Fervidobacterium changbaicum sp. nov., a novel thermophilic anaerobic bacterium isolated from a hot spring of the Changbai Mountains, China

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A thermophilic, obligately anaerobic, rod-shaped bacterium (strain CBS-1^T) was isolated from a hot spring mixture of water and mud of the Changbai Mountains, China. Strain CBS-1^T was found to be non-sporulating, Gram-negative, with optimal growth at 75–80 °C. It grew on a wide range of carbon sources, including glucose, lactose, maltose, starch, sorbitol and pyruvate amongst others. The DNA G + C content of strain CBS-1^T was 31.9 mol%. The 16S rRNA gene sequence analysis indicated that the strain was a member of the genus *Fervidobacterium*. The high concentration of C_{16:0} (52.2%) in the fatty acid profile of the cell envelope supported its inclusion as a member of the genus *Fervidobacterium*. On the basis of the low values of DNA–DNA hybridization (25.8 and 20.5%) and phenotypic features, strain CBS-1^T represents a novel species of the genus *Fervidobacterium*, for which the name *Fervidobacterium changbaicum* sp. nov. is proposed. The type strain is CBS-1^T (=DSM 17883^T=JCM 13353^T).

Based on the morphological, physiological and biochemical characteristics, the genus *Fervidobacterium* was proposed in 1985 (Patel *et al.*, 1985). Currently, the genus comprises four species with validly published names: *Fervidobacterium nodosum* (Patel *et al.*, 1985), *Fervidobacterium islandicum* (Huber *et al.*, 1990), *Fervidobacterium gondwanense* (Andrews & Patel, 1996) and *Fervidobacterium pennivorans* (Friedrich & Antranikian, 1996). All species of the genus *Fervidobacterium* have been isolated from geothermal waters. The Changbai Mountains (42° N, 128° 1' E; 2189 m altitude) of China is a resting volcano, and possesses many hot springs with temperatures ranging from 60 to 83 °C. The high temperature, sulphur-enriched and remote geographical location makes these hot springs a new habitat for searching for thermophiles. To date, no thermophilic microorganism from these hot springs has been reported. In this study, we describe a novel thermophilic anaerobic bacterium isolated from a hot spring of the Changbai

Mountains, and propose a novel species, *Fervidobacterium changbaicum* sp. nov.

A sample mixture of water and mud (approx. 400 ml) was collected from the edge of a hot spring of the Changbai Mountains, China. The pH of the water was 7.2. The enrichment and isolation procedures were performed anaerobically at 80 °C in TYE medium with sulphur (1%, w/v) and Na₂S·9H₂O (0.05%, w/v). TYE medium contained (l⁻¹): (NH₄)₂SO₄ (1.3 g), KH₂PO₄ (0.28 g), MgSO₄·7H₂O (0.25 g), CaCl₂ (0.07 g), FeSO₄·7H₂O (0.028 g), MnCl₂ (1.8 mg), Na₂B₄O₇ (4.5 mg), ZnSO₄·7H₂O (0.22 mg), CuSO₄·2H₂O (0.05 mg), NaMoO₄·2H₂O (0.03 mg), CoCl₂ (0.01 mg), yeast extract (1 g), tryptone (2 g) and 0.1% (v/v) resazurin. The medium was adjusted to pH 7.0 with 1 M NaOH. Positive cultures were transferred to the same fresh medium at least three times. Purification was done using the end-point dilution method (Patel *et al.*, 1985) and Hungate roll tubes with TYE agar [3% (w/v) of agar] at 65 °C. A single colony was picked and cultivated. The purified strain was designated CBS-1^T.

F. islandicum DSM 5733^T and *F. pennivorans* DSM 9078^T purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were used as the reference strains for determination of

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CBS-1^T is AY878719.

An electron micrograph of cells of strain CBS-1^T, graphs showing the influence of temperature, pH and salt concentration on growth of cells of strain CBS-1^T and fatty acid compositions of strain CBS-1^T are available as supplementary material with the online version of this paper.

DNA G+C content and DNA hybridization, and anaerobically incubated at 65 °C (Huber *et al.*, 1990). The physiological tests were performed according to Bergey's manual (Buchanan *et al.*, 1974). Unless otherwise indicated, all physiological experiments were performed in triplicate. The optimal conditions for growth were determined in TYE medium. To determine the pH range for growth, TYE medium was adjusted from pH 4.0 to 10.0 by injecting different volumes (with 50 µl steps) of sterile 1 M HCl or 1 M NaOH. Cell morphology was examined by light microscopy Hitachi S-570. Substrate utilization was determined in the modified TYE medium without yeast extract, and the final concentration of substrates was 0.5 % (w/v). Antibiotics were added from filter-sterilized stock solutions to sterile TYE medium under anaerobic conditions to give a final concentration of 10 and 100 µg ml⁻¹.

Cellular fatty acid profile was studied as described previously (Nazina *et al.*, 2001). Genomic DNA was prepared by using the method of Marmur (1961) and the purity was checked spectrometrically. The G+C content of genomic DNA was determined by thermal denaturation (T_m) (Marmur & Doty, 1962). The 16S rRNA gene was amplified using 27f (5'-AGAGTTTGATCCTGGCTCAG-3') (Sakai *et al.*, 2003) and 1541r (5'-AAGGAGGTGATCCAGCCGCA-3') primers (Peña *et al.*, 2004). The almost-complete 16S rRNA gene sequence of strain CBS-1^T was determined and aligned with other 16S rRNA gene sequences of closely related strains, available in the GenBank database, using CLUSTAL W version 1.8 (Thompson *et al.*, 1994). The phylogenetic tree was constructed from a distance matrix based on the neighbour-joining method (Saitou & Nei, 1987) in the TREECON program (Van de Peer & De Wachter, 1995) (Fig. 1). DNA-DNA hybridization was performed by the thermal denaturation and renaturation method (De Ley *et al.*, 1970). Temperature of optimum renaturation was defined as the temperature 25 °C lower than the T_m value. The highest and lowest values obtained in each sample were excluded and the remaining three values were used to calculate similarity values. The DNA relatedness values quoted are the mean of the three values.

Cells of strain CBS-1^T stained Gram-negative, rod-shaped. Cell sizes were approximately 0.5–0.6 × 1.0–8.0 µm after 48 h anaerobic cultivation at 80 °C on TYE medium (Supplementary Fig. S1 available in IJSEM Online). All of the colonies appeared smooth, ivory-white round and were about 1 mm in diameter after 4 days of anaerobic cultivation at 65 °C on TYE agar medium. In TYE medium, no spore formation was observed at any temperature or pH values. Spheroid structure, the protuberances of the outer cell wall that consisted in the strains of the genus *Fervidobacterium*, was also observed in strain CBS-1^T after 24 h incubation at 75 °C (data not shown). In the presence of sulphur powder, the growth of strain CBS-1^T was stimulated and H₂S was generated, which is similar to other members of the genus *Fervidobacterium*. The substrates that supported the growth of strain CBS-1^T included glucose, lactose, D-fructose, sucrose, maltose, starch, sorbitol, cellobiose, trehalose dihydrate, D-galactose, meso-erythritol, dulcitol, chrysanthanol, melibiose, pyruvate, dextrin and glycerin, but D-raffinose, D-glucuronic acid, agarose, L-rhamnose, D-gluconic acid lactone, sodium malate, mannose, mannitol, L-arabinose and ribose do not support growth. Growth of strain CBS-1^T was completely inhibited by streptomycin, chloramphenicol, carbenicillin, kanamycin, ampicillin and tetracycline (10 µg ml⁻¹), and 100 µg neomycin ml⁻¹.

The G+C content of genomic DNA of strain CBS-1^T was 31.9 ± 0.3 mol% (T_m), and that of *F. islandicum* DSM 5733^T was determined as 41.3 mol% in our study when used as a control; however, a value of 40.0 mol% was reported previously (Huber *et al.*, 1990). The fatty acid profile of strain CBS-1^T was mainly composed of C_{16:0} (52.2 %), iso-C_{14:0} (11.7 %), C_{18:0} (6.4 %) and C_{18:1ω9c} (4.5 %) (Supplementary Table S1 available in IJSEM Online). For the composition of fatty acids, C_{16:0} (52.2 %) was the dominant fatty acid similar to *Fervidobacterium* species (46.7–81.8 %, Patel *et al.*, 1991; Huber *et al.*, 1990). However, there was considerable variation in the saturated and branched saturated fatty acid composition in *Fervidobacterium* species as described previously. The saturated fatty acid content of *F. nodosum*

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence showing the relationship of strain CBS-1^T to members of the family *Thermotogaceae*. The dendrogram was constructed by using the neighbour-joining method. Jukes–Cantor evolutionary distance matrix data was obtained from a comparison of 1300 unambiguous nucleotides as implemented in PHYLIP (Felsenstein, 1988). The sequences used in the analysis were obtained from the GenBank database. Bar, 1 substitution at any nucleotide position per 100 nucleotide positions.

ATCC 35602^T (87.1 %) and *F. islandicum* DSM 5733^T (86.7 %) was higher than that of *F. gondwanense* DSM 13020^T (78.5 %) and strain CBS-1^T (61.1 %). Branched saturated fatty acid content of strain CBS-1^T was 24.8 %, obviously different from *Fervidobacterium* species (not detected in *F. nodosum* ATCC 35602^T). The 16S rRNA gene sequence similarity between strain CBS-1^T and *Fervidobacterium* species was 93.0–97.5 %. Strain CBS-1^T was closer to *F. pennivorans* DSM 9078^T and *F. islandicum* DSM 5733^T (97.5 and 97.2 % sequence similarity, respectively). However, the DNA–DNA hybridization result also exhibited a very low level of similarity (25.8 and 20.5 % hybridization values, respectively) between strain CBS-1^T and the two relative species. This data suggests that strain CBS-1^T is not related at species level to *F. pennivorans* and *F. islandicum*. In addition, strain CBS-1^T showed significant physiological characteristic differences from *Fervidobacterium* species, including temperature and salinity for growth amongst others (Table 1). The growth temperature of strain CBS-1^T ranged from 55 to 90 °C, with the optimum growth temperature at 75–80 °C (Supplementary Fig. S2 available in IJSEM Online), while other species could barely grow at 80 °C, indicating that strain CBS-1^T was more thermophilic than the other four *Fervidobacterium* species. Furthermore, the genomic DNA of strain CBS-1^T exhibits the lowest G+C content of the genus *Fervidobacterium*.

Overall, our data indicate that strain CBS-1^T represents a novel species of the genus *Fervidobacterium*, for which the name *Fervidobacterium changbaicum* sp. nov. is proposed.

Description of *Fervidobacterium changbaicum* sp. nov.

Fervidobacterium changbaicum (chang.bai'cum. N.L. neut. adj. *changbaicum*, pertaining to the isolation of the organism from the Changbai Mountains, China).

Rod-shaped, non-sporulating, Gram-negative bacterium. Growth occurs at 55–90 °C (optimum 75–80 °C), at pH 6.3–8.5 (optimum 7.5), at NaCl concentration less than 10.0 g l⁻¹ and at MgCl₂ concentration less than 3.0 g l⁻¹ (optimum 0.5 g l⁻¹). Cell size is 0.5–0.6 × 1.0–8.0 µm. Can utilize glucose, lactose, D-fructose, sucrose, maltose, starch, sorbitol, cellobiose, trehalose dihydrate, D-galactose, *meso*-erythritol, dulcitol, chrysanthanol, melibiose, pyruvate, dextrin and glycerin, but not D-raffinose, D-glucuronic acid, agarose, L-rhamnose, D-gluconic acid lactone, sodium malate, mannose, mannitol, L-arabinose or ribose. Sulphur powder can stimulate growth. Growth is inhibited by streptomycin, chloramphenicol, carbenicillin, kanamycin, ampicillin and tetracycline (10 µg ml⁻¹), and 100 µg neomycin ml⁻¹. The fatty acid profile is mainly composed of C_{16:0} (52.2 %), iso-C_{14:0} (11.7 %), C_{18:0}

Table 1. Differential characteristics of members of the genus *Fervidobacterium*

Taxa: 1, *F. gondwanense* DSM 13020^T (Andrews & Patel, 1996); 2, *F. nodosum* ATCC 35602^T (Patel *et al.*, 1985); 3, *F. islandicum* DSM 5733^T (Huber *et al.*, 1990); 4, *F. pennivorans* DSM 9078^T (Friedrich & Antranikian, 1996); 5, *F. changbaicum* CBS-1^T (this study). +, Positive; –, negative; NR, not reported. All strains utilized glucose, starch and maltose.

Characteristic	1	2	3	4	5
Habitat	Geothermal artesian waters of Australia	Volcanic hot spring of New Zealand	Volcanic hot spring of Iceland	Hot spring of the Island San Miguel	Volcanic hot spring of China
Cell sizes (µm)	0.5–0.6 × 4.0–40	0.5 × 1.0–2.5	0.6 × 1.0–4.0	0.5 × 2.0–20.0	0.5–0.6 × 1.0–8.0
Temperature range (optimum) (°C)	45–80 (65–68)	47–80 (70)	50–80 (65)	30–80 (70)	55–90 (75–80)
pH range (optimum)	6.0–8.0 (7.0)	6.0–8.0 (7.0)	6.0–8.0 (7.2)	5.5–8.0 (6.5)	6.4–8.5 (7.5)
NaCl range (optimum) (%)	0.0–0.6 (0.1)	<1.0 (NR)	<1.0 (0.2)	0.0–4.0 (0.4)	0.0–1.0 (0.0)
Generation time (min)	79	105	150	126	99
Utilization of:					
Lactose	–	+	–	–	–
Dextrin	+	+	–	NR	+
Fructose	+	–	–	+	+
Xylose	+	–	–	+	–
Arabinose	–	+	+	–	–
Pyruvate	+	–	+	–	+
Galactose	+	+	–	NR	+
Cellulose	–	NR	+	NR	–
Raffinose	–	+	+	NR	–
DNA G+C content (mol%)	35.0	33.7	40.0	40.0	31.9

(6.4%) and C_{18:1ω9c} (4.5%). The G+C content of genomic DNA is 31.9±0.3 mol% (*T_m*).

The type strain, CBS-1^T (=DSM 17883^T=JCM 13353^T), was isolated from a hot spring mixture of water and mud of the Changbai Mountains, China.

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