Fervidobacterium thailandense sp. nov., a novel extreme thermophilic bacterium isolated from a hot spring in Northern Thailand

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Abstract

Strain FC2004^T, a strictly anaerobic, extreme thermophilic heterotroph was isolated from a hot spring in Thailand. Typical cells of strain FC2004^T are rod shaped (0.5 – 0.6 x 1.1 – 2.5 μm) with an outer membrane swelling out over an end. Filaments (10 – 30 μm-long) and membrane bound spheroids containing ≥2 cells inside (3 – 8 μm-diameter) were observed. Temperature range for growth was 60 to 88 °C (optimum temperature 78 – 80 °C), pH range was 6.5 to 8.5 (optimum pH 7.5), and the growth range for NaCl concentration was 0 to <5 g/L (optimum concentration 0.5 g/L). S^o stimulated growth yield. $S_2O_3^{-2}$ and NO_3^- did not influence growth. Glucose, maltose, sucrose, fructose, cellobiose, carboxymethyl cellulose and starch were utilized for growth. Membrane was composed mainly of the saturated fatty acids $C_{16:0}$ (71.6%) and $C_{18:0}$ (10.7%). 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* H-21^T (95%), *F. nodosum* Rt17-B1^T (95%), *F. riparium* 21445t^T (95%) and *F. gondwanense* AB39^T (93%). Phylogenetic analysis of *16S rRNA* gene sequences and ANI analysis suggest strain FC2004^T as a novel species within the genus *Fervidobacterium*, The name *Fervidobacterium thailandense* sp. nov. is proposed. The type strain is FC2004^T, which is equivalent to *Fervidobacterium* sp. strain FC2004 (JCM 18757) or *Fervidobacterium thailandense* strain FC 2004 (ATCC BAA-2483).

Keywords: *Fervidobacterium thailandens*e, extreme thermophile, hot springs, Thermotogales, Thermotogae

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 4 orders containing 5 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 formers, although cells in stationary phase of *Pseudothermotoga subterranea* (or *Thermotoga subterranea*) as well as the golf club producing *Thermotoga* sp. strain PD524 were revealed highly resistant to several hazardous chemicals and conditions (Jeanthon *et al*., 1995; Kanoksilapatham *et al.,* 2015). Members of the genus *Fervidobacterium* (belonging to fam. Fervidobacteriaceae) share common morphological characteristics of short rigid rods harboring a terminal balloon-like toga. Seven described members including *Fervidobacterium nodosum* Rt17-B1^T (Patel *et al*., 1985), *F. islandicum* H-21^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 extreme thermophilic bacterium was isolated and characterized.

A sediment sample was collected at a geothermal hot spring (N19°57'59.60" E99°9' 21.53") located in Mae Fang National Park, Northern Thailand. Temperature at the sampling site was 90 ºC. The 480G medium contains (per L): NaCl (0.5 g), NH_4Cl (0.33 g), $CaCl_2{\cdot}2H_2O$ (0.15 g), $MgCl_2{\cdot}6H_2O$ (0.35 g), KCl (0.3 g), KH_2PO_4 (0.3 g), pancreatic digestion of casein (1 g) (Criterion, CA, USA), yeast extract (0.5 g) (Criterion, CA, USA), A5 solution (1 mL), resazurin solution (0.5 mL of 0.2 g/L solution) and 3 mL $Na_2S{\cdot}9H_2O$ solution [25% (w/v), pH 7]. The pH was adjusted to 7.2-7.5 using 1N NaOH or 1N HCl. A5 stock solution (per L) was composed of $Co(NO_3)_2{\cdot}6H_2O$ (0.00494 g), $CuSO_4{\cdot}5H_2O$ (0.0079

68g), H_3BO_3 (0.286 g), $MnCl_2 \cdot 4H_2O$ (0.181 g), $Na_2MoO_4 \cdot 2H_2O$ (0.039 g) and $ZnSO_4 \cdot 7H_2O$ (0.0222 g).
69The isolation procedure was performed anaerobically at 80 ºC in 480G medium amended with 1 %
70(w/v) S^o. Strain FC2004^T was obtained in pure culture using end point dilution technique performed
71three consecutive times. Cell morphology was examined using a phase-contrast microscope (Nikon
72eclipse 50i) and a scanning electron microscope (CamScanMX-2000). The Schaeffer–Fulton staining
73method was employed with slight modifications. Briefly, cell pellets were fixed with equal volume of
7410% glutaraldehyde for 20 min at room temperature. The fixed cells were smeared, air dried and heat
75fixed. The smear was stained with malachite green dye solution at 80 ºC for 20 min. Dye solution was
76rinsed off with water and counter staining with safranin for 1 min. Growth kinetics on the
77temperature, NaCl concentration and pH were determined in 480G medium (triplicate bottles of 100
78ml). Carbohydrate utilization was tested at 80 ºC in a basal medium amended with 0.1 % (w/v) of the
79tested carbohydrate (in triplicates). Composition of the basal medium is similar to the 480G medium
80except that 0.1 g/L of pancreatic digestion of casein and 0.05 g/L of yeast extract were employed. Cell
81numbers were enumerated using direct count technique. At least doubling the cell density in the
82control (basal medium) was required to record the assay as positive. Effects of $S_2O_3^{-2}$ and NO_3^- (20
83mM ea.) amended in 480G medium were studied as described previously (Kanoksilapatham *et al*.,
842015). The effect of S^o on growth was tested in 1 % (w/v) S^o containing 480G medium. Sulfide
85production was determined from samples collected before and after incubation following the
86methylene blue method (Askew & Smith**,** 2005). Hydrolysis of keratin in native feathers was
87determined at 80 ºC in a modified I-medium (Friedrich & Antranikian, 1996). The trace elements and
88vitamin solutions amended in the I-medium were replaced by NaCl (0.3 g/L) and A5 solution (1
89mL/L). Genomic DNA was purified by phenol-chloroform extraction. DNA was precipitated using
90cold absolute ethanol (-20 °C). RNA was digested using DNAse-free RNAse (10 mg/ml in TE buffer)
91at 37°C for one hour. The ethanol precipitation step was repeated. A genomic G+C content of 44.0
92mol% was estimated using thermal denaturation method (Marmur & Doty, 1962) and agreed with the
93G+C content value of 45.8 mol% from prospective genome sequences. Nucleotide sequences
94including the *16S rRNA* gene of strain FC2004^T were obtained from prospective genome sequencing
95(Accession Number LWAF01000000) in a GS FLX Pyrosequencer (Roche, Basel, Switzerland). A

phylogenetic tree was constructed using the program MEGA 6 (Tamura *et al.,* 2013). Average nucleotide identity (ANI) analyses were performed using the ANI Calculator (http://ww.enve-omics.ce.gatech.edu/ani/) according to Goris *et al*. (2007). Analysis of fatty acid composition of membrane of strain FC2004^T growing in 480G medium (80 ºC) was identified 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 analyzed by gas chromatography on a Hewlett-Packard 5890 Series II (Hewlett-Packard, Avondale, EEUU) equipped with a flame ionization detector. Separation was carried out in a SGE column BPX70 (10 m length, 0.1 mm internal diameter, 0.2 um particle size) using H_2 as carrier gas. The injector and detector temperatures were 250 ºC and 270 ºC and a temperature gradient from 50 ºC to 250 ºC was established for 45 min. FAMEs were identified by comparison of retention times with known standards.

Typical rods (0.5 – 0.6 x 1.1 – 2.5 µm) of strain FC2004^T were encapsulated by an outer membranous toga, a characteristic structure ballooning over an end (Fig. 1(a)). Two distinctive forms of the rods were revealed; a rugby and a barrel shaped rods harboring a terminal spheroid (Fig. 1(b), (c)). Filaments (>10 to 30 µm long) were observed (Fig. 1(d)). Rotund bodies (diameter range of 3 to 8 µm) or membrane bound spheroids containing as many as 10 cells or more inside were detectable (Fig. 1(a)). Under a phase-contrast microscope, a refractile oval body surrounded by a thick coat appearance (Fig. 1(e)), named in this study “refractile structure”, was identified at central position of some particular cells in stationary phase. Some cells from stationary phase were stained by malachite green dye using Schaeffer–Fulton staining method (Supplementary Fig. S1). Strain FC2004^T was able to degrade fragment of feathers (Supplementary 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 to 88 ºC (optimum temperature 80 ºC), pH range was 6.5 to 8.5 (optimum pH 7.5), and NaCl concentration range was 0 to <5 g/L (optimum concentration 0.5 g/L). No growth was detected at 90 ºC (Supplementary Fig. S3). Unlike the other

related species, strain FC2004^T failed to grow at temperatures ≤55 ºC (Table 1). At the optimal growth condition, 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, carboxymethyl cellulose and starch. No growth was detected on lactose, galactose, trehalose, arabinose, mannose, xylose, sorbitol, mannitol and cellulose (Table 1). Like the other described species within the genus, strain FC2004^T ferments glucose, maltose and fructose. Unlike *F. islandicum* H21^T and *F. riparium* 1445t^T, strain FC2004 does not utilize cellulose. However, strain FC2004^T utilizes carboxymethyl cellulose. Growth of strain FC2004^T in 480G medium (3.7 x 10^7 ± 4.3 x 10^6 cells/mL) was determined. Growth yield of strain FC2004^T (2.5 x 10^8 ± 1.9 x 10^7 cells/mL) was increased in S^o containing 480G medium (Supplementary Fig. S4). Addition of $S_2O_3^{-2}$ (3.7 x 10^7 ± 3.2 x 10^6 cells/mL) and NO_3^- (3.9 x 10^7 ± 2.6 x 10^6 cells/mL) did not influence growth yields. Sulfide formation was detected in both 480G and S^o containing 480G media.

Membrane fatty acids of strain FC2004^T were extracted from cells in mid-exponential phase growing 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 amount 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 (Supplementary Table S1). Organic acids released to the culture medium by strain FC2004^T growing exponentially in 480G medium at 80ºC were those with 5, 9, 10, 11 and 12 carbon atoms. BlastN analysis of *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* H-21^T (95%), *F. nodosum* Rt17-B1^T (95%), *F. riparium* 1445t^T (95%) and *F. gondwanense* AB39^T (93%). Phylogenetic analysis suggests the strain FC2004^T as the deepest branch in the clade formed by *Fervidobacterium* (Fig. 2). ANI analyses resulted in average percentages of nucleotide identity between strain FC2004 and the *Fervidobacterium* species well below the threshold for a single species (Goris *et al.* 2007), *F. nodosum* (78.24%, sd 7.75%), *F. pennivorans* (79.03%, sd 8.78%) and

F. islandicum (78.88%, sd 8.87%) (Supplementary Table S2). These results support the proposal of strain FC2004^T as a novel species within the genus *Fervidobacterium*, for which the name *Fervidobacterium thailandense* 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 of strain FC2004^T are rugby and barrel shaped rods (0.5 – 0.6 x 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 to 8 μm-diameter) can be detectable. Strain FC2004^T produces a refractile structure that appears (under phase-contrast microscope) as an encased bright oval body at central position. Some oval shaped cells staining with malachite green dye can be detected during stationary phase. Extreme thermophile grows at 60 to 88 ºC (optimum temperature 80 ºC), pH 6.0 to 8.5 (optimum pH 7.5) and 0 to <5 g/L NaCl (optimum concentration 0.5 g/L). Obligately anaerobic organotroph ferments proteins and several carbohydrates including glucose, maltose, sucrose, fructose, cellobiose, carboxymethyl cellulose and starch. However, the strain FC2004^T does not ferment lactose, galactose, trehalose, arabinose, mannose, xylose, sorbitol, mannitol and cellulose. Elemental sulfur stimulated growth. Thiosulfate and nitrate do not influence growth. The G+C content of genome is 45.8 mol%. The type strain FC2004^T (JCM 18757 or ATCC BAA-2483) was isolated from a sediment collected at a hot spring in Northern Thailand.

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

239 *Fervidobacterium*.

Organisms	1 (FC2004^T)	2 (Rt17-B1^T)	3 (H21^T)	4 (AB39^T)	5 (DSM9078^T)	6 (CBS-1^T)	7 (1445t^T)
Habitats	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 sizes (µm)	0.5 – 0.6 x 1.1 – 30	0.5-0.55 x 1 – 2.5	0.6 x 1 – 4	0.5 – 0.6 x 4 – 40	0.5 x 2 – 20	0.5 – 0.6 x 1 – 8	0.4 – 0.5 x 1 – 3
Temp range (optimum) (ºC)	60 to 88 (78 – 80)	47 to 80 (70)	50 to 80 (65)	>45 to <80 (65 – 68)	50 to 80 (70)	55 to 90 (75 – 80)	46 to 80 (65)
pH range (optimum)	6.0 to 8.5 (7.5-8)	6.0 to 8.0 (7.0)	6.0 to 8.0 (7.2)	6.0 to 8.0 (7.0)	5.5 to 8.0 (6.5)	6.3 to 8.5 (7.5)	5.7 to 9 (7.8)
NaCl range (optimum) (g/L)	0 to <5 (0-1.0)	<10 (nr)	<10 (2.0)	0 to 6 (1.0)	0 to 40 (4.0)	0 to 10 (0.0)	0 to 10 (0)
Generation time (min)	85	105	150	79	126	99	55
Utilization of							
Glucose	+	+	+	+	+	+	+
Sucrose	+	+	nr	nr	nr	+	+
Maltose	+	+	+	+	+	+	+
Lactose	-	+	-[b]	+	-	+	-
Fructose	+	+	+[b]	+	+	+	+
Xylose	-	-	-[b]	+	+	-	+
Arabinose	-	+(slow)	+[b]	-	-	-	-
Galactose	-	+	+[b]	+(slow)	+[b]	+	nr
Mannose	-	+	+[b]	+	+[b]	-	nr
Sorbitol	-	+	nr	nr	nr	+	nr
Mannitol	-	nr	nr	nr	nr	-	nr
Trehalose	-	nr	nr	nr	nr	+	nr
Starch	+	+	+	+	+	+	+
Cellobiose	+	nr	nr	+	nr	+	+
Carboxymethyl cellulose	+	nr	nr	+(slow)	nr	nr	+
Cellulose	-	-	+	-	-[b]	-	+
Feather hydrolysis	+	-[b]	-[b]	-[b]	+	nr	nr
G+C content (mol%)	45.8[a]	33.7	41.0	35.0	40.0	31.9	31

Taxa: 1, *Fervidobacterium thailandense* 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* DSM9078^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.

[a] Calculated from prospective genome sequencing (Accession Number LWAF01000000).
[b] Data from Nam *et al.* (2002).

240**Figure legends**

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242**Fig. 1** Morphology of strain FC2004^T. **(a)** A phase-contrast micrograph revealing a typical rod shaped 243cell with a characteristic toga at an end and a rotund body. Arrow head indicates the rotund body. 244Scale indicates 5 μm. (**b)** A SEM micrograph showing rugby shaped cell harboring a large terminal 245sac-like toga. Scale indicates 1 μm. **(c)** A SEM micrograph presenting a barrel shaped cell harboring a 246large terminal balloon-like toga. Scale indicates 1 μm**. (d)** A phase-contrast micrograph reveals a 247filament harboring a balloon-like toga. Scale indicates 5 μm. **(e)** A phase-contrast micrograph showing 248a rod shaped cell with a refractile structure. Scale indicates 2 μm.

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250**Fig. 2** Neighbor joining tree of *16S rRNA* gene sequences of family Fervidobacteriaceae showing the 251relationship of strain FC2004^T to the 6 described species belonging to genus *Fervidobacterium*. *16S* 252*rRNA* sequence of *Thermotoga maritima* MSB8^T was employed as an outgroup lineage. A bootstrap 253value of 1000 is presented as percentage.

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