Reclassification of *Thermoanaerobium acetigenum* as *Caldicellulosiruptor acetigenus* comb. nov. and emendation of the genus description

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Although the type species of the genus *Thermoanaerobium*, *Thermoanaerobium brockii*, was transferred to *Thermoanaerobacter*, *Thermoanaerobium acetigenum* was not transferred. Therefore, *Thermoanaerobium acetigenum* should be reclassified. Based on 16S rRNA gene sequence analysis and re-examination of physiological properties of the type strain, X6B^T (=DSM 7040^T=ATCC BAA-1149^T), we propose that *Thermoanaerobium acetigenum* should be reclassified as *Caldicellulosiruptor acetigenus* comb. nov. Strain X6B^T contains two separate 16S rRNA genes bracketing another species in the phylogenetic 16S rRNA gene-based tree.

Thermoanaerobium acetigenum strain X6B^T, an anaerobic, thermophilic bacterium, was isolated by Nielsen *et al.* (1993) using xylan as the substrate. This bacterium, a Gram-type positive (Wiegel, 1981), low-G + C content rod, has many characteristics of a typical member of the *Firmicutes* (Gibbons & Murray, 1978). Based on its physiological properties alone, it was placed in the genus *Thermoanaerobium*, the type species of which is *Thermoanaerobium brockii* (Zeikus *et al.*, 1979).

Because the 16S rRNA gene sequence for *Thermoanaerobium acetigenum* X6B^T had not been determined previously, the classification of *Thermoanaerobium acetigenum* X6B^T was therefore based only on some physiological similarities. Although the type species of *Thermoanaerobium*, *Thermoanaerobium brockii*, was reclassified as *Thermoanaerobacter brockii* by Lee *et al.* (1993) and, subsequently, as *Thermoanaerobacter brockii* subsp. *brockii* (type strain HTD4^T) by Cayol *et al.* (1995), *Thermoanaerobium acetigenum* X6B^T was not transferred to the genus *Thermoanaerobacter* (Wiegel & Ljungdahl, 1981) because of the lack of 16S rRNA gene sequence analysis. Here we report on the assignment of the type strain of *Thermoanaerobium acetigenum* to the genus *Caldicellulosiruptor* as *Caldicellulosiruptor acetigenus* comb. nov., based on 16S rRNA gene sequence, DNA–DNA hybridization analysis and retesting of its properties. Special attention was given to cellulose degradation, as all other presently known *Caldicellulosiruptor* species are cellulolytic, whereas strain X6B^T has been described as being non-cellulolytic.

Strain X6B^T was obtained as a freeze-dried culture of strain DSM 7040^T from the DSMZ (Braunschweig, Germany). To determine the 16S rRNA gene sequence, *Thermoanaerobium acetigenum* DSM 7040^T was grown under anaerobic conditions (Ljungdahl & Wiegel, 1986; Angelidaki *et al.*, 1990). A basal salts medium (final pH 7.3–7.4) was prepared as described by Nielsen *et al.* (1993). Strain DSM 7040^T was grown in basal salts medium supplemented with yeast extract (0.3%), tryptone (1.0%) and glucose (0.5%), and subjected to two rounds of isolation of single colonies using yeast extract, tryptone, glucose salts medium solidified with 2.2% Gelrite (colonies became visible after incubation at 65 °C for 48–72 h). Because the initial 16S rRNA gene sequence analysis yielded two different 16S rRNA species, which bracketed another *Caldicellulosiruptor* species, it became necessary to confirm the purity of the culture further. Therefore, strain DSM 7040^T was grown using three different media (substrate conditions as described below), and each culture was then subjected to three subsequent rounds of single-cell colony isolation. To establish three lines of cultures, strain DSM 7040^T was grown in the above-described basal salts medium, supplemented with yeast extract, tryptone, glucose and brain heart infusion (0.2%) (termed BYTG medium). From this culture, three parallel cultures were inoculated (0.1% inoculum) using the following media: (i) basal salts plus 0.2% arabinose medium, (ii) basal salts plus 0.2% raffinose medium and (iii) BYTG medium. Arabinose- and raffinose-supplemented basal salts media were used because the closest *Caldicellulosiruptor* species to strain X6B^T on the phylogenetic tree (Fig. 1) are unable to use these substrates (Table 1). After checking microscopically that the cultures were suspensions of individual cells and did not contain any clumps or associations

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain DSM 7040^T are AY772476 and AY772477.

Fig. 1. Neighbour-joining tree showing the estimated phylogenetic relationships of *Caldicellulosiruptor acetigenus* X6B^T based on 16S rRNA gene sequence data with maximum-likelihood correction for synonymous changes. The 16S rRNA gene data used represent *Escherichia coli* DSM 30083^T nucleotide positions 42–1424. Numbers at nodes indicate bootstrap support percentages for 1000 replicates. Bar, 0.02 nucleotide substitutions per site. GenBank accession numbers are given in parentheses.

of cells, each of the above cultures was used to inoculate dilution series of Gelrite shake-roll tubes (Ljungdahl & Wiegel, 1986), with 2.2 % (w/v) Gelrite, to obtain single-cell colonies. The Gelrite shake-roll tubes were incubated at 65 °C for 48–72 h before colonies became visible. Colonies were picked in an anaerobic chamber (Coy Products) and resuspended in a tube containing 0.3–0.4 ml of the corresponding medium, which was then used to inoculate the next round of Gelrite shake-roll tubes. This process of colony picking was repeated for three rounds of colony isolation with three colonies being picked from each of the arabinose, raffinose and BYTG media after the third and final round. Each of the picked final colonies was reinoculated into a

fresh tube of the medium from which it was isolated, resulting in nine cultures: three with the arabinose medium, three with the raffinose medium and three with BYTG medium.

Subsequent extraction of DNA from the nine cultures was performed using a DNeasy Tissue kit (Qiagen). The DNA was then amplified using a bacterial domain-specific primer set for 16S rRNA, 27 forward and 1492 reverse (Lane, 1991). PCR was carried out as described previously (Lee *et al.*, 2005). The PCR products were purified using a QIAquick PCR Purification kit (Qiagen) and sequenced by Macrogen (Seoul, Korea). PCR products from the colonies were cloned

Table 1. Differential characteristics of *Caldicellulosiruptor acetigenus* X6B^T, *Caldicellulosiruptor kristjanssonii* I77R1B^T and *Caldicellulosiruptor lactoaceticus* 6A^T

Strains: 1, *Caldicellulosiruptor acetigenus* X6B^T; 2, *Caldicellulosiruptor kristjanssonii* I77R1B^T; 3, *Caldicellulosiruptor lactoaceticus* 6A^T.

Characteristic	1	2	3
Temperature range for growth (optimum) (°C)*	50–78 (65–68) ^a	50–82 (78) ^b	50–78 (68) ^b
pH range for growth (optimum)†	5.2–8.5 (7.0) ^a	5.8–8.0 (7.0) ^b	5.8–8.2 (7.0) ^b
Substrate utilization‡:			
Arabinose	+	–	–
Fructose	+	+	–
D-Galactose	+	+	–
D-Glucose	+	+	–
Mannose	+	+	–
Raffinose	+	–	–
Sucrose	+	+	–
Trehalose	+	+	–
Avicel	–	+	+
Growth inhibition:			
H ₂ (0.5 atm)	+	–	–
NaCl§	+	–	–
Lactate as a major fermentation product	–	+	+

*Determined at: *a*, pH 7.0 with xylose as substrate; *b*, pH 7.0 with cellobiose as substrate.

†Determined at: *a*, 68 °C with xylose; *b*, 70 °C with cellobiose.

‡Substrate concentrations were 2.0 g l⁻¹.

§NaCl concentrations tested were 0.2 % for strains X6B^T and I77R1B^T and 0.5 % for strain 6A^T.

using a TOPO TA Cloning kit (Invitrogen). Clones were randomly chosen, from which plasmid DNA was extracted by using an Eppendorf FastPlasmid Mini kit (Brinkman). The DNA was subsequently amplified, purified and sequenced. The sequence similarities were determined using Sequencher v4.1.4 (Gene Codes). Ten clones were sequenced, resulting in two similar sets of sequences (Fig. 1; termed T6 and T4, GenBank accession nos AY772477 and AY772476, respectively). Three clones with the same 16S rRNA gene sequence were never obtained from a single culture, suggesting that an even distribution of clones with the different sequences existed. Analysis of the 16S rRNA gene sequence using nucleotide to nucleotide BLAST (BLASTN) at NCBI (<http://www.ncbi.nlm.nih.gov/blast/>) to retrieve the most significant homologues of the query 16S rRNA gene sequence revealed that the most significant sequence alignments of *Thermoanaerobium acetigenum* X6B^T were with *Caldicellulosiruptor* species (Sissons *et al.*, 1987; Rainey *et al.*, 1994), with *Caldicellulosiruptor lactoaceticus* 6A^T (Mladenovska *et al.*, 1995) and *Caldicellulosiruptor kristjanssonii* I77R1B^T (Bredholt *et al.*, 1999) as the closest relatives (Fig. 1). Subsequently, 16S rRNA gene sequence-based phylogenetic trees were generated using CLUSTAL_X (Thompson *et al.*, 1997) for sequence alignments, and phylogeny inference package (PHYLIP) software (Felsenstein, 1989) and neighbour-joining algorithms (Saitou & Nei, 1987) to look at differing tree constructions and to generate distance matrices. TreeExplorer (Kumar *et al.*, 1994), a supplemental program of MEGA, was used to view the tree. The phylogenetic trees generated (the neighbour-joining tree is shown in Fig. 1) showed clearly that *Thermoanaerobium acetigenum* X6B^T belongs to the clade of *Caldicellulosiruptor* and not to the genus *Thermoanaerobacter* or *Thermoanaerobacterium*. Each of the repurified strains exhibited the two different 16S rRNA sequences. In contrast to other reported cases, e.g. *Clostridium paradoxum* (Rainey *et al.*, 1996), the two sequences were not juxtaposed, but rather separated by a sequence of another species, *Caldicellulosiruptor kristjanssonii*. A comparable situation has been reported by Amann *et al.* (2000), i.e. single-cell-derived pure cultures contained two different 16S rRNA genes with about 5% inferred difference in substitutions and bracketing a different species. The relative distance between the two identified *Thermoanaerobium acetigenum* X6B^T 16S rRNA gene sequences was about 2% and the distance to the closest neighbours *Caldicellulosiruptor lactoaceticus* 6A^T and *Caldicellulosiruptor kristjanssonii* I77R1B^T was around 1%. Therefore, DNA–DNA hybridization experiments were performed using the method described by De Ley *et al.* (1970) and modified by Huß *et al.* (1983). Chromosomal DNA for DNA–DNA hybridization was extracted and purified using a Maxi Genomic [0]DNA Prep kit (A&A Biotechnology).

DNA–DNA hybridization values between *Thermoanaerobium acetigenum* DSM 7040^T and *Caldicellulosiruptor owensensis* OL^T, *Caldicellulosiruptor lactoaceticus* 6A^T and *Caldicellulosiruptor kristjanssonii* I77R1B^T were 34.3, 50.9

and 53.1%, respectively. These values are all significantly below the 70% relatedness mark that would indicate a relationship at the species level (Wayne *et al.*, 1987) and clearly distinguish *Thermoanaerobium acetigenum* X6B^T from these three *Caldicellulosiruptor* species that are its closest neighbours in the 16S rRNA gene-based phylogenetic tree (Fig. 1). The repeated isolation of single-cell colonies after growth in different media, the observed homogeneity of the colony and cell morphologies with and among cultures (using microscopy) and the fact that all isolated colonies gave rise to the two different 16S rRNA sequences indicate that it is unlikely that the two sequences are due to a mixed culture having been analysed. These results indicate that *Thermoanaerobium acetigenum* X6B^T belongs in the genus *Caldicellulosiruptor*, a member of the order *Clostridiales*, and not in the genus *Thermoanaerobacter*, order 'Thermoanaerobacteriales' (Garrity *et al.*, 2002).

Members of the genus *Caldicellulosiruptor* have the characteristic trait of coupling cellulose degradation to growth (Rainey *et al.*, 1994). However, *Thermoanaerobium acetigenum* X6B^T was characterized previously (Nielsen *et al.*, 1993) as being incapable of cellulose degradation.

Thermoanaerobium acetigenum DSM 7040^T was retested for the ability to degrade cellulose using Whatman no. 1 filter paper and carboxymethylcellulose (1.0% w/v, CMC 7LT or 7M; Hercules). In addition, cellulase activity was determined by the use of the reducing sugar assay employing *p*-hydroxybenzoic acid hydrazide and glucose as a standard (Lever, 1973). *Thermoanaerobium acetigenum* DSM 7040^T was incapable of degrading Whatman no. 1 filter cellulose with or without 0.05% (w/v) yeast extract, but utilized CMC, exhibiting moderate growth with 1.0% of the low substitution (substitution level 0.7 of 3) form Hercules 7LT or 7M. Eleven and 10 µmol ml⁻¹, respectively, of reduced sugar residues was released from the cultures after 4 days of incubation, with a requirement for yeast extract (0.05%, w/v) for growth. Growth was not observed with only CMC 7LT/7M present. More highly substituted (e.g. 1.2 out of 3) CMCs (Hercules 12M or 12L) did not serve as substrates.

The substrate utilization spectrum of *Thermoanaerobium acetigenum* DSM 7040^T, as performed by Nielsen *et al.* (1993), was re-examined by adding various carbohydrates (to a final concentration of 2 g l⁻¹) from autoclaved stock solutions (pyruvate was filter-sterilized) to the basal media. Cultures were incubated at 73 °C for 48–72 h. Growth of cultures with insoluble substrates was determined by cell counts (Olympus model Vanox microscope with a Petroff-Hausser counting chamber). The results confirmed the previously published data.

The 16S rRNA gene sequence analysis, CMC-cellulase activity and growth observed on low-substituted CMC indicate that *Thermoanaerobium acetigenum* belongs to the genus *Caldicellulosiruptor*, and we propose the name *Caldicellulosiruptor acetigenus* comb. nov.

Emended description of the genus *Caldicellulosiruptor* Rainey *et al.* 1995

The description is the same as that given by Rainey *et al.* (1994) with the addition that some members do not possess the capacity to degrade crystalline cellulose or filter paper and cannot use cellulose as a carbon and energy source, but can hydrolyse CMC.

Description of *Caldicellulosiruptor acetigenus* comb. nov.

Caldicellulosiruptor acetigenus (a.ce.ti.ge'nus. L. n. *acetum* vinegar; L. v. *genere*, *gignere* to produce; N.L. masc. adj. *acetigenus* vinegar- or acetic acid-producing).

Basonym: *Thermoanaerobium acetigenum* Nielsen *et al.* 1994.

The description is based mainly on that given by Nielsen *et al.* (1993). Cells stain Gram-negative but have a Gram-type positive cell wall structure, occur singly or in pairs, and are about 3·6–5·9 by 0·7–1·0 µm in size. Sometimes occur as chains of up to eight cells. On solidified xylan-containing medium, off-white, milky-coloured colonies are observed. Strictly anaerobic chemoorganoheterotroph. At pH 7·0, growth occurs between 50 and 78 °C (optimum 65–68 °C). Growth occurs at pH 5·2–8·6 (optimum 7·0). Doubling time under optimal conditions is approximately 4 h. Arabinose, cellobiose, fructose, D-galactose, D-glucose, lactose, maltose, mannose, raffinose, soluble starch, sucrose, trehalose, D-xylose and xylan support growth. Growth and CMC-cellulase activity is observed when grown on carboxymethylcellulose (Hercules CMC, 7LT or 7M) in the presence of traces of yeast extract, but not with filter paper or crystalline (Avicel) cellulose. Acetate, CO₂, H₂, ethanol and traces of isobutyric acid (but not lactate) are formed during growth with glucose or D-xylose. The DNA G+C content of the type strain is 35·7±0·8 mol% (chromatographic method).

The type strain is X6B^T (=DSM 7040^T=ATCC BAA-1149^T), which was isolated from a combined biomat and sediment sample taken from a slightly alkaline hot spring at Hverðagerdi, Iceland.

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