent the levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets. GenBank accession numbers are given in parentheses. Bar, 2% sequence divergence.

submitted to GenBank to search for the similar sequences using the BLAST algorithm. The 16S rRNA sequences of strains D3RC-2^T and reference strains retrieved from GenBank were aligned using the CLUSTAL_X program (v1.83) (Thompson *et al.*, 1997). Phylogenetic trees were constructed using the neighbour-joining, UPGMA, minimum-evolution and maximum-parsimony methods implemented in the MEGA3 program (Kumar *et al.*, 2001). The stability of the trees was evaluated by bootstrap analysis of 1000 datasets (Felsenstein, 1985). To ascertain the phylogenetic position of the new isolates, the complete 16S rRNA gene sequences were compared with the most similar sequences retrieved from GenBank. On the basis of a consensus 1354 bp of 16S rRNA gene sequences, an unrooted phylogenetic tree revealed that the two strains represented a new phyletic sublineage within the family *Clostridiaceae*, with <93.8% 16S rRNA gene sequence

similarity to recognized species (Fig. 2). The phylogenetic position of the two strains was also confirmed by an additional phylogenetic tree which included all of the recognized species of the genus *Clostridium* as well as species of other genera of the family *Clostridiaceae* (see Supplementary Fig. S1 in IJSEM Online). The large sequence divergence indicated that the novel strains isolated in this study represented a new genus in the family *Clostridiaceae*.

The two novel strains also showed distinct phenotypic features which enabled them to be distinguished from all of the genera already assigned to the family *Clostridiaceae* (see Table 1). They did not utilize carbohydrates and so differed from members of the genera *Alkaliphilus*, *Anaerobacter*, *Anoxynatronum*, *Caloramator*, *Caloranaerobacter*, *Caminiella*, *Oxobacter*, *Sarcina*, *Thermobrachium* and *Thermohalobacter*. The novel strains could be differentiated from members of the

Table 1. Characteristics that differentiate strain D3RC-2^T from recognized genera of the family *Clostridiaceae*

Taxa: 1, strain D3RC-2^T; 2, *Alkaliphilus* (Takai *et al.*, 2001; Cao *et al.*, 2003); 3, *Anaerobacter* (Duda *et al.*, 1987); 4, *Anoxynatronum* (Garnova *et al.*, 2003); 5, *Caloramator* (Patel *et al.*, 1987; Christostomos *et al.*, 1996); 6, *Caloranaerobacter* (Wery *et al.*, 2001); 7, *Caminiella* (Alain *et al.*, 2002); 8, *Clostridium* (Hippe *et al.*, 1992); 9, *Natronincola* (Zhilina *et al.*, 1998); 10, *Oxobacter* (Krumholz & Bryant, 1985); 11, *Sarcina* (Canale-Parole, 1986); 12, *Thermohalobacter* (Cayol *et al.*, 2000); 13, *Thermobrachium* (Engle *et al.*, 1996); 14, *Tindallia* (Kevbrin *et al.*, 1998). +, Positive; -, negative; +/-, variable; w, weakly positive.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Cell motility	-	+	-	+	+/-	+	+	+	+	+	-	+	+	+
Gram reaction	-	+	+	+	-	-	-	+/-	+	+	+	-	+	+
Spore formation	-	+	+	-	+/-	-	+	+	+/-	+	+	-	-	+/-
NaCl requirement	-	-	-	+	-	+	+	+/-	+	-	-	+	-	+
Substrates utilized:														
Carbohydrates	-	+	+	+	+	+	+	+/-	-	+	+	+	+	-
Proteolysis products	+	+	-	+	+/-	+	+	+/-	+	-	-	-	+	+
Amino acids	+	-	-	+	+/-	+	w	+/-	+	-	-	-	+	-
DNA G + C content (mol%)	41.0	30-36	29.0	48.4	25.6-39.0	27.0	24.2	21-54	31.9	38.0	28.6-30.6	33.0	31.0	26.9-40

genus *Anaerobacter* by the latter's ability to form up to five endospores per cell. The novel strains differed from members of the genus *Clostridium* by the latter's motility and spore formation. The two novel strains were Gram-staining negative and non-spore-forming which differentiated them from members of the genera *Natronincola* and *Tindallia*.

On the basis of the distant phylogenetic relationship with related taxa, unique chemotaxonomic characteristics and the physiological and biochemical traits described above, it is evident that strains D3RC-2^T and D3RC-3r represent a novel species of a new genus of the family *Clostridiaceae*, for which the name *Proteiniclasticum ruminis* gen. nov., sp. nov. is proposed.

Description of *Proteiniclasticum* gen. nov.

Proteiniclasticum [Pro.te.i.ni.cla.sti'cum. N.L. neut. n. *proteinum* protein; N.L. adj. *clasticus* (from Gr. adj. *klastos*) breaking; N.L. neut. n. *Proteiniclasticum* a bacterium able to digest proteins].

Cells are Gram-negative, rod-shaped, non-spore-forming and non-motile. Obligately anaerobic. Mesophilic and neutrophilic. Proteolytic. Do not utilize carbohydrates. Soya peptone, tryptone and amino acids are used as carbon and nitrogen sources. The major fermentation products from PY medium are acetate, propionate and iso-butyrate. The cellular fatty acids mainly comprise iso-branched fatty acids. The type species is *Proteiniclasticum ruminis*.

Description of *Proteiniclasticum ruminis* sp. nov.

Proteiniclasticum ruminis (ru.mi'nis. L. neut. gen. n. *ruminis* of the rumen).

Displays the following properties in addition to those given in the genus description. Cells are 0.6–2.0 µm in length and 0.5–0.8 µm in width. Spores are not observed. The temperature range for growth is 24–46 °C, optimal growth occurs at 38–39 °C. The pH range for growth is 5.6–8.7, optimum growth occurs at pH 7.0–7.3. The generation time is 1.8 h in PY medium at 39 °C. Utilizes soya peptone, tryptone, L-phenylalanine, L-leucine, L-methionine, L-serine, L-valine, L-threonine and L-histidine, but not cellobiose, glucose, xylose, arabinose, maltose, salicin, mannitol, lactose, glycerol, melezitose, raffinose, rhamnose, L-alanine, L-cysteine, L-aspartic acid, L-glutamic acid, L-glycine, L-isoleucine, L-lysine, L-asparagine, L-proline, L-glutamine, L-arginine or L-tryptophan. Propionate is produced from L-methionine and L-leucine. Iso-butyrate is produced from L-threonine. Liquefies gelatin but does not reduce nitrate. Aesculin is hydrolysed. Does not produce H₂S or indole. The Voges–Proskauer test is negative. Milk is curdled. The cellular fatty acids of strain D3RC-2^T are mainly composed of iso-branched fatty acids, predominantly iso-C_{14:0}, iso-C_{13:0}, iso-C_{15:0}, C_{14:0}, ante-iso-C_{13:0} and ante-iso-C_{15:0}. The G+C content of the genomic DNA is 41.0 ± 0.1 mol%.

The type strain, D3RC-2^T (=AS 1.5057^T=JCM 14817^T), was isolated from yak rumen.

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