Thermoanaerobacterium aciditolerans sp. nov., a moderate thermoacidophile from a Kamchatka hot spring

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An anaerobic, moderately thermoacidophilic bacterium, strain 761-119^T, was isolated from an acidic hot spring in the Orange Field of the Uzon Caldera (Kamchatka, far-eastern Russia). Cells were spore-forming, Gram-positive rods, possessing one polar flagellum. Growth of strain 761-119^T was observed between 37 and 68 °C and in the pH^{20 °C} range 3.2–7.1. No growth was observed within 5 days of incubation at or below 35 °C and at or above 70 °C, as well as at or below pH^{20 °C} 2.8 and at or above pH^{20 °C} 7.5. The optimal temperature and pH^{20 °C} for growth were 55 °C and pH^{20 °C} 5.7, respectively. A wide range of carbohydrates and polysaccharides were fermented, as well as peptides and proteinaceous substrates. The main products of glucose fermentation were acetate, ethanol, lactate, H₂ and CO₂. The DNA G + C content was 34 (± 0.5) mol%. 16S rRNA gene sequence analysis indicated that strain 761-119^T belonged to the genus *Thermoanaerobacterium*. The level of 16S rRNA gene sequence similarity with other *Thermoanaerobacterium* species was 86.5–97.8 %, with the only moderately acidophilic member of this genus, *Thermoanaerobacterium aotearoense*, being one of its closest relatives. DNA–DNA hybridization with *T. aotearoense* showed 33 % relatedness. Thus, morphological (one polar flagellum) and physiological characteristics (lower pH limit of growth at pH^{20 °C} 3.2 compared with *T. aotearoense*) and 16S rRNA gene sequence analyses revealed that strain 761-119^T represents a novel species in the genus *Thermoanaerobacterium*, for which the name *Thermoanaerobacterium aciditolerans* sp. nov. is proposed, with the type strain 761-119^T (= DSM 16487^T = VKM B-2363^T).

Acidic hot environments are quite common throughout the world and include anthropogenic environments such as mines, coal and coal-refuse piles and self-heated compost heaps, as well as naturally heated volcanic habitats (Brock, 1986). Most of the known thermoacidophilic prokaryotes are either aerobic archaea or micro-organisms with different types of anaerobic respiration (Johnson, 1998; Wiegel & Canganella, 2001). Thermoacidophiles with a fermentative type of metabolism are few in number. Examples are the facultatively anaerobic archaea of the genus *Thermoplasma* (Segerer *et al.*, 1988) and the obligately anaerobic archaea of the genera *Acidilobus* (Prokofeva *et al.*, 2000) and *Caldisphaera* (Itoh *et al.*, 2003). The only anaerobic thermoacidophilic representative among the bacteria is

Thermoanaerobacterium aotearoense, a member of the *Firmicutes*, an obligately anaerobic heterotroph, a moderate thermophile and a moderate acidophile, which was isolated from a hot spring in New Zealand (Liu *et al.*, 1996). Molecular and microbial analysis of the diversity of anaerobic thermophiles active at low pH in enrichment cultures obtained from terrestrial and deep-sea sources have revealed the presence of members of the genera *Acidilobus*, *Thermoplasma*, *Thermoanaerobacter*, *Thermoanaerobacterium* and *Thermococcus* (Prokofeva *et al.*, 2005). Here, we report the detailed characterization of a novel thermoacidophilic bacterium belonging to the genus *Thermoanaerobacterium*, isolated from one of these enrichment cultures inoculated with water/sediment from the Uzon Caldera in Kamchatka (far-eastern Russia).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 761-119^T is AY350594.

For enrichment of thermoacidophilic micro-organisms, the following basal medium was used (g l⁻¹): NH₄Cl, 0.33; KCl,

0.33; KH_2PO_4 , 0.33; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.33; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.33; NaCl, 25.0; yeast extract, 0.1; trace elements (Balch *et al.*, 1979), 10 ml l^{-1} ; vitamins (Wolin *et al.*, 1963) 10 ml l^{-1} . Sucrose was added as substrate, at a final concentration of

2 g l^{-1} . The medium was prepared anaerobically and reduced by adding Na_2S (600 mg l^{-1}). The medium was dispensed in 10 ml portions in 15 ml Hungate tubes; CO_2 was used as the gas phase. The pH of the medium, measured at 20°C using a pH meter calibrated at 20°C , was adjusted using anoxic 3 M HCl . The pH and cultivation temperature used were approximately those from the sampling site. From the sample from Orange Field, Uzon Caldera, Kamchatka (coordinates $54^\circ 30.237' \text{ N } 160^\circ 00.038' \text{ E}$, pH 3.8, 48°C), an enrichment culture was obtained, growing at 60°C and pH $^{20^\circ\text{C}}$ 4.0. The dominating micro-organism from this culture was isolated from colonies from the highest positive dilution of the second round of serial dilutions into 1.5 % agar shakes. Isolated colonies were transferred to liquid medium. One of the isolated strains was strain 761-119^T, described below.

Cells of strain 761-119^T were spore-forming rods, $0.4 \mu\text{m}$ wide and $3\text{--}12 \mu\text{m}$ long (Fig. 1a, b). Cells were motile, with one polar flagellum, and Gram-positive (Fig. 1c). The isolate grew over a temperature range of $37\text{--}68^\circ\text{C}$, with an optimal growth temperature of 55°C . The pH $^{20^\circ\text{C}}$ range for growth was 3.2–7.1, with an optimum of pH $^{20^\circ\text{C}}$ 5.7. The doubling time under optimal conditions was 1 h. No growth was observed within 5 days of incubation at or below pH $^{20^\circ\text{C}}$ 2.8 or 35°C and at or above pH $^{20^\circ\text{C}}$ 7.5 or 70°C . After 2 days of growth at pH $^{20^\circ\text{C}}$ 7.0, a high degree of cell lysis occurred (after abundant growth), but not in medium with pH $^{20^\circ\text{C}}$ below 5.5. Growth of strain 761-119^T occurred at NaCl concentrations of 0–3 %. No growth was observed at 4 % NaCl. Isolate 761-119^T was able to ferment various mono- and disaccharides including glucose, fructose, xylose, ribose, arabinose, galactose, maltose, sucrose and lactose. It also grew on yeast extract, peptone, sorbitol, starch, xylan, gelatin and albumin. Glycerol, ethanol, pyruvate and citrate did not support growth. Products of glucose

Fig. 1. Light and electron micrographs of strain 761-119^T. (a) Phase-contrast micrograph of exponentially growing cells. Bar, $10 \mu\text{m}$. (b) Electron micrograph of negatively stained (Bonch-Osmolovskaya *et al.*, 1990), exponentially growing cells. Bar, $1 \mu\text{m}$. (c) Electron micrograph of a thin section (Bonch-Osmolovskaya *et al.*, 1990) of exponentially growing cells. Bar, $1 \mu\text{m}$.

Table 1. Characteristic features of strain 761-119^T and *Thermoanaerobacterium* species

Taxa: 1, *T. thermosulfurigenes* (data from Lee *et al.*, 1993); 2, *T. xylanolyticum* (Lee *et al.*, 1993); 3, *T. saccharolyticum* (Lee *et al.*, 1993); 4, *T. zeae* (Cann *et al.*, 2001); 5, *T. polysaccharolyticum* (Cann *et al.*, 2001); 6, *T. aotearoense* (Liu *et al.*, 1996; this study); 7, strain 761-119^T (this study). +, Positive; –, negative; NA, no data available; ND, not determined. All species utilize glucose, galactose and sucrose and form glucose, ethanol, acetate, H₂ and CO₂.

Characteristic	1	2	3	4	5	6	7
Spore formation	+	+	–	–	–	+	+
Flagellation	Peritrichous	Motile (no data on flagellation)	Peritrichous	Peritrichous	Flagellated (no data on type of flagellation)	Peritrichous	Single flagellum
Growth temperature (min./opt./max.) (°C)	55/60/75	45/60/70	45/60/68–70	NA/65–70/72	NA/65–68/72	35/60–63/66	37/55/68
Growth pH (min./opt./max.)	4.0/5.5–6.5/7.6	5.0/6.0/7.5	5.0/6.0/7.5	3.9/NA/7.9	5.0/6.8–7.0/8.0	3.8/5.2/6.8	3.2/5.7/7.1
Growth on:							
Maltose	+	+	+	NA	NA	+	+
Starch	+	NA	+	+	–	+	+
Xylan	+	+	+	+	–	+	+
Mannose	+	+	+	+	+	+	ND
Arabinose	+	+	NA	+	+	+	+
Lactose	–	+	+	+	+	+	+
Fructose	NA	+	+	+	+	+	ND
Citrate	NA	NA	NA	–	–	–	–
Pyruvate	–	+	NA	+	+	–	–
Pectin	+	NA	NA	–	–	+	ND
Xylose	+	NA	+	NA	NA	+	+
Ribose	NA	–	–	NA	NA	+	+
Sorbitol	NA	NA	–	NA	NA	NA	+
Glycerol	–	NA	NA	–	–	–	–
Ethanol	NA	NA	NA	NA	NA	–	–
Reduction of:							
S ⁰	NA	NA	NA	–	–	–	–
Thiosulfate (product)	+	+	+	–	+	+	+
Sulfite	Inhibits growth	NA	NA	–	–	NA	+
Sulfate	–	NA	NA	NA	NA	–	–
Inhibitory concentration of NaCl (%)	2	1	NA	NA	NA	NA	4
Growth products							
Formate	NA	NA	NA	+	+	–	ND
Lactate	+	–	+	+	+	+	+
DNA G+C content (mol%)	32.6	36.1	36	42	46	34.5–35	34 ± 0.5

fermentation (Bonch-Osmolovskaya & Miroshnichenko, 1994) were acetate, ethanol and lactate, at a ratio of 14.5:9:1, and H₂ and CO₂ (not quantified). Addition of thiosulfate (2 g l^{–1}) did not stimulate growth significantly; thiosulfate was reduced to molecular sulfur, which was deposited inside the cells. The addition of elemental sulfur (10 g l^{–1}) produced no stimulating effect; only trace amounts of hydrogen sulfide were detected. Sulfate (2 g l^{–1}) supplementation of the medium did not change the growth characteristics noticeably. Sulfate was not reduced; however, sulfite (6 mM) was reduced to hydrogen sulfide and slightly inhibited growth (cell yield was 3.7-fold lower), concomitantly causing a change in cell morphology

and in the ratio of fermentation products. The cells became much longer and formed long chains; the formation of ethanol increased 5.5-fold, from approximately 1.55 × 10^{–8} to 8.5 × 10^{–8} mmol per cell.

DNA was isolated according to the method of Marmur (1961). The G+C content of the DNA was 34 (±0.5) mol%, as measured by thermal denaturation of the DNA (Marmur & Doty, 1962).

The almost complete 16S rRNA gene sequence of strain 761-119^T (1441 nt corresponding to nt 11–1469 of *Escherichia coli* numbering) was determined as described

previously (Sokolova *et al.*, 2002). Comparison of this 16S rRNA gene sequence against the existing database using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast>) revealed that strain 761-119^T was a member of the large phylum *Firmicutes*, which includes the so-called low-G + C-content, Gram-positive bacteria. Within this subgroup, strain 761-119^T fell within the genus *Thermoanaerobacterium* in the family *Thermoanaerobacteriaceae* of the order *Thermoanaerobacteriales* of the class *Clostridia* (Garrity *et al.*, 2005). The 16S rRNA gene sequence similarity values between strain 761-119^T and the type strains of *Thermoanaerobacterium* species with validly published names were in the range 86.5–97.8%. The phylogenetic position of isolate 761-119^T was revealed by constructing a 16S rRNA gene sequence phylogenetic tree (Fig. 2), employing TREECON (Van De Peer & De Wachter, 1994) and using Jukes and Cantor corrections (Jukes & Cantor, 1969). This analysis showed that the 16S rRNA gene sequence of isolate 761-119^T had <97% similarity with those of all species of the genus *Thermoanaerobacterium* with one exception – the type strain of the most closely related species *T. aotearoense* (Liu *et al.*, 1996), with 97.8% sequence similarity. This correlation was also mirrored in a comparison of phenotypic characteristics of isolate 761-119^T and species of the genus *Thermoanaerobacterium* (Table 1). Although *T. aotearoense* and isolate 761-119^T used similar growth substrates and were both moderate thermoacidophiles, they differed in that strain 761-119^T had one polar flagellum and a lower pH limit of pH^{20°C} 3.2, while *T. aotearoense* had peritrichous flagellation and an acidic pH limit for growth of 3.8. DNA–DNA hybridization between strain 761-119^T and *T. aotearoense* was carried out using the method of Marmur (1961) and yielded 33% relatedness, indicating that the two strains belonged to different species. Thus, isolate 761-119^T is proposed to represent the type strain of a novel species, for which the name *Thermoanaerobacterium aciditolerans* sp. nov. is proposed.

Description of *Thermoanaerobacterium aciditolerans* sp. nov.

Thermoanaerobacterium aciditolerans (a.ci.di.tol'er.ans. N.L. neut. n. *acidum* an acid; L. pres. part. *tolerans* tolerating; N.L. part. adj. *aciditolerans* acid-tolerating).

Cells are rod-shaped, motile and spore-forming, 0.4 µm in diameter and 3–12 µm long, with Gram-positive cell walls and one flagellum. Obligate anaerobe. Moderate thermophile growing between 37 and 68 °C, with an optimum at 55 °C (no growth within 5 days at or below 35 °C and at or above 70 °C). Moderate acidophile growing in the pH^{20°C} range 3.2–7.1, with an optimum pH^{20°C} at 5.7 (no growth at or below pH^{20°C} 2.8 and at or above pH^{20°C} 7.5), and at NaCl concentrations of 0–3% (no growth at 4% NaCl). Grows by fermentation of glucose, maltose, fructose, sucrose, lactose, xylose, ribose, arabinose, galactose, yeast extract, sorbitol, starch, xylan, gelatin and albumin. Glycerol, ethanol, pyruvate and citrate are not utilized. Fermentation products are acetate, ethanol, lactate,

H₂ and CO₂. Thiosulfate is reduced to S⁰, which is deposited inside the cells. Sulfite is reduced to sulfide. The DNA G + C content is 34 (±0.5) mol% (thermal denaturation method).

The type strain is 761-119^T (=DSM 16487^T=VKM B-2363^T), isolated from a hydrothermal vent in the Orange Field, Uzon Caldera (Kamchatka, far-eastern Russia).

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