

Classification of '*Anaerocellum thermophilum*' strain DSM 6725 as *Caldicellulosiruptor bescii* sp. nov.

Sung-Jae Yang,^{1,5} Irina Kataeva,^{1,5} Juergen Wiegel,^{1,3} Yanbin Yin,^{1,4,5} Phuongan Dam,^{1,4,5} Ying Xu,^{1,4,5} Janet Westpheling^{2,5} and Michael W. W. Adams^{1,3,5}

Correspondence

Michael W. W. Adams
adams@bmb.uga.edu

¹Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602, USA

²Department of Genetics, University of Georgia, Athens, Georgia 30602, USA

³Department of Microbiology, University of Georgia, Athens, Georgia 30602, USA

⁴Institute of Bioinformatics, University of Georgia, Athens, Georgia 30602, USA

⁵BioEnergy Science Center, Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, USA

The thermophilic, cellulolytic, anaerobic bacterium '*Anaerocellum thermophilum*' strain Z-1320 was isolated from a hot spring almost two decades ago and deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ) as DSM 6725. The organism was classified as representing a new genus, '*Anaerocellum*', primarily on its growth physiology, cell-wall type and morphology. The results of recent physiological studies and of phylogenetic and genome sequence analyses of strain DSM 6725 of '*A. thermophilum*' obtained from the DSMZ showed that its properties differed from those originally described for strain Z-1320. In particular, when compared with strain Z-1320, strain DSM 6725 grew at higher temperatures and had an expanded range of growth substrates. Moreover, the 16S rRNA gene sequence of strain DSM 6725 fell within the *Caldicellulosiruptor* clade. It is therefore suggested that '*Anaerocellum thermophilum*' should be classified as a member of the genus *Caldicellulosiruptor*, for which the name *Caldicellulosiruptor bescii* sp. nov. is proposed (type strain DSM 6725^T=ATCC BAA-1888^T). *C. bescii* sp. nov. DSM 6725^T is the most thermophilic cellulose-degrading organism known. The strain was able to grow up to 90 °C (pH 7.2) and degraded crystalline cellulose and xylan as well as untreated plant biomass, including potential bioenergy plants such as poplar and switchgrass.

'*Anaerocellum thermophilum*' strain Z-1320 was isolated from a continental thermal spring in Kamchatka, Russia, almost two decades ago (Svetlichnyi *et al.*, 1990). It was described as an extremely thermophilic, anaerobic, asporogenous, cellulolytic bacterium (Svetlichnyi *et al.*, 1990). It was reported to grow at temperatures of up to 83 °C, to grow optimally at between 72 and 75 °C at pH 7.1–7.3, to utilize a variety of polymeric carbohydrates as well as di- and

monosaccharides, and to produce lactate, acetate, H₂ and CO₂ with trace amounts of ethanol. The growth substrates included crystalline cellulose, carboxymethylcellulose (CMC), starch, glycogen and aspen sawdust, although it did not grow on xylose or pectin. In the absence of a 16S rRNA gene sequence, at the time of its discovery strain Z-1320 was assigned to a new genus, designated '*Anaerocellum*', based on its phenotypic and physiological characteristics.

Abbreviation: CMC, carboxymethylcellulose.

The GenBank/EMBL/DDBJ accession number for the genome of strain DSM 6725^T is NC_012034.

A table comparing the morphological characteristics of strain DSM 6725^T, strain Z-1320 and representatives of the genus *Caldicellulosiruptor*, images of cellobiose-grown cultures of strain DSM 6725^T in the exponential growth phase, and a sequence comparison of 16S rRNA genes from strain DSM 6725^T and '*A. thermophilum*' strain Z-1320 are available as supplementary material with the online version of this paper.

'*A. thermophilum*' strain Z-1320 was deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and was assigned deposition number DSM 6725. Recently, we obtained a culture of '*A. thermophilum*' from the DSMZ (DSM 6725) and determined its genome sequence (Kataeva *et al.*, 2009) and examined its physiological properties (Yang *et al.*, 2009). In particular, this organism was able to utilize unprocessed plant biomass, including hardwood (poplar) and grasses with low

lignin (Napier grass, Bermuda grass) and high lignin (switch-grass) contents, as growth substrates. However, some of the properties of the strain of '*A. thermophilum*' obtained from the DSMZ (strain DSM 6725) differed from those previously reported for '*A. thermophilum*' strain Z-1320 (Yang *et al.*, 2009).

The physiological properties of '*A. thermophilum*' DSM 6725 were very similar to those reported for members of the genus *Caldicellulosiruptor*. This genus currently comprises seven species with validly published names: *C. saccharolyticus* as the type species (Rainey *et al.*, 1994), *C. lactoaceticus* (Mladenovska *et al.*, 1995), *C. owensensis* (Huang *et al.*, 1998), *C. kristjanssonii* (Bredholt *et al.*, 1999), *C. acetigenus* (Onyenwoke *et al.*, 2006), *C. kronotskyensis* and *C. hydrothermalis* (Miroshnichenko *et al.*, 2008). All members of the genus *Caldicellulosiruptor* have been isolated from continental geothermal hot springs and are thermophilic, cellulolytic and non-spore-forming anaerobes. They have a Gram-positive-type cell wall and a rod-shaped morphology, and are capable of fermenting a wide range of polymeric carbohydrates.

We propose the taxonomic assignment of '*A. thermophilum*' to the genus *Caldicellulosiruptor*. Specifically, strain DSM 6725 is proposed as representing the type strain of *Caldicellulosiruptor bescii* sp. nov., based on its physiology, phylogenetic 16S rRNA gene sequence analyses and the similarity between its genome sequence and that of *Caldicellulosiruptor saccharolyticus* DSM 8903^T, the type species of the genus *Caldicellulosiruptor* and the only member for which a genome sequence is currently available.

Comparison of strain DSM 6725^T and '*A. thermophilum*' strain Z-1320

The substrate utilization spectrum and growth temperature profile of strain DSM 6725^T were determined. This strain was revived from the freeze-dried culture designated DSM 6725 that was obtained from the DSMZ (Braunschweig, Germany). It was grown in DSMZ 516 medium with the following modification. The mineral solution contained (l⁻¹): 0.33 g NH₄Cl, 0.14 g KH₂PO₄, 0.33 g KCl, 0.33 g MgCl₂·6H₂O, 0.14 g CaCl₂·2H₂O, 0.5 g yeast extract, 0.5 mg resazurin, 5 ml vitamin solution and 1 ml trace element solution. The vitamin solution contained (l⁻¹): 4 mg biotin, 4 mg folic acid, 20 mg pyridoxine-HCl, 10 mg thiamine-HCl, 10 mg riboflavin, 10 mg nicotinic acid, 10 mg calcium pantothenate, 0.2 mg vitamin B12, 10 mg *p*-aminobenzoic acid and 10 mg lipoic acid. The trace element solution contained (l⁻¹): 1.5 g FeCl₂·4H₂O, 0.07 g ZnCl₂, 0.1 MnCl₂·4H₂O, 0.006 g H₃BO₃, 0.19 g CoCl₂·6H₂O, 0.002 g CuCl₂·2H₂O, 0.024 g NiCl₂·6H₂O and 0.036 g Na₂MoO₄·2H₂O. The medium was reduced using (l⁻¹) 0.5 g cysteine and 0.5 g N₂S and then NaHCO₃ (1 g l⁻¹) was added. The final pH was 7.2. The medium was filter-sterilized using a 0.22 µm pore filter. All soluble and insoluble substrates were used at a final concentration of 0.5% (w/v) and were added directly to sterilized culture

bottles followed by the addition of the filter-sterilized medium. The culture media containing the insoluble substrates without inoculation were used as negative controls. To investigate substrate utilization, cultures were grown at 75 °C. To determine the temperature range for growth, static cultures were grown at between 42 and 90 °C in 100 ml serum bottles with 50 ml of the medium under a gas phase of N₂/CO₂ (80:20, v/v). Growth was determined after 24 h by measuring cell counts (phase-contrast microscope with a Petroff-Hausser counting chamber) or total cell protein (Bradford assay).

Cellobiose-grown cultures of strain DSM 6725^T in the exponential growth phase contained rod-shaped cells with rounded ends (see Supplementary Fig. S1 in IJSEM Online), which were identical to the morphology of cells of strain Z-1320 as described by Svetlichnyi *et al.* (1990). Some characteristics of strain DSM 6725^T, strain Z-1320 and representatives of the genus *Caldicellulosiruptor* are compared in Supplementary Table S1 (available in IJSEM Online). The significant physiological difference between strains DSM 6725^T and Z-1320 concerned their ability to grow on xylose and pectin. Strain Z-1320 was previously described (Svetlichnyi *et al.*, 1990) as being incapable of growing on these substrates as carbon and energy sources, characteristics which made this strain distinct from members within the genus *Caldicellulosiruptor*. In contrast, strain DSM 6725^T grew well with xylose as well as with xylans from various sources (birchwood, beechwood and oat spelts) at 75 °C. After 24 h, strain DSM 6725^T reached cell densities of approximately 3.0 × 10⁸ cells ml⁻¹ when grown on xylose and xylans. It also utilized pectin efficiently as well as polygalacturonate, the main component of de-esterified pectin. The cell density after 24 h was 2.3 × 10⁸ cells ml⁻¹ for pectin and 1.0 × 10⁸ cells ml⁻¹ for polygalacturonate.

Strain DSM 6725^T could also be distinguished from strain Z-1320 on the basis of its growth temperature range. Strain Z-1320 grew optimally at 72–75 °C, but no growth was observed at temperatures above 83 °C (Svetlichnyi *et al.*, 1990). In contrast, strain DSM 6725^T reached cell densities above 1.8 × 10⁸ cells ml⁻¹ growing on crystalline cellulose (Avicel) after 24 h at 85 °C and was capable of growing at up to 90 °C (Fig. 1). The optimal growth temperature of strain DSM 6725^T was 78–80 °C. When grown on crystalline cellulose under optimal conditions, strain DSM 6725^T had a specific growth rate of 0.4 h⁻¹ and a doubling time of 1.7 h (Fig. 2). It was also able to utilize lignocellulosic plant biomass efficiently, exhibiting a specific growth rate of 0.37 h⁻¹ and a doubling time of 1.9 h using switchgrass (hot-water washed at 75 °C for 24 h) as the carbon and energy sources under optimal conditions (Fig. 2). Among characterized micro-organisms that are able to grow on crystalline cellulose, *C. kristjanssonii* was the most thermophilic bacterium previously known, growing optimally at 78 °C with a maximal growth temperature of 82 °C (Bredholt *et al.*, 1999). With an optimal growth temperature of 78–80 °C and a

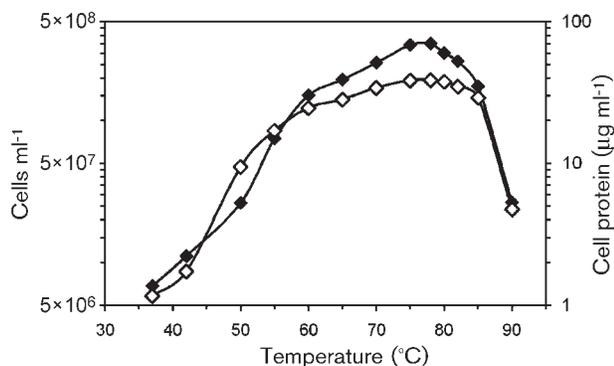


Fig. 1. Effect of temperature on the growth of strain DSM 6725^T. The strain was cultured at various temperatures for 24 h as static cultures in closed 100 ml serum bottles with 50 ml mineral medium (pH 7.2) containing 0.5% (w/v) crystalline cellulose (Avicel) using a gas phase N₂/CO₂ (80:20, v/v). Growth was monitored by measuring cell density (solid symbols) and cell protein (open symbols).

maximum of 90 °C, strain DSM 6725^T is now the most thermophilic cellulolytic (crystalline cellulose and ligno-cellulose) bacterium characterized to date.

The genome sequence of strain DSM 6725^T revealed three almost identical 16S rRNA genes showing 99.7–99.9% sequence similarities (Kataeva *et al.*, 2009). Comparison with the 16S rRNA gene of strain Z-1320 (Rainey *et al.*, 1994) showed sequence variations in several regions

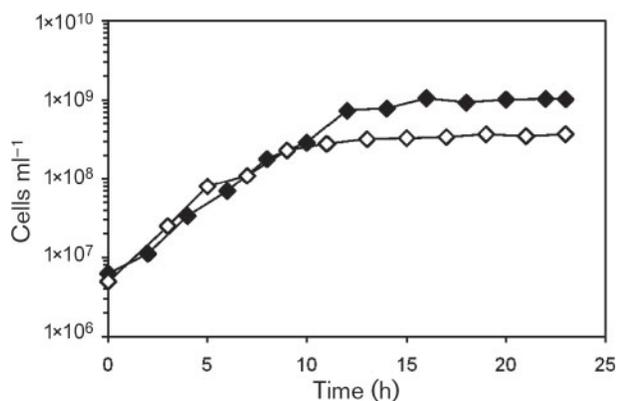


Fig. 2. Growth of strain DSM 6725^T on crystalline cellulose and the insoluble form of switchgrass. The culture was agitated (150 r.p.m.) for 23 h at 75 °C in a 600 l tower fermentation vessel with 500 l mineral medium containing 0.5% (w/v) crystalline cellulose (solid symbols) and insoluble switchgrass (open symbols). The pH was maintained at 7.2 and the gas phase was continuously flushed with N₂/CO₂ (80:20, v/v). The insoluble fraction of switchgrass was prepared by washing twice with water (5 g l⁻¹) at 75 °C for 24 h.

with 14 mismatches and two inserts/deletions. The overall sequence similarity between the two strains was 98.6% (see Supplementary Figure S2). The sequences of two other genes of the Z-1320 strain have been reported and these encode a cellulase (Zverlov *et al.*, 1998) and a xylanase (<http://www.uniprot.org/uniprot/Q59150>). These are encoded in strain DSM 6725^T by the genes Athe_1867 and Athe_0618, respectively. Compared with their Z-1320 counterparts, Athe_1876 (98.2% amino acid similarity) had 15 amino acid mismatches and lacked a 16-residue linker, and Athe_0618 (99.1% amino acid similarity) had five amino acid mismatches and one amino acid was deleted. The ‘*A. thermophilum*’ strain designated DSM 6725^T that was revived from two separate DSM 6725 cultures supplied by the DSMZ is therefore not identical to the original strain Z-1320, which was isolated, deposited and described by Svetlichnyi *et al.* (1990) and used in the studies of Rainey *et al.* (1994) and Zverlov *et al.* (1998).

Comparison of strain DSM 6725^T and representative species of the genus *Caldicellulosiruptor*

The type species of the genus *Caldicellulosiruptor*, *C. saccharolyticus*, is a thermophilic, anaerobic, asporogenous, rod-shaped, cellulolytic bacterium with a Gram-positive cell-wall type that utilizes a range of simple and complex carbohydrates. All of these features are also characteristics of strain DSM 6725^T (see Supplementary Table S1). In order to analyse the genotypic characteristics of strain DSM 6725^T, comparative sequence analysis of the 16S rRNA genes was performed. A phylogenetic tree was generated by the neighbour-joining method from the evolutionary distance matrix using the MEGA4 program (Tamura *et al.*, 2007). Bootstrap values were calculated on 1000 replicates. The phylogenetic tree obtained (Fig. 3) demonstrated that strain DSM 6725^T was taxonomically placed within the genus *Caldicellulosiruptor*. The closest relative of strain DSM 6725^T was the most recently described species of the genus, *Caldicellulosiruptor hydrothermalis* (DSM 18901^T; Miroshnichenko *et al.*, 2008), with 98.1–98.3% sequence similarity. The sequence similarity of strain DSM 6725^T to the type species, *C. saccharolyticus* (DSM 8903^T; van de Werken *et al.*, 2008), was 95.8–96.3%. The most distant relative of strain DSM 6725^T among the species of the genus *Caldicellulosiruptor* was *Caldicellulosiruptor* sp. Rt8B.4 (Dwivedi *et al.*, 1996) with 93.4–93.5% sequence similarity, although this organism has not been formally described. The overall sequence similarity between strain DSM 6725^T and the recognized species of the genus *Caldicellulosiruptor* was above 95%. *C. saccharolyticus* is the only species in this taxonomic group whose genome has been sequenced, and a comparison of the genome of strain DSM 8903^T with that of strain DSM 6725^T further demonstrated the close evolutionary relationship between these two organisms. Both genomes were 2.97 Mb in size with 35 mol% G+C content. Moreover, of the predicted 2662 open reading frames in the genome of strain

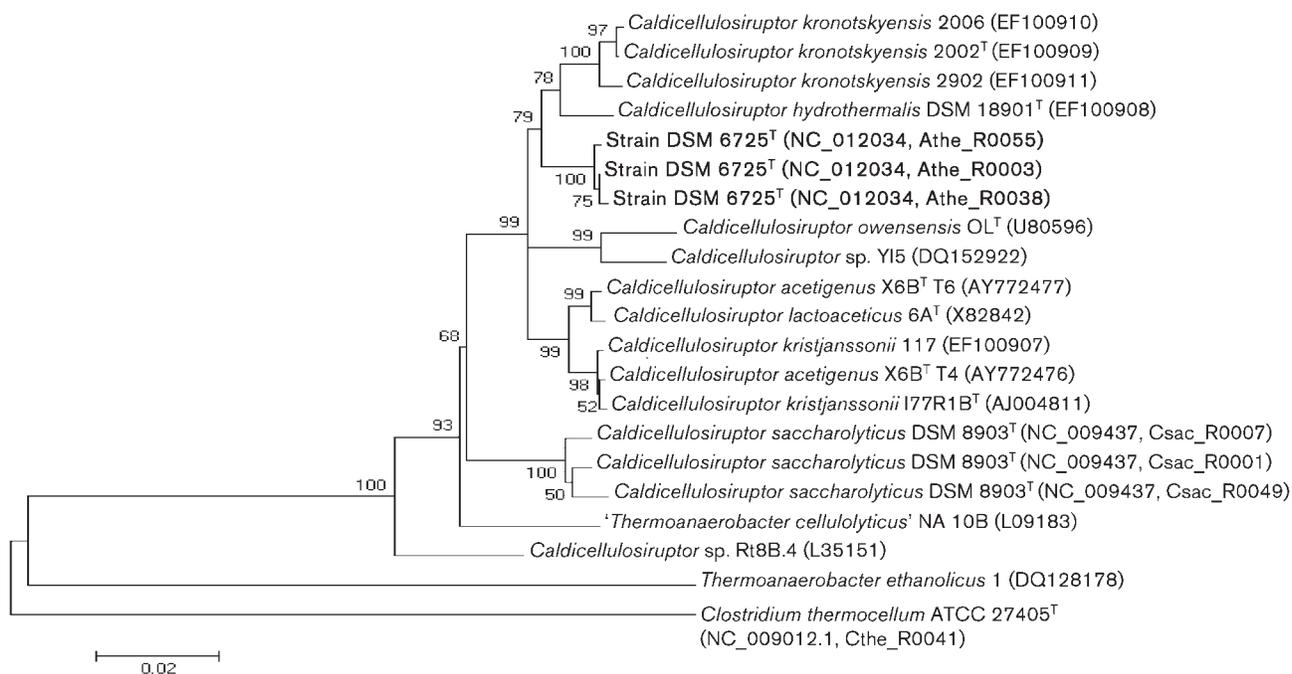


Fig. 3. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain DSM 6725^T within the genus *Caldicellulosiruptor*. The Tamura-Nei model was used with a gamma value of 1. The tree is a bootstrap consensus tree, numbers are bootstrap values for 1000 replicates. GenBank sequence accession numbers are given in parentheses. Bar, 0.02 nucleotide substitutions per site.

DSM 6725^T, 2112 of them showed their best BLAST hits (e-value of <math><1e-20</math>) in the genome of *C. saccharolyticus* DSM 8903^T.

These phylogenetic analyses, together with the phenotypic properties, unambiguously demonstrate that strain DSM 6725^T (and Z-1320) is most closely related to species of the genera *Caldicellulosiruptor* and should be classified within this genus.

Description of *Caldicellulosiruptor bescii* sp. nov.

Caldicellulosiruptor bescii (bes'ci.i. N.L. gen. masc. n. *bescii* of BESC, the BioEnergy Science Center of the US Department of Energy located in Oak Ridge, TN, USA).

An extremely thermophilic, anaerobic, rod-shaped, chemo-organotrophic bacterium with a growth temperature range of 42–90 °C (T_{opt} 78–80 °C and T_{max} 90 °C) with no growth at or below 37 °C or at or above 92 °C (at pH 7.2). Utilizes arabinose, CMC, cellobiose, crystalline cellulose (Avicel and sigmacell-20), β -cyclodextrin, filter paper (Whatman), fructose, galactose, glucose, glycogen, lactose, maltose, mannose, melibiose, pectin, pullulan, rhamnose, starch, sucrose, trehalose, xylan (beechwood, birchwood and oat spelts) and xylose. When grown on Avicel under optimal conditions, the specific growth rate and doubling time are 0.4 h⁻¹ and 1.7 h, respectively. Also grows on unprocessed plant biomass, including hardwood (poplar

and grasses with low lignin (Napier grass, Bermuda grass) and high lignin (switchgrass) contents (Yang *et al.*, 2009). When grown with insoluble materials of switchgrass at 75 °C (pH 7.1), doubling time is 1.9 h and the specific growth rate is 0.37 h⁻¹. Weak growth is observed on mannitol, polygalacturonate, raffinose, sorbitol and yeast extract. Does not grow on acetate, chitosan, dextran, erythritol, glycerol, inulin, lactate, mannan, pyruvate or xylitol. The major fermentation end products are acetate, lactate, H₂, and CO₂. The G+C content of its genome of 2.97 Mb is 35.14 mol% (Kataeva *et al.*, 2009).

The type strain DSM 6725^T (=ATCC BAA-1888^T) was originally deposited at the DSMZ as '*Anaerocellum thermophilum*' strain Z-1320.

Acknowledgements

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