Research Article

Diversity of Hyperthermophilic Bacteria Belonging to Order Thermotogales Thriving in Three Hot Springs in Thailand: Resources of Genes Encoding Thermostable Enzymes

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Abstract

Hyperthermophilic microorganisms belonging to order Thermotogales and thriving in high temperature habitats grow at temperatures above 70 ˚C to near 100 ˚C. In this study, 15 rod shaped bacteria with characteristic of terminal sac-like membrane were isolated from Pong Duet, Fang, and Jae Son hot springs. Differential biochemical properties of the isolates were characterized. Twelve isolates were detected utilizing carbohydrates in CT basal medium, but no growth was observed in the other three isolates. Keratin in native duck feather was degraded at 75-80 ˚C by 8 isolates. Based on morphology, physiology, and 16S rDNA sequences, 5 and 10 isolates belonging to morphological groups I and II were identified as *Thermotoga* species and *Fervidobacterium* species, respectively. The 16S rDNA type II PCR profile of isolates FC2004, FC201, FC202, FA004 and JS602 distinguish themselves from previously reported known species belonging to *Fervidobacterium*. Results obtained from this study indicate that some of Thailand's isolates are distinct, and the geothermal spring ecosystems are rich in divergent hyperthermophiles which still remain to be explored. The hyperthermophilic isolates are crucial sources of numerous thermostable enzymes with potential to be applicable in the degradation of polymers in agricultural wastes such as starch, cellulose, and keratin.

***Key Words*:** Hot spring; Hyperthermophile; Thermotogales; Thermostable Enzyme

Introduction

Hyperthermophilic archaea and bacteria have been recognized as the most primitive forms of life on earth. Among the bacterial members, the thermophiles and hyperthermophiles belonging to order Thermotogales were phylogenetically positioned in close proximity to those ancient Archea (Stetter, 1996; Woese et al., 1990; Huber et al., 1986; Fiala and Stetter, 1986; Patel et al., 1985). Typical ecosystems suitable for growth of hyperthermophilic bacteria belonging to order Thermotogales are extremely hot environments such as marine hydrothermal systems, petroleum reservoirs, and continental hot springs. Members of this order are obligately anaerobic heterotrophs growing on various complex substrates such as proteins, starch, cellulose, and xylan. Many of them have been demonstrated gaining energy from anaerobic respiration using elemental sulfur (S°) and sulfur compounds (Miranda-Tello et al., 2004; Balk

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et al., 2002; Wery et al., 2001; Ravot et al., 1995; Patel et al., 1985).

Currently, order Thermotogales comprises single family Thermotogaceae, which consists of eleven official genera. Typically, all members belonging to this order are gram-negative nonendospore forming rods. Cells are usually surrounded by a thick membranous sheath and form a sac-like structure (or a toga) at one or both terminals (Jayasinghearachchi and Lal, 2011; Feng et al., 2010; L'Haridon et al., 2002; Davey et al., 1993; Huber et al., 1989).

Most members belonging to order Thermotogales were reported as moderate thermophiles growing at temperature around 60 ˚C or below (Miranda-Tello et al., 2004; Jayasinghearachchi and Lal, 2011; Alain et al., 2002; Wery et al., 2001; Jeanthon et al., 1995). Few genera have been reported growing in the temperature range of mesophiles (Nesbo et al., 2012; Dipippo et al., 2009). Some species belonging to the genera *Thermotoga* spp. and *Fervidobacterium* spp. have been frequently reported growing at the temperature of hyperthermophiles (70 ˚C to 90 ˚C). *Thermotoga maritima* and *T. neapolitana* were first isolated from marine hydrothermal ecosystems and later were found in low salt continental hot springs. Both species were recorded to grow at the upper growth limit temperatures up to 90 ˚C (Huber et al., 1986). Both *Thermotoga petrophila* and *T. naphthophila* were discovered from a subterranean oil reservoir in Japan and grew optimally at 80 ˚C. Both of them tolerated high salt concentration of > 5% (w/v) NaCl and required at least 0.1% NaCl for growths (opt. conc. of 1% NaCl) (Takahata et al., 2001).

Fervidobacterium nodosum, the representative new genus, was first isolated from volcanic hot springs in New Zealand, and its growth temperature range of 47 to 80 ˚C (opt. temp. of 70 ˚C) was reported (Patel et al., 1985). *Fervidobacterium islandicum* (Huber et al., 1990) and *F. gondwanense* (Andrews and Patel, 1996) were isolated from a volcanic hot spring and geothermal artesian water, respectively. Both *F. islandicum* (opt. temp. of 65 ˚C) and *F. gondwanense* (opt. temp. of 65-68 ˚C) failed to grow at ≤45 ˚C; however, a maximum growth temperature close to 80 ºC was reported. All isolates were reported growing in media with dilute NaCl concentration (opt. conc. of 0.1-0.2 % NaCl), and not growing at concentrations of > 0.6 - 1% NaCl. In addition, two hyperthermophiles growing optimally at >70 ˚C named *Fervidobacterium pennivorans* (opt. temp. of 70 ˚C) and *F. changbaicum* (opt. temp. of 80 ˚C) were previously isolated from volcanic hot springs. Both species required diluted NaCl concentration (0-0.1 % NaCl) for optimal growths (Cai et al., 2007; Friedrich and Antranikian, 1996).

In this study, hyperthermophilic microorganisms were successfully isolated from three hot springs located at different angular distances in Northern Thailand. The isolates are sources of genes encoding numerous thermostable enzymes. The attempt to culture microorganisms growing at a temperature near the boiling point of water is considered groundbreaking in Thailand.

Materials and Methods

Sample Collection and Isolation of Hyperthermophiles

Sediment samples were collected near thermal sources of Pong Duet (19° 7′ N, 98° 56′ E), Fang (19° 58′ N, 99° 12' E), and Jae Son (18° 50′ N, 99° 28′ E) hot springs in Northern Thailand. The temperatures measured *in situ* at the thermal exits were 80 to 100 ˚C. The sediments were transported to a laboratory in an ice box. Approx. 1 g of samples were inoculated into 480G medium in serum bottles and incubated anaerobically at 75 to 80 ˚C for 1 to 2 days or until turbidity was observed. Isolation of pure cultures was performed using serial tube

dilution technique for at least triplicate times. Pure cultures obtained were named after the initial letter of the hot springs' names. Briefly, FA0, FC0, FC1, FC2 and FC3 indicate the wells located at Fang hot spring. PD5 stands for well 5 located at Pong Duet hot spring. JS4, JS5 and JS6 stand for wells 4, 5 and 6 located at Jae Son hot spring. The last two or three digits indicate isolate numbers. All cultures were stored in 480G medium at 4 ˚C.

480G Medium and Preparation

A liter of 480G medium was composed of 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), yeast extract (0.5 g), A5 solution (1 ml), resazurin solution (0.5 ml of 0.2 g/l solution) and $Na_2S.9H_2O$ solution [3 ml of 25% (w/v), pH 7]. pH was adjusted to 7.2-7.5 at room temperature using 1N NaCl or 1N HCl before sterilization. The medium was prepared anaerobically in serum bottles under N_2 atmosphere. Sterilization was performed at 100 ˚C for 1 h. A liter of the A5 solution was composed of $Co(NO_3)_2.6H_2O$ (0.00494 g), $CuSO_4.5\ H_2O$ (0.0079 g), H_3BO_3 (0.286 g), $MnCl_2.4\ H_2O$ (0.181 g), $Na_2MoO_4.2H_2O$ (0.039 g) and $ZnSO_4.7H_2O$ (0.0222 g).

Carbohydrate Utilization Test

CT medium, a basal medium for testing carbohydrate utilization, was developed in this study. Compositions of the medium were similar to those of the 480G medium except that 0.1 g/l of pancreatic digestion of casein and 0.05 g/l of yeast extract were employed. Glucose, sucrose, maltose, lactose, starch, cellobiose and carboxymethyl cellulose (CMC) were tested at a final concentration of 1 g/l. Briefly, overnight inoculum was diluted 100 times using the basal medium prior to the inoculation (to obtain an approx. conc. of 10^5cells/ml) into the triplicate bottles of a test sugar. Cell yields compared with those of the controls were determined at 48 h. Cells were counted using the direct count technique or measured optical density (OD_{660nm}).

Ability to Degrade Duck Feather

A medium named FD was modified from medium I described by Friedrich and Antranikian (1996). The medium was employed to test the feather degradation capacity. A liter of the FD medium was composed of $K_2HPO_4.3H_2O$ (2.09 g), $NaH_2PO_4.2H_2O$ (1.29 g), $(NH_4)_2SO_4$ (1.5 g), $CaCl_2.2H_2O$ (0.1 g), NaCl (0.3 g), $MgSO_4.7H_2O$ (0.3 g), $NaHCO_3$ (1 g), pancreatic digestion of casein (1 g), yeast extract (1 g), cysteine hydrochloride (0.5 g), $FeCl_2.6H_2O$ (6 mg), A5 solution (1 ml), resazurin solution (0.5 ml of 0.2 g/l solution) and $Na_2S.9H_2O$ solution [3 ml of 25% (w/v), pH 7]. pH was adjusted to 7.2-7.5 at room temperature using 1N NaCl or 1N HCl before sterilization. The medium was prepared in a Hungate tube (15 ml) that contained a piece of duck feather (15 mg). Cultures were incubated at 75-80 ˚C for 48 h.

Primers and PCR Conditions

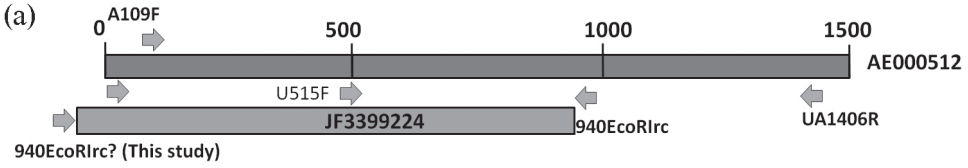
16S DNA fragments were amplified using the following primers: THER3F, A109F, U515F, 940EcoRIrc and UA1406R. The THER3F primer was designed in this study and has the nucleotide sequence of 5' AGGGTTTGATCMTGG 3'. Nucleotide sequence of 940EcoRIrc (5' CGGCGTGAATTCCAATTAAACCGCACGC 3') was previously described (Kanoksilapatham et al., 2012). Nucleotide sequences of A109F (5' ACKGCTCAGTAACACGT 3'), U515F (5' GTGCCAGCMGCCGCGGTAA 3') and UA1406R (5' ACGGGCGGTGWGTRCAA 3') were described elsewhere (Baker and Cowan, 2004). Relative binding positions of these primers on a 16S rDNA sequence (GenBank AE000512) are shown in Figure 1. Predicted from the binding sites of the 940EcoR1rc primer, either a 1000 bp-long PCR product or none (Figure 1a) might be amplified. The A109F, an archaeal specific primer, is anticipated to bind more strongly on the 16S rDNA sequences from *Thermotoga* than *Fervidobacterium* (Figure 1b).

All PCR reactions were performed as follows: 1 cycle of 95˚C for 4 min then 30 cycles of 95˚C for 1 min, 57˚C for 1 min and 72˚C for 1 min; and a final extension at 72˚C for 10 min. The products were separated using 1% agarose gel electrophoresis.

Results and Discussion

Isolation and Morphology

Fifteen strictly anaerobic heterotrophs growing at temperature of 80 ˚C were isolated from three hot springs (Pong Duet, Fang, and Jae Son hot springs) located in Northern Thailand. They include 3 isolates from Pong Duet (PD501, PD502 and PD522), 9 isolates from Fang (FA002, FA003, FA004, FC1002, FC2004, FC201, FC202, FC203 and FC303), and 3 isolates from Jae Son (JS401, JS504 and JS602) hot springs. Phase contrast micrographs reveal that the cells of these isolates share common characteristics of rod shaped with a terminal toga at one or both ends (Figure 2), enclosed in membranous sheath, stained with Gram-negative or Gram-nonreactive, usually arranged singly and rarely in pairs to short chain. Occasionally filamentous cells were detected on some isolates (Figures 2c-2d). No endospore was observed on all isolates. Based on characteristic of toga and shape,



(b)

Microorganisms	GenBank Nos.	Nt. Nos.	Sequences	Nt. Nos.
			A109F Primer : *ACKGCTCAGTAACACGT→*	
Thermotoga maritima	(AE000512)	104	: ACGGGTGAGTAACACGT	:120
Thermotoga neapolitana	(CP000916)	507416	: ACGGGTGAGTAACACGT	:507390
Thermotoga petrophila	(CP000702)	1755135	: ACGGGTGAGTAACACGT	:755118
Thermotoga elfii	(DQ374393)	95	: ACGGGTGAGTAACACGT	:111
Thermotoga subterranean	(NR_025969)	77	: ACGGGTGAGTAACACGT	:93
Fervidobacterium nodosum	(CP000771)	257	: ACGGGTGCGTAACACGT	:240
Fervidobacterium gondwanense	(NR_036997)	83	: AGCGGTGCGTAACACGT	:99
Fervidobacterium pennivorans	(EF565822)	100	: ACGGGTGCGTAACGCGT	:116
Fervidobacterium changbaicum	(NR_043248)	101	: ACGGGTGCGTAACGCGT	:117
Fervidobacterium islandicum	(AF434670)	101	: ACGGGTGCGTAACGCGT	:117
Isolate no.FC2004	(JF339224)	129	: ACGGGTGCGTAACGCGT	:145

Figure 1 Relative binding positions of primers on 16S rDNA sequences (1.5 kb). (a) Diagram shows relative binding positions of primers on the *16S rRNA* gene of *T. maritima* (GenBank AE000512). Nt. no. 1 corresponds to the nt. no. 188970 of the AE000512. GenBank JF339224 which was amplified by a 940EcoR1rc is shown. An inverted sequence of 940EcoR1rc (named 940EcoR1rc?) located at upstream of the JF339224 is identified in this study. Binding site of THER3 primer begins at nt. no.23 of the JF339224 and the nt. no. 6 of the AE000512. Relative binding sites of universal primers (U515F, 940EcoR1rc and UA1406R) are indicated at relative positions. (b) Alignment of A109F primer sequence (5' ACKGCTCAGTAACACGT 3') *versus* sequences from order Thermotogales was conducted using MEGA5.5. Symbols: Horizontal boxes indicate 16S rRNA genes. Arrows represent primers and arrow heads indicate direction of PCR polymerization. Underline indicates identical bases.

they could be categorized into two morphological groups. The first group includes the isolates FA002, FA003, FC203, FC1002 and PD522. Cells of the first group are long slender rods (an average size range of 0.4-0.5x1.5-5 µm) with a toga at both terminals; this characteristic is a typical hallmark of the genus *Thermotoga* spp. (Figures 2a and 3a). The second group includes the isolates PD501, PD502, FA004, FC2004, FC201 FC203, FC303, JS401, JS504 and JS602. Cells of the second group are short rods (size range of 0.5-0.6x1-2.5 µm) with a balloon-like toga presenting at a terminal (Figures 2b-2d and 3b-3c). Few long filaments (an average size range of 10-40 µm) were rarely observed on some isolates (Figures 2c-2d).

Biochemical Properties

Ability to utilize carbohydrate of the isolates was tested in CT basal medium containing 1 g/l of test carbohydrates. In general, slight growths (with a magnitude of 10^6 cells/ml) were observed in the CT basal medium (controls), but no growth was detected on the isolates FA003, PD522 and FC202 (data not shown). In addition, no growth on all carbohydrates tested was also observed in these three isolates, implying this medium might not be suitable for their growths (Table 1). Therefore, these isolates are categorized into a separate group named in this study as "biochemical subgroup I", and the remaining 12 isolates are grouped as "biochemical subgroup II". Members of the subgroup II were

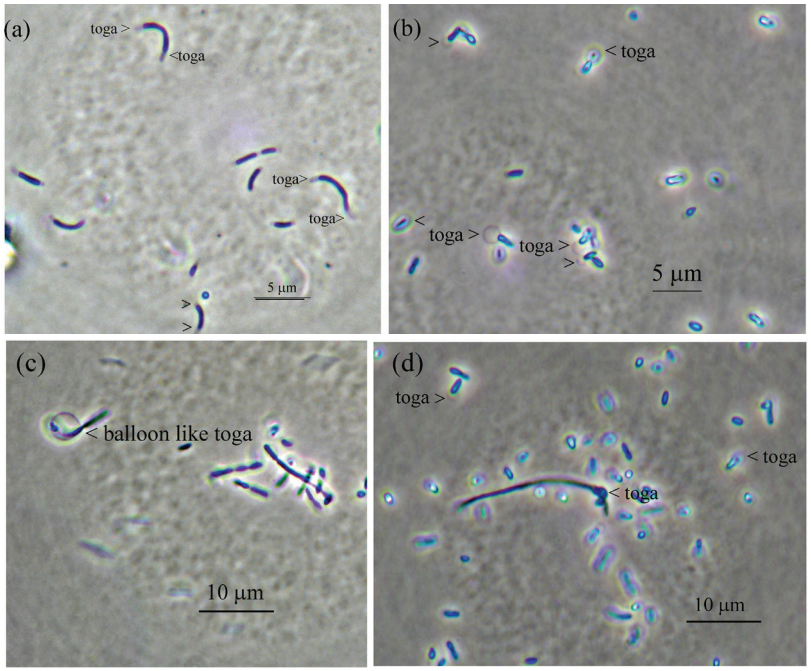


Figure 2 Phase contrast micrographs of some isolates belonging to order Thermotogales. (a) Isolate FA002 shows slender rod shaped cells with a toga at both terminals. Cells occur singly sized of 0.4-0.5x2.5-5 µm. Arrow heads indicate toga. (b) Isolate FC2004 shows short rod shaped cells with a single large terminal toga. Cells are usually encased in thick sheath-like membrane, occurring singly or in pairs. Arrow heads indicate toga. (c) Isolate JS401 shows short rod shaped cells with a toga at one terminal. Filament with a terminal spheroid toga is occationally detected. Cells arrange singly and short chain. Arrow head indicates a filamentous cell with a balloon-like toga. (d) Isolate FC2004 shows a long filamentous and short rod shaped cells with a terminal toga. Arrows indicate toga.

detectable growing at least on glucose, sucrose and maltose (with a magnitude of 10^7 cells/ml). No growth on lactose was detected in all isolates, except that slight growth was observed on isolate FC1002. Little to remarkable growths on soluble starch and carboxymethyl cellulose (CMC) were demonstrated on isolates FC1002, FC2004, FA004, FC201, JS602 and FC303 (Table 1).

Degradation of Duck Feather

Previous reports suggested that some strains of hyperthermophilic *Fervidobacterium pennavorans* (Friedrich and Antranikian, 1996), *Fervidobacterium changbaicum* (Cai et al., 2007), and *Fervidobacterium islandicum* (Nam et al., 2002) digested keratin in feather. Ability to degrade native feather of the 15 isolates was tested at 75 ˚C and 80 ˚C. Results reveal that all of the 5 isolates belonging to the morphological group I were found unable to degrade the feather. In contrast, 8 from 10 isolates belonging to the morphological group II significantly degraded keratin in the feather (Table 1). Although isolate FC202 was inert on the test carbohydrates, it was observed degrading the feather in the medium.

Table 1 Biochemical properties of hyperthermophilic isolates belonging to order Thermotogales. All tests were performed in triplicates and compared with controls.

Isolates numbers	Morphological groups	Biochemical subgroup	Carbohydrate utilization[1]								Types of 16S rDNA profiles
			Glucose	Sucrose	Maltose	Lactose	Cellobiose	Soluble starch	CMC	Degradation of native feather[2]	
FC1002	I	II	+	+	+	+	+	+	+	-	I
FA002	I	II	±	+	+	-	+	-	-	-	I
FA003	I	I	-	-	-	-	-	-	-	-	I
FC203	I	II	+	+	+	-	+	+	-	-	I
PD522	I	I	-	-	-	-	-	-	-	-	I
FC2004	II	II	+	+	+	-	+	+	+	+	II
FA004	II	II	+	+	+	-	-	+	±	+	II
FC201	II	II	+	+	+	-	-	+	+	-	II
FC202	II	I	-	-	-	-	-	-	-	+	II
JS602	II	II	+	+	+	-	-	+	±	+	II
FC303	II	II	+	+	+	-	+	+	+	-	III
PD501	II	II	+	+	+	-	±	+	-	+	III
PD502	II	II	+	+	+	-	-	+	-	+	III
JS401	II	II	+	+	-	-	+	+	-	+	III
JS504	II	II	+	+	+	-	-	+	-	+	III

[1] + = growth, ± = slight growth, - = no growth, (n=3).

[2] + = degrade duck feather within 48 h, - = not degrade duck feather within 48 h, (n=3).

16S rDNA Profiles

16S rDNA profiles were constructed using three primer pairs (U515F/UA1406R, A109F/940EcoR1rc and THER3F/UA1406R). Approx. sizes of 900 and 1400 bp-long PCR products were anticipated from the binding positions of the U515F/UA1406R and THER3F/UA1406R, respectively (Figures 1a). Analysis of the A109F priming sequences (length at 3'end) suggests that the primer might bind more strongly to the sequences of *Thermotoga* spp. than *Fervidobacterium* spp. (Figure 1b).

Experimental results reveal three distinguishable profiles generated using these primer pairs (Figure 4). As expected, the bands with approx. size of 900 and 1400 bp-long were obtained from all isolates when amplified using the universal primer pairs of U515F/UA1406R and THER3F/UA1406R, respectively. On the other hand, 3 distinct PCR profiles (Figure 4) named "16S type I", "16S type II", and "16S type III" were revealed using the A109F/940EcoR1rc.

The 16S type I is identified by a faint PCR product size of 850 bp-long amplified using the A109F/940EcoR1rc (lane 3 in Figure 4), and it was disclosed on the isolates belonging to genus *Thermotoga* spp. (FC1002, FA002, FA003, FC203 and PD522) mentioned above (see also alignment of the A109F in Figure 1b). The 16S rDNA sequence of isolate FC1002 (GenBank JF339227) reveals the highest similarity (94-97%) to several known sequences of *Thermotoga* spp. (GenBank nos. AE000512, AJ401024, CP001839, CP000702 and NR_024751). The results confirm that the isolates with a toga at both terminals share the same characteristic as genus *Thermotoga* species (Figure 3a).

The 16S type II is depicted by a prominent DNA fragment size of 1000 bp-long amplified using the A109F/940EcoR1rc (lane 6 in Figure 4), and it was demonstrated on 5 isolates belonging to genus *Fervidobacterium* (FC2004, FA004, FC201,

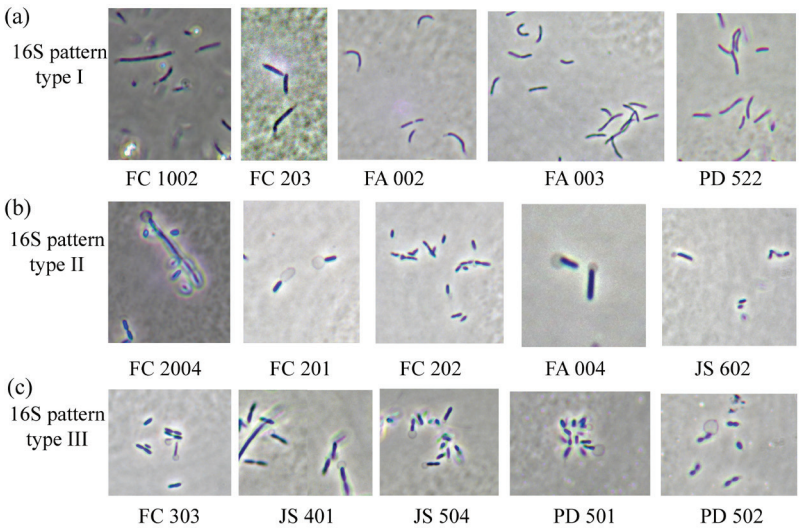


Figure 3 Classification based on morphology and 16S profiles. (a) Cells with morphological group I and 16S type I profile are classified as genus *Thermotoga* species. Isolate numbers were listed beneath the pictures. (b) Cells with morphological group II and 16S type II profile are classified as genus *Fervidobacterium* species. Isolate numbers were listed beneath the pictures. (C) Cells with morphological group II and 16S type III profile are classified as genus *Fervidobacterium* species. Isolate numbers were listed beneath the pictures.

FC202 and JS602). The 16S rDNA sequence of the isolate FC2004 (GenBank JF339226) reveals 96% similarity to *Fervidobacterium changbaicum* strain CBS-1 (GenBank EF138832), *F. islandicum* strain AW-1 (GenBank AF434670) and *F. nodosum* Rt17-B1 (GenBank CP000771) and 92% similarity to *F. gondwanense* strain AB39 (GenBank NR_036997). The results confirm these isolates as *Fervidobacterium* spp. (Figure 3b). In addition, a reverse priming site of the 940EcoR1rc (named 940EcoR1rc?) is identified in this study (Figures 1a). In order to confirm the presence of this priming site, PCR amplification reactions using the single 940EcoR1rc primer and DNA templates from the 16S type II isolates reveals 1000 bps PCR product (data not shown). However, this additional priming site (940EcoR1rc?), at the adjacent sequences of the *16S rRNA* genes, was absent in the complete genome sequences of *F. nodosum* (GenBank CP000771) and *F. penivorans* (GenBank CP003260). The results imply that these 5 isolates are differentiated from *F. nodosum* and *F. penivorans*.

The 16S type III is recognized by lacking PCR product when amplified using the A109F/940EcoR1rc (lane 9 in Figure 4), and it was determined on the other 5 isolates belonging to genus *Fervidobacterium* (FC303, PD501, PD502, JS401 and JS504). The missing DNA band might result from a weak bonding (where the 3' hydroxyl end of the A109F primer) on several reported 16S rDNA sequences from *Fervidobacterium* species including the *F. nodosum* and *F. penivorans* mentioned above (Figure 1b).

Diversity of the Hyperthermophilic Bacteria across the Hot Springs

Five isolates belonging to *Thermotoga* spp. were discovered from Fang and Pong Duet hot springs (four and one isolates), respectively (Figure 3a). However, no *Thermotoga* sp. was obtained from Jae Son hot spring. Unlike the isolate FC1002, the isolate FA002 is unable to utilize lactose, soluble starch and CMC, and the isolate FC203 is unable to utilize lactose and CMC (Table 1). Although, the isolates FA003 and PD522, which are classified

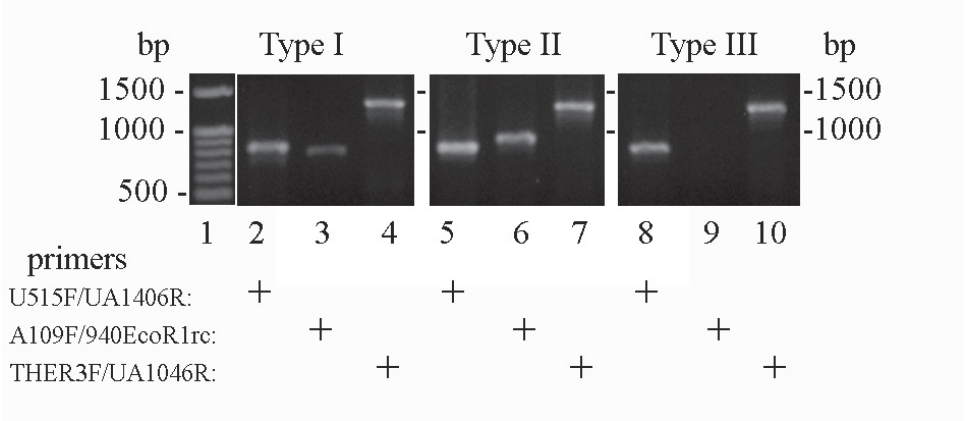


Figure 4 16S rDNA profiles generated using 3 primer pairs (U515F/UA1406R, A109F/940EcoR1rc and THER3F/UA1406R). Lane 1 indicates 100 bp ladder size markers. Lanes 2-4 represent profile type I that was amplified from the isolates FC203, FC1002, FA002, FA003 and PD522. Lanes 5-7 represent a profile type II that was amplified from the isolates FC2004, FA004, FC201, FC202 and JS602. Lanes 8-10 represent profile type III that was amplified from the isolates FC303, PD501, PD502, JS401 and JS504.

within the biochemical subgroup I, are inert on carbohydrate utilization, they were isolated from remote habitats. The results suggest that all 5 isolates belonging to *Thermotoga* spp. are distinct in their biochemical characteristics.

Ten isolates with the morphological group II are identified as *Fervidobacterium* spp. (Figures 3b and 3c). They include 5 isolates from Fang (FC2004, FA004, FC201, FC202 and FC303), 2 isolates from Pong Duet (PD501 and PD502), and 3 isolates from Jae Son hot springs (JS401, JS504 and JS602). Excluding the isolates FC201 and FC303, all *Fervidobacterium* spp. were keratin-degrading detectable in native feather. Unlike isolate FC2004, isolates FA004, FC201 and JS602 are unable to utilize cellobiose. In contrast to isolate PD501, isolate PD502 is unable to utilize cellobiose. In contrast to isolate JS401, isolate JS504 utilizes maltose, but not cellobiose. Among isolates belonging to *Fervidobacterium* spp., isolate FC202 was determined inert on carbohydrate utilization (Table 1). However, it degrades keratin in native feather. The 16S type II and III profiles imply diverse sequences of these isolates belonging to *Fervidobacterium*. In this study, the 16S type II profile is uniquely determined only in some Thai strains.

Conclusions

Two morphological groups of hyperthermophilic bacteria belonging to order Thermotogales were isolated from various sediment samples collected from Pong Duet (3 isolates), Fang (9 isolates), and Jae Son (3 isolates) hot springs. All grew at temperatures around 80 ^{o}C by gaining carbon and energy from pancreatic digest of casein and yeast extract. Strain differentiation was revealed based on biochemical properties and 16S rDNA fragment profiles. Twelve isolates were determined, utilizing carbohydrates including soluble starch and CMC. Five isolates (FC1002, FA002, FA003, FC203 and PD522) were identified as the genus *Thermotoga* species (Figure 3a), and ten isolates as the genus *Fervidobacterium* species (Figures 3b and 3c). It is noticed that the isolates belonging to the genus *Thermotoga* species do not degrade native feather. In contrast, 8 of the 10 isolates belonging to the genus *Fervidobacterium* species substantially degraded keratin excluding the isolates FC201 and FC303 (Table 1).

In conclusion, geothermal hot spring ecosystems in Thailand are rich in cultured hyperthermophilic species belonging to *Thermotoga* and *Fervidobacterium*. Strain differentiation among these isolates using conserved sequences from *tRNA* genes and an arbitrarily primed PCR based technique is under investigation (Welsh and McClelland, 1991; Patlada et al., 2011). Among the three hot springs examined, Fang hot spring might be a unique habitat and suitable for growths of divergent hyperthermophiles. The 16S type II observed among the 50% of strains belonging to *Fervidobacterium* indicates that they are differentiated from those reported known *Fervidobacterium nodosum* and *F. pennivorans* (Friedrich and Antranikian, 1996; Patel, et. al., 1985). The hyperthermophilic isolates obtained from this study are crucial sources of thermostable enzymes with potential to be applicable in degrading polymers such as starch, cellulose and insoluble keratin.

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