Caldicellulosiruptor kronotskyensis sp. nov. and *Caldicellulosiruptor hydrothermalis* sp. nov., two extremely thermophilic, cellulolytic, anaerobic bacteria from Kamchatka thermal springs

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Five novel strains (2002^T, 2902, 2006, 108^T and 117) of cellulose-degrading, anaerobic, thermophilic bacteria were isolated from terrestrial hot springs of Kamchatka (Far East, Russia). Strains 2002^T and 108^T were non-spore-forming bacteria with a Gram-positive type cell wall and peritrichous flagella. Optimum growth of strains 2002^T and 108^T occurred at pH 7.0 and at temperatures of 70 and 65 °C, respectively. The G + C contents of the DNA of strains 2002^T and 108^T were 35.1 and 36.4 mol%, respectively. Comparative 16S rRNA gene sequence analysis revealed that the isolates belonged to the genus *Caldicellulosiruptor*. However, DNA–DNA hybridization experiments indicated that the levels of relatedness between strains 2002^T and 108^T and those of recognized members of the genus *Caldicellulosiruptor* ranged between 32 and 54 %. Based on both phenotypic and genomic differences, strains 2002^T and 108^T are considered to represent two novel species of the genus *Caldicellulosiruptor*. The names proposed for these organisms are *Caldicellulosiruptor kronotskyensis* sp. nov. (type strain 2002^T=DSM 18902^T=VKM B-2412^T) and *Caldicellulosiruptor hydrothermalis* sp. nov. (type strain 108^T=DSM 18901^T=VKM B-2411^T).

Cellulolytic capability is widely distributed across the domain *Bacteria* and is displayed by a physiologically and phylogenetically diverse group of micro-organisms that differ in growth temperature, salt tolerance and relation to oxygen. Extremely thermophilic cellulose-degrading micro-organisms are of particular fundamental and biotechnological interest owing to the presence of highly thermostable enzymes. The group of thermophilic cellulolytic prokaryotes includes two aerobic species, *Rhodothermus marinus* and *Acidothermus cellulolyticus*, and numerous anaerobes of the genera *Caldicellulosiruptor*, *Clostridium*, *Spirochaeta*, *Fervidobacterium* and *Thermotoga* (reviewed by Bergquist *et al.*, 1999). At the time of writing, the genus *Caldicellulosiruptor* comprised five species with validly published names: *Caldicellulosiruptor saccharolyticus*

(Rainey *et al.*, 1994), *Caldicellulosiruptor lactoaceticus* (Mladenovska *et al.*, 1995), *Caldicellulosiruptor owensensis* (Huang *et al.*, 1998), *Caldicellulosiruptor kristjanssonii* (Bredholt *et al.*, 1999) and *Caldicellulosiruptor acetigenus* (Onyenwoke *et al.*, 2006). All members of the genus *Caldicellulosiruptor* are extremely thermophilic, cellulolytic, non-spore-forming anaerobes with Gram-positive type cell wall, and are capable of fermenting a wide spectrum of carbohydrates. These bacteria have been isolated mostly from neutral or slightly alkaline geothermal springs in New Zealand, Iceland and California.

The hot springs of Kamchatka have also provided the source of isolation of anaerobic, thermophilic, cellulolytic bacteria. '*Dictyoglomus turgidus*' was isolated from Uzon caldera (Svetlichnii & Svetlichnaya, 1988) and '*Anaerocellum thermophilum*' was isolated from the Valley of Geysers (Svetlichnii *et al.*, 1990). 16S rRNA gene sequence analysis subsequently placed the latter organism

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 2002^T and 108^T are EF100909 and EF100908, respectively.

within the genus *Caldicellulosiruptor* (Mladenovska *et al.*, 1995). In the present study, we describe two novel thermophilic, cellulose-degrading, anaerobic bacteria isolated from hot springs of Kamchatka (Russia).

Five strains of cellulose-degrading micro-organisms were isolated from samples collected from thermal springs of the Kamchatka peninsula, at Geyser Valley (Kronotsky National Park), Moutnovsky Volcano and the Pauzhetka group of thermal springs. The sampling sites, with temperatures varying from 55 to 65 °C and pH 4.5–8.0, were rich in dead plant material. Enrichment cultures were made by inoculating 10 % (w/v) of 12 samples into Hungate tubes with anaerobically prepared sterile Pfennig medium (Pfennig, 1965), supplemented with microcrystalline cellulose (10 g l⁻¹), yeast extract (0.2 g l⁻¹; Difco), resazurin (1 mg l⁻¹), trace element solution (1 ml l⁻¹; Pfennig & Lippert, 1966) and vitamin solution (1 ml l⁻¹; Wolin *et al.*, 1963). N₂ was used as gas phase. Within 3–4 days of incubation at 70 °C, turbidity and gas bubbles caused by intensive microbial growth were observed in five tubes. Positive enrichment cultures were subcultured three times and then serially diluted in agar shake tubes, containing the same medium but without cellulose, solidified with 2 % agar (Difco) and supplemented with cellobiose (1 g l⁻¹; Sigma). Agar shake tubes were incubated at 60 °C for 1 week. Single colonies from the

highest positive dilutions were picked and inoculated into liquid medium containing microcrystalline cellulose. Colonies were 1–1.2 mm in diameter, spherical and milk or slightly creamy white in colour. The purification procedure was repeated twice. Finally, five pure cultures were obtained and these were designated strains 108^T and 117 (both from Pauzhetka), 2902 (from Moutnovsky Volcano), and 2006 and 2002^T (both from Geyser Valley).

For amplification and sequencing of 16S rRNA genes, DNA was obtained by standard phenol/chloroform extraction. The 16S rRNA genes were selectively amplified by using primers 5'-AGAGTTGATCCTGGCTCAG-3' (forward) and 5'-TACGGTTACCTTGTACGACTT-3' (reverse) and the PCR products were purified by using a Wizard PCR-Prep kit (Promega) according to the manufacturer's instructions. Sequencing was performed by using a BigDye Terminator v. 3.1 sequencing reaction kit and the sequencing reactions were run on an ABI 3730 DNA automatic sequencer (Applied Biosystems). Positions that had not been sequenced in one or more reference organisms were omitted from analysis. Pairwise evolutionary distances were computed by using the correction of Jukes & Cantor (1969). Preliminary phylogenetic analysis of the new sequences was done with the NCBI BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST/>). Nucleotide sequences were aligned with sequences from GenBank by

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strains 108^T, 117, 2002^T, 2006 and 2902 among members of the genus *Caldicellulosiruptor*. Bootstrap values (expressed as percentages of 100 replications) are shown at branch points; values of >70 % were considered to be significant. Bar, Jukes–Cantor distance (5 nucleotide substitutions per 100 nucleotides).

using CLUSTAL W. The unrooted phylogenetic tree was constructed according to the neighbour-joining method by using the TREECONW program package (Van de Peer & De Wachter, 1994).

Almost-complete (nearly 1500 nt) sequences of the 16S rRNA genes of strains 108^T, 117, 2902, 2006 and 2002^T were determined. In initial analyses, the 16S rRNA gene sequences of these strains were compared with corresponding GenBank sequence data. Preliminary analysis revealed that the new sequences grouped with those of members of the genus *Caldicellulosiruptor* (Rainey *et al.*, 1994) within the family *Syntrophomonadaceae* of Gram-positive bacteria (Garrity *et al.*, 2001). The phylogenetic tree generated (Fig. 1) showed clearly that the novel strains fell within the radiation of recognized *Caldicellulosiruptor* species. The 16S rRNA gene sequence of strain 117 was almost identical to that of the type strain of *Caldicellulosiruptor kristjanssonii* (99.5% similarity) and *Caldicellulosiruptor acetigenus* (99.7%). The 16S rRNA gene sequences of strains 2902, 2006 and 2002^T were almost identical to each other (99.2–99.9% similarity) and formed a single separate cluster among recognized *Caldicellulosiruptor* species, with levels of similarity of 94.8–97.7% to the type strains of these latter species. The 16S rRNA gene sequence of strain 108^T was closely related to this cluster, with sequence similarity values of 98.1–98.2%, whereas it showed lower levels of similarity to the type strains of recognized *Caldicellulosiruptor* species (94.5–97.6%).

The new isolates were compared with each other and all recognized species of the genus *Caldicellulosiruptor* based on DNA–DNA hybridization experiments (Miroshnichenko *et al.*, 1994). The following reference strains were used: *Caldicellulosiruptor saccharolyticus* DSM 8903^T, *Caldicellulosiruptor kristjanssonii* DSM 12137^T, *Caldicellulosiruptor lactoaceticus* DSM 9545^T, *Caldicellulosiruptor owensensis* DSM 13100^T and *Caldicellulosiruptor acetigenus* DSM 7040^T. The level of DNA–DNA relatedness between strain 117 and *Caldicellulosiruptor kristjanssonii* DSM 12137^T was 83%. Strain 117 was thus considered to represent *Caldicellulosiruptor kristjanssonii*. The high level of DNA–DNA relatedness (80–92%) found between strains 2902, 2006 and 2002^T indicated that they were strains of the same species. Levels of DNA–DNA relatedness between strain 2002^T and the above reference *Caldicellulosiruptor* strains were 46% (with *Caldicellulosiruptor lactoaceticus* DSM 9545^T) or lower, and between strain 108^T and the same reference strains were 54% (with *Caldicellulosiruptor lactoaceticus* DSM 9545^T) or lower. By contrast, the level of DNA–DNA relatedness between strains 2002^T and 108^T (40%) clearly revealed a lack of relationship at the species level. Based on these genomic DNA studies, strains 2002^T and 108^T were chosen for further characterization.

Cells of the new isolates as revealed by transmission electron microscopy (Bonch-Osmolovskaya *et al.*, 1990) were morphologically similar, being short ($3\text{--}3.2 \times 0.5\text{--}$

$0.7\text{ }\mu\text{m}$) straight rods with rounded ends and peritrichous flagella (Fig. 2a). Thin-section analysis revealed Gram-positive type cell walls (Fig. 2b). Cells occurred singly, in pairs or in short chains. Spores were not observed.

The results of phenotypic characterization of isolates 2002^T and 108^T are presented in Table 1. Growth experiments were performed by using the same basal medium as used for enrichment and isolation, but supplemented with maltose (20 mM) as the growth substrate. Other substrates tested were added at concentrations of 20 mM (soluble substrates) or 10 g l^{-1} (cellulose, xylan, dextran). Isolates 2002^T and 108^T grew under strictly anaerobic conditions, but were able to grow well on the anaerobically prepared medium without any reducing agent. The temperature range for growth was between 45 and 82 °C (optimum at 70 °C) for strain 2002^T and between 50 and 80 °C (optimum at 65 °C) for strain 108^T. The two strains grew over the pH range 6.0–8.0 with optimum growth at pH 7.0. The generation time for strains 108^T and 2002^T growing on maltose under optimal conditions was 2 and 2.5 h, respectively. Substrates utilized by strain 2002^T included cellulose, carboxymethylcellulose (CM-cellulose), filter paper, starch, dextran, xylan, pectin, cellobiose, glucose, fructose, galactose, sucrose, maltose, lactose, arabinose, xylose, trehalose, mannitol, yeast extract and peptone.

Fig. 2. Electron micrographs of (a) negatively stained and (b) thin-sectioned cells of strain 108^T. Bars, 0.5 μm .

Strains 2902 and 2006 shared with strain 2002^T the ability to use peptone and trehalose as carbon sources. Strain 108^T grew well on cellulose, CM-cellulose, filter paper, starch, dextran, xylan, pectin, cellobiose, glucose, fructose, galactose, sucrose, maltose, lactose, arabinose, xylose, mannitol and yeast extract. Strains 108^T and 2002^T were unable to grow with pyruvate, chitin, raffinose or ribose.

The main end products of glucose fermentation by strains 2002^T and 108^T were determined by GLC by using a Stayer chromatograph, UV/VID detector (wavelength 220 nm), REZEX ROA 300 × 7.8-mm column (Phenomenex), maintained at 35 °C, and 0.2 % H₃PO₄ solution as a solvent at a flow rate of 0.5 ml min⁻¹. The main end products found were lactate and acetate.

Table 1 details differential characteristics of strains 2002^T and 108^T as compared with recognized species of the genus *Caldicellulosiruptor*. Based on DNA–DNA hybridization experiments, strains 2002^T and 108^T are most closely related to the type strain of *Caldicellulosiruptor lactoaceticus* (46 and 54 %, respectively). However, strains 2002^T and 108^T could be differentiated from *Caldicellulosiruptor lactoaceticus* based on a much wider spectrum of utilized

substrates (Table 1). At the same time, the low level of DNA–DNA relatedness between strains 2002^T and 108^T (40 %) and differences between these two strains in the ability to utilize peptone and trehalose indicate that they represent two different species. We therefore suggest that strains 2002^T and 108^T represent two novel species of the genus *Caldicellulosiruptor*, for which the names *Caldicellulosiruptor kronotskyensis* sp. nov. and *Caldicellulosiruptor hydrothermalis* sp. nov. are proposed, respectively.

Description of *Caldicellulosiruptor kronotskyensis* sp. nov.

Caldicellulosiruptor kronotskyensis (kro.no.t'sky.en.sis. N.L. masc. adj. *kronotskyensis* pertaining to Kronotsky National Park, Kamchatka, Russia, from where the type strain was isolated).

Cells are short, straight rods (3–3.2 × 0.5–0.7 µm) with flagella. Cell wall is of Gram-positive type. Non-spore-forming. Strictly anaerobic. Temperature range for growth is 45–82 °C, with optimum growth at 70 °C; pH range for growth is 6.0–8.0, with optimum growth at pH 7.0.

Table 1. Differential characteristics between strain 108^T, strain 2002^T and members of the genus *Caldicellulosiruptor*

Taxa: 1, *Caldicellulosiruptor saccharolyticus* (data from Rainey *et al.*, 1994); 2, *Caldicellulosiruptor lactoaceticus* (Mladenovska *et al.*, 1995); 3, *Caldicellulosiruptor owensensis* (Huang *et al.*, 1998); 4, *Caldicellulosiruptor kristjanssonii* (Bredholt *et al.*, 1999); 5, *Caldicellulosiruptor acetigenus* (Onyenwoke *et al.*, 2006); 6, strain 2002^T (*Caldicellulosiruptor kronotskyensis* sp. nov.); 7, strain 108^T (*Caldicellulosiruptor hydrothermalis* sp. nov.). +, Positive; –, negative; NR, not reported.

Characteristic	1	2	3	4	5	6	7
Cell size (µm)	3–4 × 0.4–0.6	1.5–2.8 × 0.7	2–5 × 0.5–0.8	2.8–9.4 × 0.7–1.0	3.6–5.9 × 0.7–1.0	3–3.2 × 0.5–0.7	3–3.2 × 0.5–0.7
pH range for growth (optimum)	5.5–8.0 (7.0)	5.8–8.2 (7.0)	5.5–9.0 (7.5)	5.8–8.0 (7.0)	5.2–8.5 (7.0)	6.0–8.0 (7.0)	6.0–8.0 (7.0)
Temperature range for growth (optimum) (°C)	45–80 (70)	50–78 (68)	50–80 (75)	50–82 (78)	50–78 (65–68)	45–82 (70)	50–80 (65)
Generation time (h)	NR	2.0	7.3	2.0	4.0	2.5	2.0
Utilization of:							
Sucrose	+	–	+	+	+	+	+
Galactose	+	–	+	+	+	+	+
Ribose	–	–	+	–	NR	–	–
Raffinose	–	–	+	–	+	–	–
Arabinose	+	–	+	–	+	+	+
Fructose	+	–	+	+	+	+	+
Glucose	+	–	+	+	+	+	+
Trehalose	+	–	–	+	+	+	–
Mannitol	–	–	+	–	NR	+	+
Yeast extract	NR	NR	+	–	+	+	+
Peptone	–*	–	–	–	+	+	–
DNA G + C content (mol%)	37.5	35.2	36.6	35	35.7	35.1	36.4
NaCl tolerance (%)	NR	1	1	>0.2	0.2	1	1
Major fermentative product	Acetate	Lactate	Acetate	Acetate	Lactate	Lactate	Lactate

*Data from this study.

Chemo-organoheterotroph. Utilizes cellulose, CM-cellulose, filter paper, starch, xylan, dextran, pectin, cellobiose, glucose, fructose, sucrose, xylose, galactose, maltose, lactose, arabinose, trehalose, mannitol, yeast extract and peptone. Does not grow with pyruvate, chitin, raffinose or ribose. Fermentation products are lactate and acetate. The G + C content of the DNA of the type strain is 35.1 mol%.

The type strain, 2002^T (=DSM 18902^T=VKM B-2412^T), was isolated from a terrestrial neutral geothermal spring in Geyser Valley, Kamchatka. Strains 2902 and 2006 are reference strains.

Description of *Caldicellulosiruptor hydrothermalis* sp. nov.

Caldicellulosiruptor hydrothermalis (hy.dro.ther'ma.lis. N.L. masc. adj. *hydrothermalis* pertaining to hydrothermal vents).

Cells are short, straight rods (3–3.2 × 0.5–0.7 µm) with flagella. Cell wall is of Gram-positive type. Non-spore-forming. Strictly anaerobic. Temperature range for growth is 50–80 °C, with optimum growth at 65 °C; pH range for growth is 6.0–8.0, with optimum growth at pH 7.0. Chemo-organoheterotroph. Utilizes cellulose, CM-cellulose, filter paper, starch, xylan, dextran, pectin, cellobiose, glucose, fructose, sucrose, xylose, maltose, galactose, lactose, arabinose, mannitol and yeast extract. Does not grow with pyruvate, chitin, raffinose, trehalose, ribose or peptone. Main fermentation products are lactate and acetate. The G + C content of the DNA of the type strain is 36.4 mol%.

The type strain, 108^T (=DSM 18901^T=VKM B-2411^T), was isolated from a terrestrial neutral geothermal spring in the southern region of Kamchatka.

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