Research Article

Isolation of *Thermotoga* spp. and *Fervidobacterium* spp., and Characterization of *16S rRNA* Genes of Order Thermotogales: Unique Lineage of Hyperthermophiles Thriving in 3 Hot Springs in Thailand

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Abstract

Order Thermotogales comprise mostly strictly anaerobic thermophilic and hyperthermophilic bacteria which thrive in high temperature habitats throughout the world. In order to study diversity of hyperthermophiles in Thailand's hot springs, culture dependent technique was performed at 80 °C. Pure cultures of 19 isolates obtained from 3 hot springs were characterized based on *16S rRNA* genes of order Thermotogales. Twelve and 7 isolates were identified as deepest blanches within two clades formed by divergent encapsulated rod shaped bacteria belonging to *Fervidobacterium* and *Thermotoga*, respectively. Phylogenetic evidences suggest that the Thailand strains from both branches are possible candidates for two novel species. Closely related strains within both genera were identified regardless of their habitats and years of sample collection, implying that these native inhabitants found in Thailand's hot springs might have evolved from a unique common ancestor. Because all isolates require extremely high temperatures for growth, these isolates are likely sources of genes encoding numerous thermostable enzymes.

***Key Words*:** *Fervidobacterium*; Hot spring; *Hyperthermophile*; *Thermotoga*; Thermotogales

Introduction

Members of order Thermotogales include divergent thermophilic and hyperthermophilic bacterial lineages which possess a characteristic outer sheath-like structure, the so-called toga. Most members of this order thrive in diverse geothermal environments ranging from hot springs, subterranean, off shore oil fields to complex shallow and deep marine hydrothermal ecosystems. Most thermophilies and barophiles which require high hydrostatic pressure for growths, *Marinitoga* spp. and *Thermosipho* spp. were found associated tightly with deep sea hydrothermal vents (Huber et al., 1989; Antoine et al., 1997; Takai and Horikoshi, 2000; L'Haridon et al., 2001; Wery et al., 2001; Alain et al., 2002; Miranda-Tello et al., 2004; Postec et al., 2005; Nunoura et al., 2007). Species assigned to *Fervidobacterium* are mostly isolated from freshwater hot springs and are sensitive to low NaCl concentrations (Patel et al., 1985; Huber et al., 1990; Andrews and Patel, 1996; Friedrich and Antranikian, 1996; Nam et al., 2002; Cai et al., 2007; Podosokorskaya et al., 2011). Described species of *Petrotoga*, *Geotoga* spp. and *Oceanotoga teriensis* were discovered only on land and offshore petroleum production sites (Davey et al., 1993; Lien et al., 1998; Miranda-Tello et al., 2004; Miranda-Tello et al., 2007; Jayasinghearachchi and Lal, 2011). On the other hand, *Thermotoga* were found dispersing in diverse geothermal ecosystems including petroleum reservoirs (*T. petrophila*, *T. naphthophila*, *T. elfii*, *T. subterranean* and *T. hypogea*), marine hydrothermal vents (*T. maritima* MSB8^T and *T. neapolitana* NES^T),

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solfataric hot springs (*T. profunda*, *T. caldifontis* and *T. thermarum*) and an anaerobic bioreactor (*T. lettingae*, a possible contaminant) (Huber et al., 1986; Jannasch et al., 1988; Windberger et al., 1989; Jeanthon et al., 1995; Ravot et al., 1995; Duckworth et al., 1996; Fardeau et al., 1997; Takahata et al., 2001; Balk et al., 2002; Mori et al., 2014). *Kosmotoga olearia* (DiPippo et al., 2009) is a moderate thermophile (opt. temp. 65 °C), and able to grow in a broad temperature range (20-80 °C) that can support the growth of most mesophiles and hyperthermophiles. Recently, a true mesophile (opt. temp. 37 °C), *Mesotoga prima* MesG1.Ag.4.2^T, was isolated (Nesbø et al., 2012). However, a close relative *Mesotoga infera* which grows optimally at 45 °C is considered a moderate thermophile (Ben Hania et al., 2013).

In this study, pure cultures of hyperthermophiles were isolated from 3 different hot springs located in Northern Thailand. Diversity of the isolates was studied with respect to their *16S rRNA* genes and habitats. Phylogenetic relationships within order Thermotogales were examined. Thresholds for differential species and genera were estimated.

Materials and methods

Media

Isolation and enrichment media were 480G, 480GM5 and YE5. A liter of 480GM5 medium (an isolation medium) was composed of 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 (5 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 7.0]. pH was adjusted to 7.2 to 7.5 at room temperature using 1N NaOH or 1N HCl before sterilization. The medium was prepared anaerobically in serum bottles under a 1 atm N_2 headspace. The A5 stock solution was composed of $Co(NO_3)_2{\cdot}6H_2O$ (0.00494 g), $CuSO_4{\cdot}5\ H_2O$ (0.0079 g), H_3BO_3 (0.286 g), $MnCl_2{\cdot}4\ H_2O$ (0.181 g), $Na_2MoO_4{\cdot}2H_2O$ (0.039 g), and $ZnSO_4{\cdot}7H2O$ (0.0222 g) per L. The 480G and YE5 media have similar composition to the 480GM5 medium except that 1 g/L of pancreatic digestion of casein and 5 g/L of yeast extract were employed, respectively. Sterilization condition of 105 °C for 1 h was reported previously (Keawram and Kanoksilapatham, 2013). In this study, sterilization condition was modified and performed at 121 °C for 20 min to avoid low temperature sterilization.

Habitats and sample collection

A map depicting relative locations of the hot springs is shown in Figure 1. Sediment samples were collected from Pong Duet (PD), Fang (F) and Jae Sawn (JS) hot springs in May 2008, June 2009, and June 2011, respectively as described by Keawram and Kanoksilapatham (2013). Briefly, wet sediment samples were collected and filled up in containers that were capped to prevent incoming air. The closed containers were transported to a laboratory in an ice bath. Prior to being stored at 4 °C in a cold room, the samples were then immediately aliquot into 15-mL vials (ea. with minimal head space) filled with N_2 atmosphere, capped with butyl rubber stoppers, and sealed with aluminum caps.

Figure 1 Map of Thailand depicting Pong Duet, Fang and Jae Sawn hot springs. Symbols: PD represents Pong Duet hot spring, F represents Fang hot spring and JS represents Jae Sawn hot spring.

Isolation of *Thermotoga* and *Fervidobacterium*

Enrichment was performed anaerobically at 80 °C in serum bottles containing 100 mL of isolation media (480G, 480GM5 and YE5 media). Generally, complex cultures are obtained from primary isolations, using natural samples (Mori, 1990; Erbeznik et al., 1997; Dotzauer et al., 2002). One positive culture per sample was chosen for further purification. Pure cultures were obtained using tube dilution technique for at least 3 consecutive times. Briefly, one mL of appropriate dilutions (usually 10^{-7}-10^{-9} tenfold dilutions) was aliquot (0.1 mL ea.) and inoculated into 10 tubes of medium. An end point dilution is defined as the highest dilution that supports growth <10 tubes per mL, and thus growth in each tube

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is likely to be derived from single cell. Pure cultures obtained were named according to the names of the hot springs followed by isolate numbers as previously described (Keawram and Kanoksilapatham, 2013). All pure cultures were stored at 4 °C.

Microorganisms

Isolate numbers PD501, PD502, PD522, JS401, JS504, JS602, FA002, FA003, FA004, FC201, FC202, FC203, FC303, FC1002, and FC2004 were recently reported in a previous study (Keawram and Kanoksilapatham, 2013). However, their SEM images and *16S rRNA* gene sequences were not identified. In this study, 4 additional isolates from Fang hot spring (FC302) and Pong Duet hot spring (PD504, PD517 and PD524) were included.

Morphology

Cell morphology was examined using a phase-contrast microscope (Nikon eclipse 50i) and a Cam ScanMX 2000 scanning electron microscope (UK).

Specimen preparation for scanning electron microscopy

Cell pellets were harvested from 24 h cultures by centrifugation. The pellets were fixed using fresh 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h. The fixed cells were dropped on a cover slide and dried at 80 °C (repeat for 8-10 successive times). The cover slides were rinsed (30 sec), using 0.1 M phosphate buffer pH 7.4 for 3-5 times, and air dried at room temperature. The specimens were then dehydrated for 20 min using 30, 50, 70, 75, 90, 95 and 100% ethanol rinses in succession. The dried specimens were sputter coated with gold and kept in a desiccator.

Genomic DNA preparation

Genomic DNAs were extracted from overnight cultures (300 mL) following phenol chloroform method (Charbonnier and Forterre, 1995). DNAs were precipitated using cold absolute ethanol (-20 °C). DNA pellets were washed three times at room temperature using 70% (v/v) ethanol. RNAs in the samples were digested using DNAse-free RNAse (10 mg/mL in TE buffer) at 37 °C for at least one hour. The DNA solutions were repeated once with the ethanol precipitation step.

Sequencing and phylogenetic analysis of *16S rRNA* gene sequences

Nucleotide sequences of THER3F primer (5' AGGGT TTGAT CMTGG 3') and UA1406R (5' ACGGG CGGTG WGTRC AA 3') were as previously described (Keawram and Kanoksilapatham, 2013; Baker and Cowan, 2004). A standard PCR with annealing temperature of 50 °C was performed. DNA fragments (1300-1400 bp-long) were TA cloned into pTG19-T plasmid. Plasmid DNAs were sequenced using forward and reverse primers of the pTG19-T by AITbiotech (Singapore). Nucleotide sequences were aligned using CLUSTAL W (Thompson et al., 1994). Pairwise distances were computed using the program MEGA 5.1 (Tamura et al., 2011). A Neighbor Joining phylogenetic tree was constructed using a bootstrap value of 1000.

Statistical analysis

16S rRNA gene sequences (total of 50 sequences) representing 11 official genera belonging to phylum Thermotogae are available from GenBank nucleotide data base. These include *Fervidobacterium* sp. FC2004 (KJ473436) (this study), *F. nodosum* Rt17-B1^T (NR_074093.1), *F. gondwanense* AB39^T (NR_036997), *F. pennavorans* DSM9078^T (EF565822), *F. islandicum* AW-1 (AF434670), *F. islandicum* H-21^T (NR_044730), *F. riparium* 1445t^T (NR_108234.1) and *F. changbaicum* CBS-1^T (NR_043248); *Thermotoga* sp. PD524 (KF164213) (this study), *T. maritima* MSB-8^T (NR_029163.1), *T. neapolitana* DSM 4359^T (NR_027530), *T. petrophila* RKU-1^T (NR_074991.1), *T. naphthophila* RKU-10^T (NR_074952.1), *T. subterranea* SL1^T (NR_025969), *T. thermarum* DSM 5069^T (NR_024751), *T. hypogea* SEBR 7054^T (NR_029205), *T. elfii* SM-2^T (EU276416), *T. lettingae* TMO^T (NR_027542), *T. caldifontis* AZM44c09^T (AP014509.1) and *T. profunda* AZM34c06^T (AP0145010.1); *Geotoga petraea* ATCC 51226^T (HM037999.1), *G. subterranea* CC-1T (NR_029145) and *G. aestuarianus* T3B^T (AF509468); *Oceanotoga teriensis* OCT74^T (EU588727.1) and *Oceanotoga* sp. ST186^T (HF548344.1); *Kosmotoga olearia* TBF 19.5.1^T (NR_044583.1), *K. arenicorallina* S304^T (AB530679.1), *K. pacifica* SLHLJ1^T (KC119212.1), *K. shengliensis* 2SM-2^T (EU276414.2); *Marinitoga camini* MV1075^T (NR_028907.1), *Ma. piezophila* KA3^T (NR_027541), *Ma. hydrogenitolerans* AT1271^T (NR_042320.1) and *Ma. okinawensis* JCM 13303^T (NR_041466); *Meotoga prima* MesG1.Ag.4.2^T (NR_102952.1) and *Me. infera* VNs100^T (NR_117646); *Petrotoga halophila* MET-B^T (NR_043201), *P. mexicana*

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MET12^T (AY125964), *P. miotherma* SJ95t^T (L10657.1), *P. mobilis* SJ95^T (NR_074401.1), *P. olearia* SL24^T (AJ311703.1) and *P. siberica* SL25^T (AJ311702.1); *Thermopallium natronophilum* (X91822.1); *Thermosipho globiformans* DSM 19918^T (AB257289), *Tsp. japonicus* IHB1^T (NR_024726), *Tsp. africanus* Ob7^T (NR_043912), *Tsp. melanesiensis* BI429^T (NR_027617), *Tsp. ferriphilus* (AF491334.1), *Tsp. geolei* DSM 13256^T (NR_025389.1) and *Tsp. atlanticus* DV1140^T (NR_029020.1); and *Defluviitoga tunisiensis* SulfLac1^T (FR850164.1). Multiple alignments of queries were performed across the sequences, using the software Align Two or More Sequence available at the NCBI webpage. Blast results obtained were analyzed using descriptive statistics and Kruskal-Wallis Test. A species index is defined as a grand mean that was calculated from similarities of all species tested within the 11 genera. A generic index is defined as a grand mean that is estimated from mean similarities obtained from all possible combination among the genera within the phylum Thermotogae.

Results and discussion

Microorganisms

A total of 19 pure cultures were obtained including 6 isolates from Pong Duet hot spring (PD501, PD502, PD504, PD517, PD522 and PD524), 10 isolates from Fang hot spring (FA002, FA003, FA004, FC201, FC202, FC203, FC302, FC303, FC1002 and FC2004) and 3 isolates from Jae Sawn hot spring (JS401, JS504 and JS602). Although several attempts were made, no isolate belonging to *Thermotoga* was successfully isolated from the Jae Sawn samples. The isolate numbers FC1002 and FC2004 were deposited to the American Type Culture Collection and the Japan Collection of Microorganisms. Reference numbers ATCC BAA-2482 and ATCC BAA-2483, and JCM 18756 and JCM 18757 were assigned, respectively. The isolate PD524 was deposited to the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany, and DSM 28089 was assigned. A previous study reported that the isolates FC2004, FA004, FC202, JS602, PD501, PD502, JS401 and JS504 were able to degrade native feather, a solid waste from avian slaughter house (Keawram and Kanoksilapatham, 2013). The isolates thus have potential applications.

Morphology

In this study, the isolates were categorized based on morphology into two distinguishable characteristics belonging to *Fervidobacterium* (Figure 2) and *Thermotoga*, respectively (Figure 3). Figure 2 shows phase contrast and SEM images of the isolates belonging to *Fervidobacterium* spp. including PD501, PD502, PD504, JS401, JS504, JS602, FC2004, FC201, FC202, FC303 and FA004. In general, all isolates share common characteristics of short rods encapsulated by outer membranous structure forming a big balloon-like protuberance at one end and thus called "terminal spheroid" in this study. The diameters of the terminal spheroids varied, ranging from 1 to >3 μm. However, few cells harboring a tube balloon-like structure were rarely observed on isolates PD502, PD504, and FC201 (the 2nd and 3rd images in the top row and the 2nd image in the bottom row of Figure 2). A size range of typical rods (0.6 to 0.7 x 1.1 to 3 μm) was determined. A filament sized of >10 μm-long was frequently detectable on many isolates (as indicated by arrow head-f in the images of JS401, PD502, JS504, FC2004, FC202 in Figure 2). Cells usually arrange singly and doubly, but long chain arrangement was observed on some isolates (the 5th image in the third row in Figure 2). Spheroid appears as empty spherical membranous structure was usually developed in a stationary growth phase (as indicated by arrow head-s in the image of JS504). Rotund bodies which contain ≥2 to 10 cells were frequently observed (as indicated by arrow head-r in the image of FC201). A large spherical membranous structure as named in this study "giant spheroid" was frequently identified on FC302 (as indicated by arrow head-gs in the 5th and 4th-6th images in the third and fourth rows, respectively).

Figure 3 shows phase contrast and SEM images of isolates belonging to *Thermotoga* spp. including FA002, FA003, FC203, FC1002, PD522, PD524 and PD517. In general, all isolates share morphological features of long slender rod shaped cells harboring a small terminal spheroid at both ends. Cells usually arrange singly and doubly (top row in Figure 3). A size range of typical rods (0.4 to 0.5 x 1.5 to 10 μm) was determined. A filament sized of >10 μm-long was frequently identified (middle row in Figure 3). Bundles of flagella were observed on

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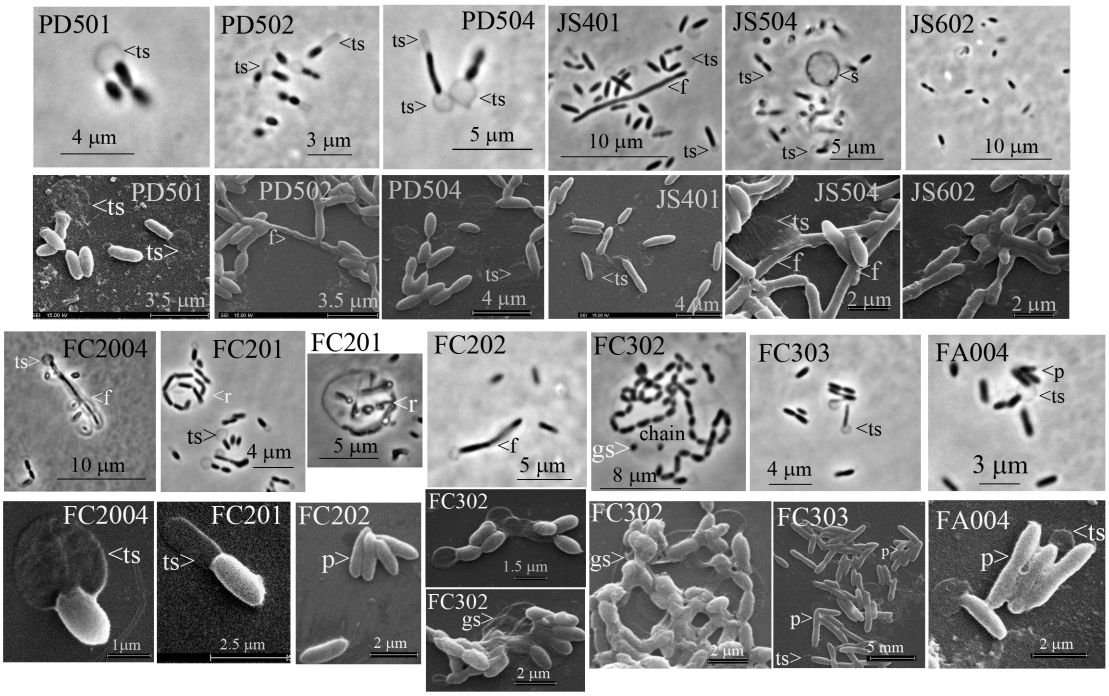

Figure 2 Phase contrast and SEM images of *Fervidobacterium* show typical rigid rod shaped cells with a balloon-like structure at an end. The top and second rows (from left to right) show phase contrast and SEM images of PD501, PD502, PD504, JS401, JS504 and JS602, respectively. The third row (from left to right) shows an encapsulated filament harboring a terminal spheroid of FC2004, typical cells and a rotund body of FC201, a rotund body of FC201, cells and a filament of FC202, chain arrangement and a giant spheroid of FC302, and typical cells of FC303 and FA004. The bottom row shows a rod shaped cell harboring a large terminal spheroid of FC2004, a rod shaped cell harboring a tube balloon-like protuberance of FC201, palisade arrangement of FC202, outer membrane fusion of 5 cells from FC302 indicating initial formation of giant spheroid (upper image), outer membrane fusion of several cells from FC302 (lower image), a giant spheroid with a diameter of >10 μm formed by cells of FC302 (as named according to size), and palisade arrangements of FC303 and FA004, respectively. Symbols: arrow heads-ts,-f, -s, -r, -p, and -gs indicate terminal spheroid, filament, spheroid, rotund body, palisade arrangement, and giant spheroid, respectively.

isolate FC1002 (the 4th image from left in the middle row in Figure 3). Cells in the stationary phase appear pleomorphic (bottom row in Figure 3). Free spheroids (the 2nd and 3rd images in the bottom row in Figure 3) and amoeboid cells or amorphous cells (the 4th and 6th images in the bottom row in Figure 3) were detectable in many isolates including PD524 and PD517. However, abundant numbers of amoeboid cells (>50%) were particularly observed in the late exponential phase of PD517 (the 4th and 5th images in the bottom row in Figure 3). A novel spherical protuberance or a "golf club structure" was observed only on PD524 (the 6th image in the middle row in Figure 3).

***16S rRNA* gene sequencing and phylogenetic analysis**

16S rDNA sequence of FC1002 (JF339227.1) was deposited and is available from NCBI nucleotide data base. In this study, stretches of 1339 and 1380 bp-long DNA fragments amplified using a THER3F and UA1406R primer pairs were cloned and sequenced. The 18 nucleotide sequences obtained were deposited to NCBI, and GenBank numbers assigned were listed in Table 1. Approx. 83% sequence similarity was observed indicating far beyond the threshold, and thus implying two differential species (Stackebrandt and Goebel, 1994; Martens et al., 2008; Tindall et al., 2010). The shorter fragments (KM361907 to KM361914, KM361918 to KM361920 and KJ473436) share high similarity to *Fervidobacterium changbaicum*

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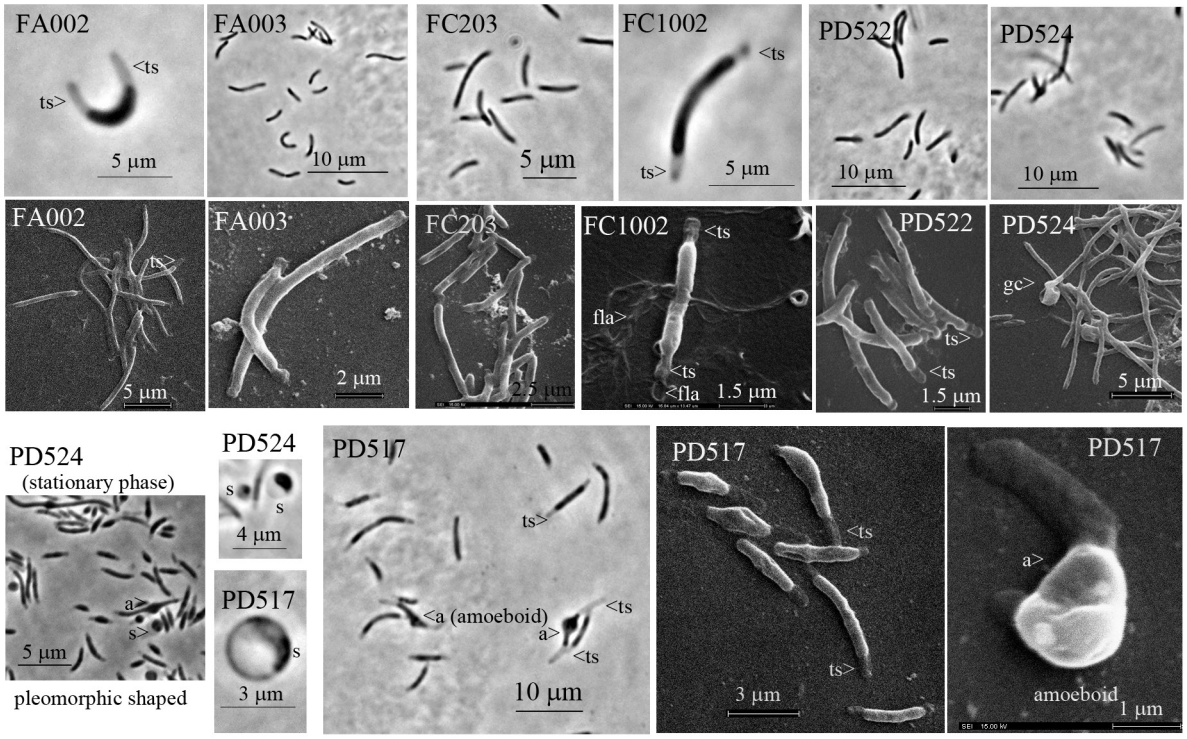

Figure 3 Images of *Thermotoga* spp. show long slender rods harboring a small terminal spheroid at both ends. Top row (from left to right) shows phase contrast images of FA002, FA003, FC203, FC1002, PD522 and PD524. Middle row (from left to right) shows SEM images of FA002, FA003, FC203, FC1002, PD522 and PD524. Bottom row (from left to right) shows pleomorphic shaped cells in the stationary phase of PD524, two spheroids of PD524, a spheroid of PD517, pleomorphic shaped cells of PD517 in the late exponential phase (phase contrast), pleomorphic shaped cells of PD517 (SEM) and an amoeboid cell of PD517. Symbols: arrow heads -ts, -s, -fla, -a, and -gc indicate terminal spheroid, spheroid, flagella, amoeboid cell, and golf club structure, respectively.

CBS-1^T (96%), *F. pennavorans* DSM9078^T (96%), *F. islandicum* AW-1 (96%), *F. islandicum* H-21^T (95%), *F. nodosum* Rt17-B1^T (94%), *F. riparium* 1445t^T (94%) and *F. gondwanense* AB39^T (92%), respectively. The longer fragments (KM361915 to KM361917, KM361921 to KM361922 and KF164213) and the JF339227 share the highest similarity (96%) to the four described hyperthermophilic species of *Thermotoga*, including *T. maritima* MSB8^T, *T. neapolitana* NES^T, *T. petrophila* RKU-1^T, and *T. naphthophila* RKU-10^T.

Statistical analysis and phylogenetic analysis of phylum Thermotogae

Parameters obtained including species and generic indices, similarities within species and among combination of genus, as well as 95% confident interval limits for % similarities are summarized in Table 2. In Table 2, a threshold for species differentiation (species index) 94.5% similarity {95%-CI limit (92.4, 96.6)}, and for genus differentiation (generic index) 81.9% similarity {95%-CI limit (81.8, 82.1)} were presented. Remarkably high similarities compared to the generic index were observed between *Petrotoga* spp. vs *Defluviitoga tunisiensis* (90.7%), *Fervidobacterium* spp. vs *Thermopallium natronophilum* (89.5%), and *Kosmotoga* spp. vs *Mesotoga* spp (88.5%), suggesting a high degree of phylogenetic relationships between these genera. This finding was confirmed by a phylogenetic analysis as shown in Figure 4.

Moreover, a possible inconsistency of species identification was observed within the genus *Geotoga* (Figure 4). In Figure 4, *Geotoga aestuarianus* T3B^T was positioned within the clade formed by *Oceanotoga* sp.

Table 1 List of GenBank numbers assigned to the 19 isolates (including the JF339227.1). Both fragment sizes of 16S rDNA (1339 and 1380 bp-long) were amplified using the THER3F and UA1406R primer pairs. GenBank nos. KM361907 to KM361922, KJ473436 and KF164213 were sequenced in this study.

Genus	GenBank Numbers	Sizes (bp)	Similarity to (%similarity)	References
Fervidobacterium sp. JS401	KM361907	1339	*Fervidobacterium changbaicum*	Cai et al., 2007;
Fervidobacterium sp. JS504	KM361908	1339	CBS-1^T (96%),	
Fervidobacterium sp. JS602	KM361909	1339		
Fervidobacterium sp. FA004	KM361910	1339	*F. pennavorans* DSM9078^T (96%),	Friedrich and Antranikian,
Fervidobacterium sp. FC201	KM361911	1339	and	1996;
Fervidobacterium sp. FC202	KM361912	1339		
Fervidobacterium sp. FC302	KM361913	1339	*F. islandicum* H-21^T (96%)	Huber et al., 1990
Fervidobacterium sp. FC303	KM361914	1339		
Fervidobacterium sp. PD501	KM361918	1339		
Fervidobacterium sp. PD502	KM361919	1339		
Fervidobacterium sp. PD504	KM361920	1339		
Fervidobacterium sp. FC2004	KJ473436	1339		
Thermotoga sp. FC1002	JF339227.1	1281	*Thermotoga maritima* MSB8^T (96%),	Huber et al., 1986;
Thermotoga sp. FA002	KM361915	1380	*T. neapolitana* NES^T (96%),	Jannasch et al., 1988;
Thermotoga sp. FA003	KM361916	1380	*T. petrophila* RKU-1^T (96%) and	Takahata et al., 2001;
Thermotoga sp. FC203	KM361917	1380	*T. naphthophila* RKU-10^T (96%)	Takahata et al., 2001
Thermotoga sp. PD517	KM361921	1380		
Thermotoga sp. PD522	KM361922	1380		
Thermotoga sp. PD524	KF164213	1380		

JC186 and *O. teriensis* OCT74^T rather than formed by *Geotoga petraea* ATCC 51226^T and *G. subterranea* CC-1^T. The results correspond to a remarkably low similarity value (91.0%) compared to the species index as mentioned above (Table 2).

For Thailand isolates, they were classified based on phylogenetic of *16S rRNA* genes as two deepest branches within the clades formed by members of *Fervidobacterium* (bootstrap score of 99) and *Thermotoga* (bootstrap score of 90). In clade formed by *Fervidobacterium*, the 12 Thailand isolates (JS401, JS504, JS602, FC303, FC201, FC202, FA004, FC302, FC2004, PD501, PD502 and PD504) were grouped as a cluster that forms a distinctive branch within this clade. Regardless of their habitats and years of sample collection, these strains share 99% similarity. The results imply that the Thailand's lineage might have had a common ancestor.

Similarly, the remaining isolates of FC1002, FA002, FA003, FC203, PD517, PD522, and PD524 were classified within the clade formed by *Thermotoga*, and their *16S rRNA* genes were revealed sharing 99% similarity suggesting that the strains from the Fang and Pong Duet inhabitants are closely related. In addition, two distinctive branches within this clade, thermophilic and hyperthermophilic *Thermotoga* are revealed (Figure 4). The thermophilic branch is formed by *Thermotoga elfii, T lettingae, T. subterranea, T. profundus, T. thermarum,* and *T. caldifontis* (all species with optimal temperatures of < 70 ^oC), and the hyperthermophilic branch is formed by the Thailand strains and 4 described hyperthermophilic *Thermotoga* including *T. maritima, T. neapolitana, T. petrophila* and *T. napthophila* (all species with optimal temperatures of ≥80 ^oC) (Figure 4). Approx. 90.6% similarity identified among the members within both

Table 2 Predicted mean similarities within and among genera of phylum Thermotogae. Taxa: F, *Fervidobacterium*; T, *Thermotoga*; G, *Geotoga*; O, *Oceanotoga*; K, *Kosmotoga*; Ma, *Marinitoga*; Me, *Mesotoga*; P, *Petrotoga*; Tpal, *Thermopallium*; Tsp, *Thermosipho*; D, *Defluviitoga*; T-h, hyperthermophilic branch of *Thermotoga*; T-t thermophilic branch of *Thermotoga*. Mean values of species within a genus are shown in diagonal boxes. Mean values of thermophilic and hyperthermophilic branches of *Thermotoga* were highlighted in grey. Mean similarity between genera are listed in columns below the diagonal boxes. Thresholds represent indices are bolded. Numbers in parenthesis represent numbers of species in a particular genus. 95%CI limit represents 95% confidence interval limit.

Taxa	Mean similarity (%)												
	F	T	T		G	O	K	Ma	Me	P	Tpal	Tsp	D
			T-h	T-t									
	(8)	(12)	(5)	(7)	(3)	(2)	(4)	(4)	(2)	(6)	(1)	(7)	(1)
F	95.4												
T	83.3	92.6											
T-h	83.6		98.0										
T-t	83.2		90.6	94.5									
G	79.4	78.1			91.0								
O	79.4	78.5			91.8	98.0							
K	83.0	83.8			79.3	78.9	94.2						
Ma	80.5	81.2			83.1	83.7	82.0	95.5					
Me	79.8	80.3			78.0	78.5	88.5	80.5	97				
P	78.5	76.7			83.7	84.2	79.3	81.5	77.9	97.5			
Tpal	89.5	82.3			80.3	80.5	83.0	81.6	79.0	79.0	[a]100		
Tsp	85.1	88.1			79.3	79.4	84.8	81.3	81.1	80.5	84.3	95.7	
D	78.9	78.0			84.0	85.0	79.8	81.0	79.0	90.7	80.0	79.9	[a]100
											Threshold for species index (% similarity)	**Mean:**	**94.5**
												95% CI limit:	92.4, 96.6
											Threshold for generic index (% similarity)	**Mean:**	**81.9**
												95% CI limit:	81.8, 82.1

[a] Only one representative sequence was employed.

branches indicates their remarkably differential genomes in these two different physiological groups.

In conclusion, this study presents phylogenetic evidences highlighting unique lineages of two deepest hyperthermophilic bacteria which are possible candidates for two novel species (Figure 4). However, additional complex analyses are required to meet standard characterizations for taxonomic purposes including classical phenotypic characterizations, metabolic end products, fatty acid analysis using Sherlock MIS based system, and genome sequence analysis including nucleic acid hybridization, multilocus sequence analysis, or complete genome sequencing (Tindall et al., 2010). Highly related strains were evidenced among the Thailand isolates regardless of their diverse ecosystems, implying that these native inhabitants of Thailand might have evolved from a common ancestor. Moreover, the isolates obtained from this study are sources of genes encoding thermostable enzymes that can be further studied for future applications.

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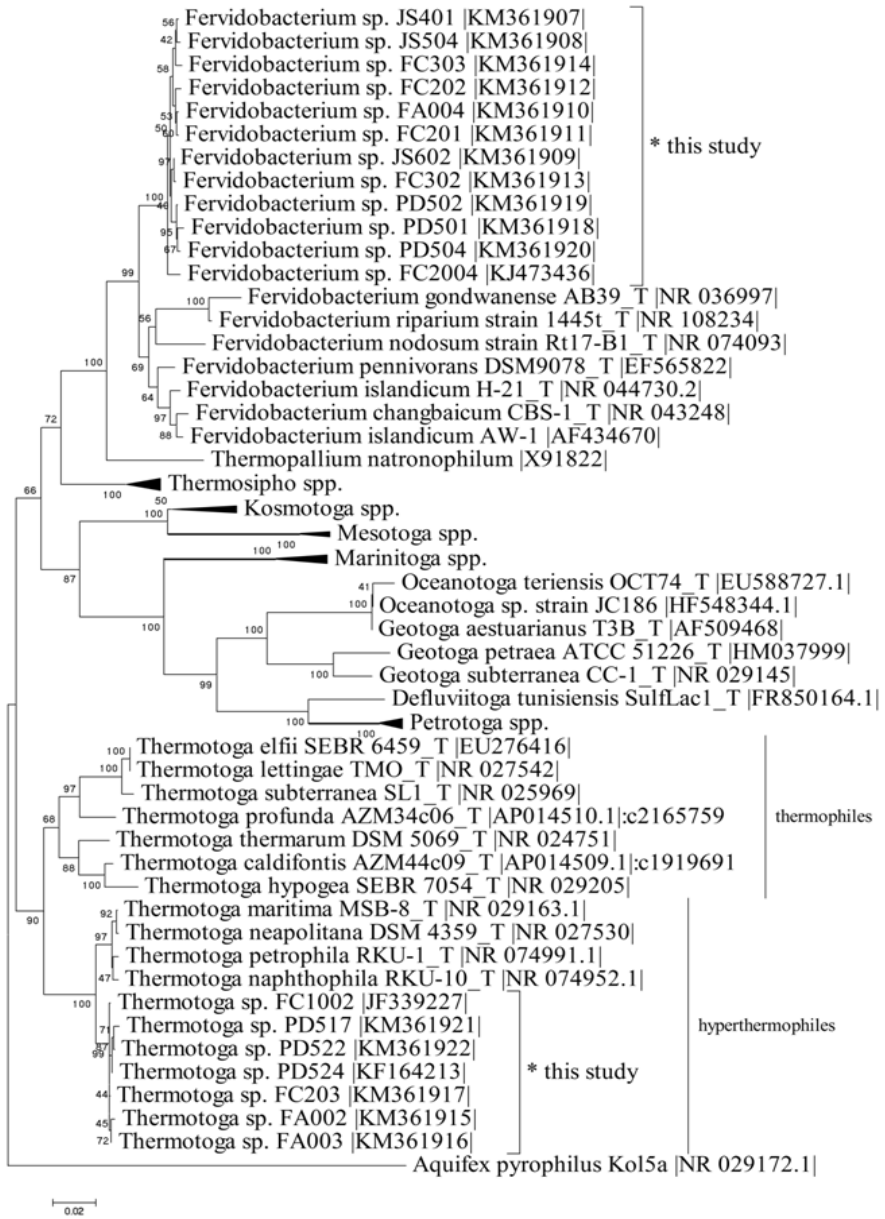

Figure 4 Phylogenetic analysis of *16S rRNA* genes of Thailand isolates and members belonging to phylum Thermotogae.

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