Fervidobacterium thailandense sp. nov., an extremely thermophilic bacterium isolated from a hot spring

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Strain FC2004^T, a strictly anaerobic, extremely thermophilic heterotroph, was isolated from a hot spring in Thailand. Typical cells of strain FC2004^T were rod shaped (0.5–0.6×1.1–2.5 μm) with an outer membrane swelling out over an end. Filaments (10–30 μm long) and membrane-bound spheroids containing two or more cells inside (3–8 μm in diameter) were observed. The temperature range for growth was 60–88°C (optimum 78–80°C), pH range was 6.5–8.5 (optimum pH 7.5) and NaCl concentration range was 0 to <5 g l^{-1} (optimum 0.5 g l^{-1}). S^0 stimulated growth yield. $S_2O_3^{2-}$ and NO_3^- did not influence growth. Glucose, maltose, sucrose, fructose, cellobiose, CM-cellulose and starch were utilized for growth. The membrane was composed mainly of the saturated fatty acids $C_{16:0}$ and $C_{18:0}$. The DNA G+C content was 45.8 mol%. The 16S rRNA gene sequence of strain FC2004^T revealed highest similarity to species of the genus *Fervidobacterium*: *F. pennivorans* DSM 9078^T (97–96 %), *F. islandicum* AW-1 (96 %), *F. changbaicum* CBS-1^T (96 %), *F. islandicum* H21^T (95 %), *F. nodosum* Rt17-B1^T (95 %), *F. riparium* 1445t^T (95 %) and *F. gondwanense* AB39^T (93 %). Phylogenetic analysis of 16S rRNA gene sequences and average nucleotide identity analysis suggested that strain FC2004^T represented a novel species within the genus *Fervidobacterium*, for which the name *Fervidobacterium thailandense* sp. nov. is proposed. The type strain is FC2004^T (=JCM 18757^T=ATCC BAA-2483^T).

The phylum *Thermotogae* comprises divergent mesophilic, thermophilic and hyperthermophilic, obligately anaerobic, heterotrophic bacteria which all possess a characteristic outer sheath-like membranous structure, the so-called toga. Currently, members of this phylum have been reclassified into four orders containing five families: *Thermotogales* (*Thermotogaceae* and *Fervidobacteriaceae*), *Kosmotogales* (*Kosmotogaceae*), *Petrotogales* (*Petrotogaceae*), and *Mesoaciditogales* (*Mesoaciditogaceae*) (Reysenbach *et al.*, 2013; Bhandari & Gupta, 2014; Itoh *et al.*, 2016). The phylum *Thermotogae* is described unambiguously to date as non-spore-forming bacteria, although cells in stationary phase of *Pseudothermotoga subterranea* (or *Thermotoga subterranea*) as well as the golf-club-producing *Thermotoga* sp. strain PD524 were revealed as highly resistant to several hazardous chemicals and conditions comparing to cells in exponential phase (Jeanthon *et al.*, 1995; Kanoksilapatham *et al.*, 2015). Members of the genus *Fervidobacterium* (family *Fervidobacteriaceae*) share common morphological characteristics of

Abbreviations: ANI, average nucleotide identity; FAME, fatty acid methyl ester.

The GenBank/EMBL/DDBJ accession numbers for the prospective genome and 16S rRNA gene sequences reported in this study are LWAF01000000 and KJ473436, respectively.

Two supplementary tables and four supplementary figures are available with the online Supplementary Material.

short rigid rods harbouring a terminal balloon-like toga. Seven members of the genus, *Fervidobacterium nodosum* Rt17-B1^T (Patel *et al.*, 1985), *F. islandicum* H21^T (Huber *et al.*, 1990), *F. gondwanense* AB39^T (Andrews & Patel, 1996), *F. pennivorans* DSM 9078^T (Friedrich & Antranikian, 1996), *F. islandicum* AW-1 (Nam *et al.*, 2002), *F. changbaicum* CBS-1^T (Cai *et al.*, 2007) and *F. riparium* 1445t^T (Podosokorskaya *et al.*, 2011), have been described and were retrieved from diverse hot springs around the world. Recently, thermophilic and hyperthermophilic bacterial lineages belonging to the phylum *Thermotogae* were revealed thriving in hot spring ecosystems in northern Thailand (Cuecas *et al.*, 2014). In this study, a novel extremely thermophilic bacterium was isolated and characterized.

A sediment sample was collected at a geothermal hot spring (19° 57′ 59.60″ N 99° 9′ 21.53″ E) located in Mae Fang National Park, northern Thailand. Temperature at the sampling site was 90 °C. The medium used for isolation was 480G medium, containing (per litre): NaCl (0.5 g), NH_4Cl (0.33 g), $CaCl_2.2H_2O$ (0.15 g), $MgCl_2.6H_2O$ (0.35 g), KCl (0.3 g), KH_2PO_4 (0.3 g), pancreatic digestion of casein (1 g; Criterion), yeast extract (0.5 g; Criterion), A5 solution (1 ml), resazurin solution (0.5 ml of a 0.2 g l^{-1} solution) and 3 ml $Na_2S.9H_2O$ solution [25 % (w/v), pH 7]. The pH was adjusted to 7.2–7.5 using 1 M NaOH or 1 M HCl. A5 stock solution was composed of (per litre): $Co(NO_3)_2.6H_2O$ (0.00494 g), $CuSO_4.5H_2O$ (0.0079 g), H_3BO_3 (0.286 g), $MnCl_2.4H_2O$ (0.181 g), $Na_2MoO_4.2H_2O$ (0.039 g) and $ZnSO_4.7H_2O$ (0.0222 g). The isolation procedure was performed anaerobically at 80 °C in 480G medium amended with 1 % (w/v) S^0. Strain FC2004^T was obtained in pure culture using the end point dilution technique performed three consecutive times.

Cell morphology was examined using a phase-contrast microscope (Nikon eclipse 50i) and a scanning electron microscope (CamScanMX-2000). The Schaeffer–Fulton staining method was used with slight modifications. Briefly, cell pellets were fixed with an equal volume of 10 % glutaraldehyde for 20 min at room temperature. The fixed cells were smeared, air dried and heat fixed. The smear was stained with malachite green dye solution at 80 °C for 20 min. Dye solution was rinsed off with water and this was followed by counterstaining with safranin for 1 min. Growth kinetics with regard to temperature, NaCl concentration and pH were determined in 480G medium (triplicate bottles of 100 ml). Carbohydrate utilization was tested at 80 °C in a basal medium amended with 0.1 % (w/v) of the tested carbohydrate (in triplicate). The composition of the basal medium was similar to the 480G medium except that 0.1 g pancreatic digestion of casein l^{-1} and 0.05 g yeast extract l^{-1} were used. Cell numbers were enumerated using a direct count technique. At least a doubling of the cell density in the control (basal medium) was required to record the assay as positive. Effects of $S_2O_3^{2-}$ and NO_3^- (20 mM each) amended in 480G medium were studied as described previously (Kanoksilapatham *et al.*, 2015). The effect of S^0 on growth was tested in 480G medium containing 1 % (w/v) S^0. Sulfide production was determined from samples collected before and after incubation following the methylene blue method (Askew & Smith, 2005). Hydrolysis of keratin in native feathers was determined at 80 °C in a modified I-medium (Friedrich & Antranikian, 1996). The trace elements and vitamin solutions amended in the I-medium were replaced by NaCl (0.3 g l^{-1}) and A5 solution (1 ml l^{-1}).

Genomic DNA was purified by phenol/chloroform extraction. DNA was precipitated using cold absolute ethanol (−20 °C). RNA was digested using DNAse-free RNAse (10 mg ml^{-1} in TE buffer) at 37 °C for 1 h. The ethanol precipitation step was repeated. A genomic DNA G+C content of 44.0 mol% was estimated using the thermal denaturation method (Marmur *et al.*, 1962) and agreed with the G+C content value of 45.8 mol% from prospective genome sequences. Nucleotide sequences including the 16S rRNA gene of strain FC2004^T were obtained from prospective genome sequencing (accession number LWAF01000000) in a GS FLX Pyrosequencer (Roche). A phylogenetic tree was reconstructed using the program MEGA 6 (Tamura *et al.*,

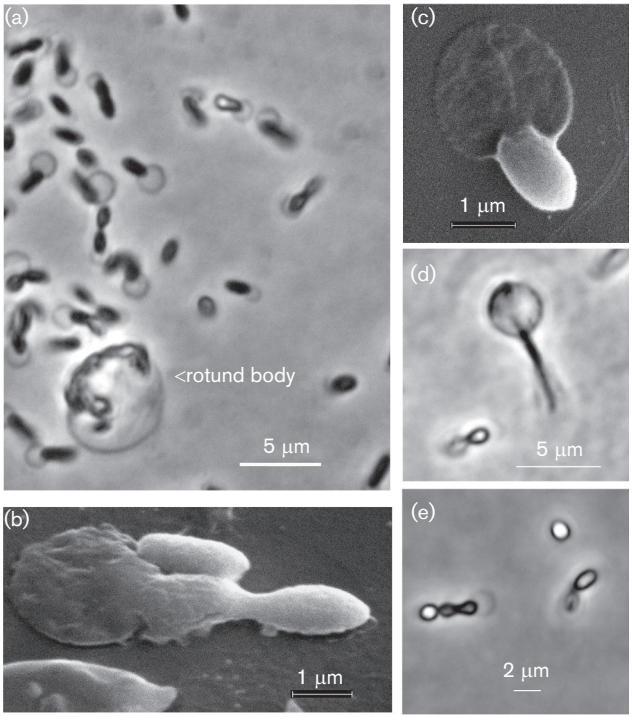

Fig. 1. Morphology of strain FC2004^T. (a) Phase-contrast micrograph revealing a typical rod-shaped cell with a characteristic toga at an end and a rotund body. Arrowhead indicates the rotund body. Bar, 5 μm. (b) Scanning electron micrograph showing a rugby-ball-shaped cell harbouring a large terminal sac-like toga. Bar, 1 μm. (c) Scanning electron micrograph showing a barrel-shaped cell harbouring a large terminal balloon-like toga. Bar, 1 μm. (d) Phase-contrast micrograph revealing a filament harbouring a balloon-like toga. Bar, 5 μm. (e) Phase-contrast micrograph showing a rod-shaped cell with a refractile structure. Bar, 2 μm.

Table 1. Characteristics of strain FC2004^T and the type strains of known species of the genus *Fervidobacterium*

Strains: 1, FC2004^T (this study); 2, *F. nodosum* Rt17-B1^T (Patel *et al.*, 1985); 3, *F. islandicum* H21^T (Huber *et al.*, 1990); 4, *F. gondwanense* AB39^T (Andrews & Patel, 1996); 5, *F. pennivorans* DSM 9078^T (Friedrich & Antranikian, 1996); 6, *F. changbaicum* CBS-1^T (Cai *et al.*, 2007); 7, *F. riparium* 1445t^T (Podosokorskaya *et al.*, 2011). +, Positive; −, negative; NR, not reported.

Characteristic	1	2	3	4	5	6	7
Isolation source	Fang Hot Spring, Thailand	Hot spring, New Zealand	Hot spring, Iceland	Geothermal artesian basin, Australia	Hot spring in the Azores Islands, Portuguese Republic	Hot spring, China	Kunashir Island, Russia
Cell size (μm)	0.5–0.6× 1.1–30	0.5–0.55 ×1–2.5	0.6×1–4	0.5–0.6×4–40	0.5×2–20	0.5–0.6×1–8	0.4–0.5× 1–3
Temperature range (°C)	60–88	47–80	50–80	>45 to <80	50–80	55–90	46–80
Optimum	78–80	70	65	65–68	70	75–80	65
pH range	6.0–8.5	6.0–8.0	6.0–8.0	6.0–8.0	5.5–8.0	6.3–8.5	5.7–9
Optimum	7.5–8	7.0	7.2	7.0	6.5	7.5	7.8
NaCl range (g l^{-1})	0–5	<10	<10	0–6	0–40	0–10	0–10
Optimum	0–1.0	NR	2.0	1.0	4.0	0	0
Generation time (min)	85	105	150	79	126	99	55
Utilization of:							
Glucose	+	+	+	+	+	+	+
Sucrose	+	+	NR	NR	NR	+	+
Maltose	+	+	+	+	+	+	+
Lactose	−	+	−*	+	−	+	−
Fructose	+	+	+*	+	+	+	+
Xylose	−	−	−*	+	+	−	+
Arabinose	−	+ (slow)	+*	−	−	−	−
Galactose	−	+	+*	+ (slow)	+*	+	NR
Mannose	−	+	+*	+	+*	−	NR
Sorbitol	−	+	NR	NR	NR	+	NR
Mannitol	−	NR	NR	NR	NR	−	NR
Trehalose	−	NR	NR	NR	NR	+	NR
Starch	+	+	+	+	+	+	+
Cellobiose	+	NR	NR	+	NR	+	+
CM-cellulose	+	NR	NR	+ (slow)	NR	NR	+
Cellulose	−	−	+	−	−*	−	+
Feather hydrolysis	+	−*	−*	−*	+	NR	NR
DNA G+C content (mol%)	45.8†	33.7	41.0	35.0	40.0	31.9	31

*Data from Nam *et al.* (2002).
†Calculated from prospective genome sequencing (accession number LWAF01000000).

2013). Nucleotide sequences were aligned with the corresponding genes of *Fervidobacterium nodosum* Rt17-B1^T (NC_009718) and *F. pennivorans* DSM 9078^T (NC_017095) using CLUSTALW (Thompson *et al.*, 1994). Average nucleotide identity (ANI) analyses were performed using the ANI Calculator (http://enve-omics.ce.gatech.edu/ani/) according to Goris *et al.* (2007).

Analysis of the fatty acid composition of the cell membrane of strain FC2004^T grown in 480G medium (80 °C) was done using the Sherlock Microbial Identification System (MIS) (Sasser, 1990). Organic acids released to the culture medium during growth of strain FC2004^T in 480G medium were converted to fatty acid methyl esters (FAMEs) by a transesterification reaction using sodium metoxide (0.5 % in methanol). The FAMEs were analysed by GC on a Hewlett Packard 5890 Series II chromatograph equipped with a flame ionization detector. Separation was carried out in an SGE column BPX70 (10 m length, 0.1 mm internal diameter, 0.2 μm particle size) using H_2 as the carrier gas. The injector and detector temperatures were 250 and 270 °C, respectively, and a temperature gradient from 50 to 250 °C was established for 45 min. FAMEs were identified by comparison of retention times with known standards.

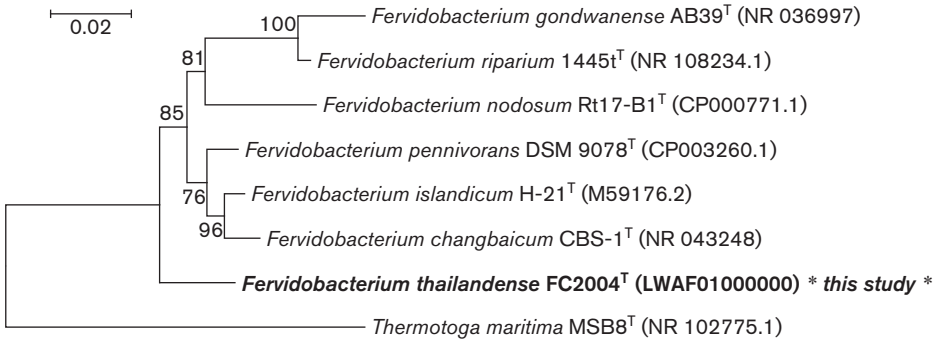

Fig. 2. Neighbour-joining tree of 16S rRNA gene sequences of members of the family *Fervidobacteriaceae* showing the relationship between strain FC2004^T and species belonging to genus *Fervidobacterium*. The 16S rRNA gene sequence of *Thermotoga maritima* MSB8^T was employed as an outgroup lineage. Bootstrap values as a percentage of 1000 replications are presented. Bar, 0.02 changes per nucleotide position.

Typical rods (0.5–0.6×1.1–2.5 μm) of strain FC2004^T were encapsulated by an outer membranous toga, a characteristic structure ballooning over one end (Fig. 1a). Two distinctive forms of the rods were revealed: rugby-ball- and barrel-shaped rods harbouring a terminal spheroid (Fig. 1b, c). Filaments (>10 to 30 μm long) were observed (Fig. 1d). Rotund bodies (diameter range 3–8 μm) or membrane-bound spheroids containing as many as 10 cells or more inside were detectable (Fig. 1a). Under a phase-contrast microscope, a refractile oval body surrounded by a thick coat (Fig. 1e), herein named 'refractile structure', was identified at a central position of some cells in stationary phase. Stationary phase cells were observed by staining with malachite green dye using the Schaeffer–Fulton staining method (Fig. S1, available in the online Supplementary Material). Strain FC2004^T was able to degrade a fragment of feathers (Fig. S2). This phenotype is similar to *F. pennivorans* DSM 9078^T and *F. islandicum* AW-1 (Friedrich & Antranikian, 1996; Nam *et al.*, 2002). For strain FC2004^T the temperature range for growth was 60–88 °C (optimum 80 °C), pH range was 6.5–8.5 (optimum pH 7.5) and NaCl concentration range was 0–5 g l^{-1} (optimum 0.5 g l^{-1}). No growth was detected at 90 °C (Fig. S3). Unlike the other related species, strain FC2004^T failed to grow at temperatures ≤55 °C (Table 1). At the optimal growth conditions, a specific growth rate constant of 0.49 h^{-1} was estimated.

Growth of strain FC2004^T was observed on glucose, maltose, sucrose, fructose, cellobiose, CM-cellulose and starch. No growth was detected on lactose, galactose, trehalose, arabinose, mannose, xylose, sorbitol, mannitol or cellulose (Table 1). Like the other described species within the genus *Fervidobacterium*, strain FC2004^T fermented glucose, maltose and fructose. Unlike *F. islandicum* H21^T and *F. riparium* 1445t^T, strain FC2004^{T}did not utilize cellulose. However, strain FC2004^T utilized CM-cellulose. Growth of strain FC2004^T in 480G medium ($3.7\times10^7\pm4.3\times10^6$ cells ml^{-1}) was determined. Growth yield of strain FC2004^T ($2.5\times10^8\pm1.9\times10^7$ cells ml^{-1}) was increased in S^0-containing 480G medium (Fig. S4). Addition of $S_2O_3^{2-}$ ($3.7\times10^7\pm3.2\times10^6$ cells ml^{-1}) and NO_3^- ($3.9\times10^7\pm2.6\times10^6$ cells ml^{-1}) did not influence growth yields. Sulfide formation was detected in both 480G and S^0-containing 480G media.

Membrane fatty acids of strain FC2004^T were extracted from cells in mid-exponential phase grown in 480G medium. Its composition included mainly $C_{12:0}$ (2.8 %), $C_{14:0}$ (7.2 %), $C_{16:0}$ (71.6 %) and $C_{18:0}$ (10.7 %) fatty acids. Small amounts of unsaturated fatty acids $C_{16:1}$ (0.4 %) and $C_{18:1}$ (2.4 %) were detectable. Approximately 2.4 % branch chain fatty acids, including iso-$C_{15:0}$ (0.3 %), iso-$C_{16:0}$ (0.8 %), iso-$C_{17:0}$ (0.3 %), anteiso-$C_{17:0}$ (0.3 %) and iso-$C_{18:0}$ (0.7 %), were identified. Low proportions of short-chain fatty acids (1.3 % $C_{9:0}$ and $C_{10:0}$) were observed (Table S1). Organic acids released to the culture medium by strain FC2004^T growing exponentially in 480G medium at 80 °C were those with five, nine, 10, 11 and 12 carbon atoms.

16S rRNA gene sequence analysis of strain FC2004^T via the BLASTN program revealed highest similarity to species of the genus *Fervidobacterium*: *F. pennivorans* DSM 9078^T (97–96 %), *F. islandicum* AW-1 (96 %), *F. changbaicum* CBS-1^T (96 %), *F. islandicum* H21^T (95 %), *F. nodosum* Rt17-B1^T (95 %), *F. riparium* 1445t^T (95 %) and *F. gondwanense* AB39^T (93 %). Phylogenetic analysis suggests strain FC2004^T as the deepest branch in the clade formed by the genus *Fervidobacterium* (Fig. 2). ANI analyses between strain FC2004^T and *Fervidobacterium* species revealed nucleotide identity well below the threshold for a single species (Goris *et al.*, 2007): *F. nodosum* (mean±SD, 78.24 ±7.75 %), *F. pennivorans* (79.03±8.78 %) and *F. islandicum* (78.88±8.87 %) (Table S2). These results support the suggestion that strain FC2004^T represents a novel species within the genus *Fervidobacterium*, for which the name *Fervidobacterium thailandense* sp. nov. is proposed.

Description of *Fervidobacterium thailandense* sp. nov.

Fervidobacterium thailandense (thai.land.en′se. N.L. neut. adj. *thailandense* pertaining to Thailand, the country where the type strain was isolated).

Cells are rugby-ball- and barrel-shaped rods (0.5–0.6×1.1–2.5 μm) with an outer membranous sheath-like toga, protruding to form a balloon-like structure over an end. Filaments (10–30 μm long) and rotund bodies (3–8 μm in diameter) are detectable. Produces a refractile structure that appears (under phase contrast microscopy) as an encased bright oval body at a central position. Some oval-shaped cells seen with malachite green dye staining can be detected during stationary phase. Extremely thermophilic. Grows at 60–88 °C (optimum 80 °C), at pH 6.0–8.5 (optimum pH 7.5) and with 0–5 g NaCl l^{-1} (optimum 0.5 g l^{-1}). Obligately anaerobic organotroph that ferments proteins and several carbohydrates including glucose, maltose, sucrose, fructose, cellobiose, CM-cellulose and starch. However, does not ferment lactose, galactose, trehalose, arabinose, mannose, xylose, sorbitol, mannitol or cellulose. Elemental sulfur stimulates growth. Thiosulfate and nitrate do not influence growth.

The type strain, FC2004^T (=JCM 18757^T=ATCC BAA-2483^T), was isolated from sediment collected at a hot spring in northern Thailand. The genomic DNA G+C content of the type strain is 45.8 mol%.

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