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ORIGINAL PAPER

Isolation, characterization, and survival strategies of *Thermotoga* sp. strain PD524, a hyperthermophile from a hot spring in Northern Thailand

Wirojne Kanoksilapatham[1] · Porranee Keawram[1,2] · Juan M. Gonzalez[3] · Frank T. Robb[4,5]

Abstract A hyperthermophilic *Thermotoga* sp. strain PD524 was isolated from a hot spring in Northern Thailand. Cells were long-curved rods (0.5–0.6 × 2.5–10 µm) surrounded by a typical outer membrane toga. Strain PD524 is aero-tolerant at 4 °C but is aero-sensitive at 80 °C. A heat resistant subpopulation was observed in late-stationary phase. Cells from late-stationary phase were revealed remarkably less sensitive to 0.001 % SDS treatment than cells from exponential phase. The temperature range for growth was 70–85 °C (opt. temp. 80 °C), pH range was 6–8.5 (opt. pH 7.5–8.0), and NaCl range of 0 to <10 g/L (opt. 0.5 g/L). Glucose, sucrose, maltose, fructose, xylose, mannose, arabinose, trehalose, starch, and cellobiose were utilized as growth substrates. Growth was inhibited by S^o. Growth yield was stimulated by $SO_4^=$ but not by $S_2O_3^=$ and NO_3^-. Analysis of *16S rRNA* gene sequence (KF164213) of strain PD524 revealed closest similarity (96 %) to *Thermotoga maritima* MSB8[T], *T. neapolitana* NES[T], *T. petrophila* RKU-1[T], and *T. naphthophila* RKU-10[T].

Introduction

The order Thermotogales comprises many thermophilic and hyperthermophilic species that thrive in geothermal environments including continental hot springs and undersea hydrothermal vents. Members of the Thermotogales all possess a characteristic outer sheath-like membranous structure, the so-called toga. Most members have optimal temperatures ranging from 50 to 90 °C including five described hyperthermophilic species, *Thermotoga maritima* MSB8[T], *T. neapolitana* NES[T], *T. petrophila* RKU-1[T], *T. naphthophila* RKU-10[T], and *Fervidobacterium changbaicum* CBS-1 (Huber et al. 1986; Jannasch et al. 1988; Takahata et al. 2001; Cai et al. 2007). Currently, 12 official genera are reported, including *Fervidobacterium* (Patel et al. 1985), *Thermotoga* (Huber et al. 1986), *Thermosipho* (Huber et al. 1989), *Geotoga* (Davey et al. 1993), *Petrotoga* (Davey et al. 1993), *Thermopallium* (Duckworth et al. 1996), *Marinitoga* (Wery et al. 2001), *Kosmotoga* (DiPippo et al. 2009), *Oceanotoga* (Jayasinghearachchi and Lal 2011), *Mesotoga* (Nesbø et al. 2012), *Defluviitoga* (Ben Hania et al. 2012), and *Mesoaciditoga* (Reysenbach et al. 2013). Based on *16S rRNA* phylogenetic, conserved signature indels in protein sequences and comparative genomic

✉ Wirojne Kanoksilapatham
kanoksilapatham_w@su.ac.th

✉ Frank T. Robb
frobb@som.umaryland.edu

1 Department of Microbiology, Faculty of Science, Silpakorn University, Nakhon Pathom 73000, Thailand

2 Western University Kanchanaburi, Huai Krachao District, Kanchanaburi 71170, Thailand

3 IRNAS-CSIC, Avda. Reina Mercedes 10, 41012 Seville, Spain

4 Institute of Marine and Environmental Technology, University of Maryland, 701 E. Pratt Street, Baltimore, MD 21202, USA

5 Department of Microbiology and Immunology, University of Maryland, Baltimore, MD 21202, USA

analyses, the members of order Thermotogales have been recently reclassified into 3 orders (Thermotogales, Kosmotogales ord. nov., and Petrotogales ord. nov.). In addition, the thermophilic combinations (a subgroup of genus *Thermotoga*) including *Thermotoga thermarum, T. lettingae, T. elfii, T. subterranea*, and *T. hypogea* were renamed as *Pseudothermotoga* gen. nov. (Bhandari and Gupta 2014). These bacteria share common morphological features of pleomorphic rod-shaped cells, except *Kosmotoga shengliensis* (Feng et al. 2010; Nunoura et al. 2010) which has coccoid shaped, and are either Gram-negative or Gram-nonreactive. The order is described categorically as nonsporeforming although survival strategies are expected in this taxonomic group. Species assigned to the genera *Thermotoga* and *Pseudothermotoga* are flexible rods containing a balloon-like membranous bleb at each cell pole (Huber et al. 1986; Jannasch et al. 1988; Windberger et al. 1989; Jeanthon et al. 1995; Ravot et al. 1995; Fardeau et al. 1997; Takahata et al. 2001; Balk et al. 2002). Cells of *Pseudothermotoga subterranea* strain SL1[T] growing in late exponential phase were proved sensitive to dissolved oxygen at growth temperature; however, cell viability was retained at 4 °C for 1 day, and approx. 100 cells/mL (from the initial 10^8 cells/mL) of oxygen-resistant cells remained after 4 weeks' exposure to air (Jeanthon et al. 1995).

In this study, a hyperthermophilic *Thermotoga* species isolated from a solfataric hot spring in Northern Thailand was characterized with respect to morphology, phylogenetic analysis of *16S rRNA* gene, growth kinetics, and physiological properties. In addition, survival mechanisms relating to resistance to oxygen (at permissive and nonpermissive growth temperatures), SDS concentrations, and thermal sensitivity of strain PD524 in exponential and stationary phases were compared.

Materials and methods

Media

The 480GM5 medium (an isolation medium) was composed of (per L) NaCl (0.5 g), NH$_4$Cl (0.33 g), CaCl$_2$·2H$_2$O (0.15 g), MgCl$_2$·6H$_2$O (0.35 g), KCl (0.3 g), KH$_2$PO$_4$ (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$_2$S.9H$_2$O solution [25 % (w/v), pH 7]. The pH was adjusted to 7.2–7.5 at room temperature using 1 N NaOH or 1 N HCl before sterilization. The medium was prepared anaerobically in serum bottles under a 1 atm N$_2$ headspace and sealed with a butyl rubber stopper. Sterilization was performed at 121 °C for 20 min. The A5 stock solution was composed of Co(NO$_3$)$_2$·6H$_2$O (0.00494 g),

CuSO$_4$·5H$_2$O (0.0079 g), H$_3$BO$_3$ (0.286 g), MnCl$_2$·4H$_2$O (0.181 g), Na$_2$MoO$_4$·2H$_2$O (0.039 g), and ZnSO$_4$·7H$_2$O (0.0222 g) per L.

The 480G and YE5 media are as described for the 480GM5 medium except that the concentration of pancreatic digestion of casein was reduced to 1 g/L and replaced by 5 g/L of yeast extract, respectively. CT and CT5 basal media are similar to the 480GM5 medium except that 0.1 g/L of pancreatic digestion of casein and 0.05 g/L of yeast extract, and 0.5 g/L of pancreatic digestion of casein were employed as major organic nitrogen sources. The CT5 medium was used to test the utilization of carbohydrates.

Isolation of strain PD524

A sediment sample was collected anaerobically from a 95 to 98 °C thermal source at Pong Duet hot spring, in Northern Thailand (N19°13′53.23″, E98°40′9.73″). Enrichment was performed at 80 °C using 480GM5 medium. Strain PD524 was obtained in pure culture using the end-point dilution technique in Hungate tubes performed three consecutive times. Strain PD524 was deposited as DSM 28089.

Morphology

Cell morphology was examined using a phase-contrast microscope (Nikon eclipse 50i) and a Cam ScanMX 2000 scanning electron microscope. Specimen for scanning electron microscopy was prepared as follows. Strain PD524 was grown in 480GM5 and YE5 media for 24 h. Cell pellets were harvested 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. The cover slide was rinsed (30 s) using 0.1 M phosphate buffer pH 7.4 for 3–5 times and air dried at room temperature. The specimen was then dehydrated using successive 20 min washes of 30, 50, 70, 75, 90, 95, and 100 % ethanol rinses. The dried specimen was sputter coated with gold and kept in a desiccator.

Growth kinetics

The effects of temperature, pH and NaCl concentration on growth were determined in 480GM5 medium modified by addition of NaCl or pH adjustment using 1 N NaOH or 1 N HCl. Triplicate serum bottles of prewarmed medium (100 mL) were inoculated to an initial density of 10^5 cells/mL. To measure growth rates, samples (1 mL) were taken at appropriate time intervals (2–3 h) during exponential phase. Cell numbers were enumerated using a Neubauer chamber under phase contrast. Specific growth rate constants (μs) were estimated from regression analysis of semilogarithmic plots.

Carbohydrate utilization

Glucose, maltose, sucrose, fructose, lactose, galactose, trehalose, arabinose, mannose, xylose, sorbose, xylitol, sorbitol, mannitol, cellobiose, carboxymethyl cellulose, and starch were tested at 0.1 % w/v final concentration in CT5 basal medium (triplicate tubes). Cell numbers were counted at 48 h and compared to controls (without added carbohydrate). Growth was determined by the increase of cell density. Doubling of the cell density of the control was recorded as positive growth and $\geq$1.5 to <2 times of the control was recorded as slight growth stimulation.

Effect of inorganic compounds and elemental sulfur on growth

The effects of $SO_4^=$, $S_2O_3^=$, and NO_3^- on growth were tested in triplicate bottles of 480G medium (100 mL). Briefly, an overnight culture was inoculated to an initial density of 10^5 cells/mL, and the cultures were incubated at 80 °C for 24 h. Then $SO_4^=$, $S_2O_3^=$, and NO_3^- were amended into the growing cultures to a final concentration of 20 mM. Sterile distilled water was added to controls. All were further incubated for an additional 24-h period. The effect of S^o on growth was tested in 2 %S° containing 480G and 480G (control) media. Cell densities (at 24 and 48 h) were determined using direct count technique. Cell yields were compared with controls. Statistical analysis was performed with One-Way ANOVA and LSD with a p value <0.05 considered as significant.

Air sensitivity test

Oxygen sensitivity of strain PD524 growing at 80 °C in mid-exponential (15–16 h) and late-stationary (>60 h) phases was tested using a modification of the method described by Jeanthon et al. (1995). Briefly, two sets of cultures growing in 100 mL of YE5 medium were flushed vigorously with sterilized air by 0.45 μm membrane filters (one inlet and one outlet filters) for a few minutes until the resazurin containing medium turned pink. Immediately, one set of the aerated samples was further incubated at 80 °C, and the other was exposed to air at 4 °C (both sets with the inserted filters). Survival over time was determined using the MPN method (5 replicates) and YE5 medium as diluent. Numbers of turbid cultures were recorded after 2 days of incubation and were employed to determine MPN values.

SDS sensitivity test

Strain PD524 growing at 80 °C (480GM5 medium) was subjected to SDS treatments. Briefly, 5 mL of 16 and 64 h cultures (approx. total of 10^7–10^8 MPN) was treated for 5 min by adding an equal volume of SDS solution (to obtain final of 0.001, 0.003 and 0.005 %). The treated cells were centrifuged (5600 rpm for 15 min) and washed twice with sterile 480GM5 medium. The washed cell pellets were suspended in 1 mL and rinsed with another 0.5 mL of the medium. Both liquid fractions were injected into 100 mL of the medium (dilution factor of 10^2). Controls were treated with sterile N_2 saturated water (5 mL) under the same conditions. Viable cells were enumerated using MPN technique and appropriate dilution. Turbid cultures were recorded after 2 days. The decrease in survival numbers relative to controls (recovery) was considered as follows: not sensitive ($\leq$magnitude of 10), slightly sensitive ($\leq$magnitude of 10^3), and sensitive (>magnitude of 10^3–10^7), respectively.

Thermal sensitivity test

Strain PD524 growing at 80 °C in mid-exponential and late-stationary phases were subjected to heat shock at 98 °C. Briefly, cells from 16 h and 66 h cultures were transferred into 100 mL of prewarmed YE medium (98 °C) to obtain approx. final concentrations of 10^7 and 10^5 MPN/mL, respectively. Bottles were immediately incubated upside down at 98 °C. For the exponential phase, three sets of experiment were conducted. For the late-stationary phase, two sets of experiment were conducted. Samples were removed after a range of heat shock exposures (0–4 min, 0–15 min, and 10–70 min). The samples were immediately diluted for MPN determination using YE5 medium as diluent at room temperature. Survival was enumerated using the MPN technique. Turbid cultures were recorded after 2 days. The decimal reduction time (D value) of cells in 16 h culture, i.e., mid-exponential phase, was determined using data collected from 0 to 4 min treatment to compare vegetative cells to dormant cultures.

Phylogenetic analysis of *16S rRNA* gene sequences

A specific primer to Order Thermotogales named "THER3F" was designed for this study. Nucleotide sequence of UA1406R (5′ ACGGGCGGTGWGTRCAA 3′) was as previously described (Baker and Cowan 2004). This primer pair was employed to amplify a 16S rDNA fragment (1380 bp). The fragment was cloned into a PCR cloning vector, pTG19-T. Plasmid DNAs were sequenced using forward and reverse primers by AITbiotech (Singapore). Closest relatives were identified by homology searches using the blastN algorithm at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). 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.

G+C content of DNA

The G+C content of DNA was determined using thermal denaturation (Marmur and Doty 1962). Increased absorbance at 260 nm was measured using a model T70 UV–VIS Spectrophotometer (PG Instruments Ltd) and a micro quartz cuvette. The genomic DNA of *Pyrococcus furiosus* DSM 3638 (40.8 mol %) was employed as a reference.

Results

DNA properties and phylogeny

The G+C content of genomic DNA of strain PD524 was 45 mol % (Table 1). A 1380 bp stretch of *16S rRNA* gene (KF164213) was sequenced. BlastN alignment accounted for 100 % of KF164213 confirming the lack of chimeras in this sequence. BlastN analysis revealed 96 % identity to *Thermotoga maritima* MSB8[T], *T. neapolitana* NES[T], *T. petrophila* RKU-1[T], and *T. naphthophila* RKU-10[T]; 93 % identity to *Pseudothermotoga thermarum* DSM 5069[T]; and 90-91 % identity to *Thermotoga caldifontis* AZM44c09[T], *T. profunda* AZM34c06[T], *Pseudothermotoga elfii* G1[T], *Pst. subterranea* SL1[T], and *Pst. lettingae* TMO[T] (Bhandari and Gupta 2014). The results suggest that *Thermotoga caldifontis* AZM44c09[T] and *T. profunda* AZM34c06[T] (Mori et al. 2014) are the closest relatives of *Pseudothermotoga* rather than the hyperthermophilic *Thermotoga*. In addition, the hyperthermophilic *Thermotoga* and thermophilic *Pseudothermotoga* were phylogenetic placed as two distinctive clades (Fig. 1). Strain PD524 formed the deepest branch of the clade shared with the four hyperthermophilic species (*Thermotoga maritima* MSB8[T], *T. neapolitana* NES[T], *T. petrophila* RKU-1[T], and *T. naphthophila* RKU-10[T]).

Morphology

Morphology was examined after growth in 480GM5 and YE5 media. Typical cells of strain PD524 are encapsulated by a "toga," a characteristic membranous structure ballooning over the ends. Endospores characterized by a light refractile appearance under phase contrast, typical of the genus *Clostridium* were not observed. Cells appeared as long-curved rods with a small terminal bleb at both termini (Fig. 2) and were stained Gram-negative. A size range of 0.5–0.6 × 2.5–10 μm was determined. Filamentous cells sized of up to 40 μm were frequently detected (Fig. 2a, e). Spheroid bodies (diameter range of 2–4 μm) were commonly observed (Fig. 2b). A novel spherical protuberance

at a subterminal position, as named in this study a "golf club structure" (Fig. 2c–e), was rarely detected in both 480GM5 and YE5 media.

Growth kinetics

A temperature range of 70 to <90 °C (opt. temp. 80 °C), a pH range of >5.5 to 8.5 (opt. pH 7.5–8.0), and a NaCl concentration range of 0 to <10 g/L (opt. 0.5 g/L) were determined (Fig. 3a–c). The optimal growth condition (80 °C, pH 7.5, 0.5 g/L NaCl) was identified. Under optimal conditions, viable cell densities of $4.6 \times 10^7 \pm 1.5 \times 10^7$ and $1.4 \times 10^5 \pm 3.6 \times 10^4$ MPN/mL were obtained from mid-exponential (16 h) and late-stationary (64 h) phases, respectively. The results reveal that cultivatable cell numbers in the late-stationary phase decreased remarkably compared to the mid-exponential phase.

Carbohydrate utilization

No growth of strain PD524 was observed in CT basal medium containing glucose or other test carbohydrates (0.1 % w/v ea.). Slight growth was observed on CT5 basal medium, and thus, the medium was employed to test carbohydrate utilization. Glucose, sucrose, maltose, fructose, xylose, mannose, arabinose, trehalose, starch, and cellobiose were utilized as growth substrates. However, lactose, galactose, sorbose, xylitol, sorbitol, mannitol, starch, and carboxymethyl cellulose were not utilized (Table 1).

Effect of inorganic compounds and elemental sulfur on growth

Growth in 480G medium ($3.0 \times 10^7 \pm 2.1 \times 10^6$ cells/mL) was inhibited by S°, and no growth was detected in S° containing 480G medium. Growth yield after addition of $SO_4^=$ ($3.5 \times 10^7 \pm 2.5 \times 10^6$ cells/mL) was enhanced significantly (p value <0.05). However, growth was not stimulated by addition of $S_2O_3^=$ ($2.9 \times 10^7 \pm 3.1 \times 10^5$ cells/mL) and NO_3^- ($2.6 \times 10^7 \pm 1.2 \times 10^6$ cells/mL).

Air sensitivity test

Strain PD524 tested negative for catalase and did not grow in aerobic medium, as indicated by a pink color of the redox indicator resazurin. Aerobic conditions at 80 °C were lethal. Initial active cell density of the 15 h culture age (1.7×10^8 MPN/mL) declined sharply within 3 h, and no survival was detected after 6 h aeration at 80 °C. A specific death rate constant (a negative μ of -1.56 h^{-1}) was determined. Under these conditions, an initial

Table 1 Characteristics of strain PD524 and type strains of known species of the genera *Thermotoga* and *Pseudothermotoga*

Characteristic	1 (PD524)	2 (MSB8^T)	3 (NES^T)	4 (RKU-1^T)	5 (RKU-10^T)	6 (SL1^T)	7 (SEBR 6459^T)	8 (TMO^T)
Habitats	Fresh water sediment, Pong Duet Hot Spring, Thailand	Marine sediment, Vulcano, Italy	Submarine thermal vent, Lucrino, Italy	Oil reservoir, Niigata, Japan	Oil reservoir, Niigata, Japan	Oil reservoir, East Paris Basin, France	Oil producing well, Africa	Sulfate reducing bioreactor
Cell size (μm)	0.5 to 0.6 × 2.5 to 10	0.6 × 1.5 to 11	0.6 × 1.5 to 11	0.7 to 1.0 × 2 to 7	0.8 to 1.2 × 2 to 7	0.5 × 3 to 10	0.5 to 1 × 2 to 3	0.5 to 1 × 2 to 3
Temp. range (opt.), °C	70 to 85 (80)	55 to 90 (80)	55 to 90 (80)	47 to 88 (80)	48 to 86 (80)	50 to 75 (70)	50 to 72 (66)	50 to 75 (65)
pH range (opt.)	6 to 8.5 (8.0)	5.5 to 9 (6.5 to 7)	5.5 to 9 (7.0)	5.2 to 9.0 (7.0)	5.2 to 9.0 (7.0)	6 to 8 (7.0)	5.5 to <9 (7.5)	6 to 8.5 (7.0)
NaCl range (opt.), g/L	0 to <10 (0.5)	2.5 to 37.5 (27)	nr	1 to 55 (10)	1 to 60 (10)	0 to 24 (12)	0 to 28 (10)	0 to 28 (10)
Generation time, min	78	75	45	55–60	50–60	285	168	240
Utilization								
Glucose	+	+	+	+	+	±	+	+
Sucrose	+	+	+	+	+	nr	+	+
Maltose	+	+	+	+	+	+	+	+
Lactose	−	+	+	+	+	nr	+	+
Fructose	+	+	nr	+	+	nr	+	+
Xylose	+	+	+	−	−	nr	−	+
Arabinose	±	nr	nr	+	+	nr	+	+
Galactose	−	+	+	+	+	nr	nr	+
Mannose	+	nr	nr	nr	nr	nr	+	+
Sorbitol	−	nr	nr	nr	nr	nr	−	nr
Mannitol	−	−	nr	−	+	nr	−	−
Trehalose	+	nr	nr	nr	nr	nr	nr	nr
Starch	±	+	+	+	+	nr	nr	+
Cellobiose	±	nr	nr	nr	nr	nr	nr	+
G+C content, mol %	45	46.2	41	46.8	46.1	40	39.6	39.2
Growth stimulation by								
S°	Inhibition	No	nr	Inhibition	Inhibition	Inhibition	Inhibition	Yes
SO$_4^=$	Yes	nr	nr	nr	nr	No	No	No
S$_2$O$_3^=$	No	nr	nr	Inhibition	No	Yes	Yes	Yes
NO$_3^-$	No	nr	nr	nr	nr	No	nr	No

Taxa: 1, *Thermotoga* sp. PD524 (this study); 2, *Thermotoga maritima* MSB8^T (Huber et al. 1986); 3, *Thermotoga neapolitana* NES^T (Jannasch et al. 1988); 4, *Thermotoga petrophila* RKU-1^T (Takahata et al. 2001); 5, *Thermotoga naphthophila* RKU-10^T (Takahata et al. 2001); 6, *Pseudothermotoga subterranea* SL1^T (Jeanthon et al. 1995); 7, *Pseudothermotoga elfii* SEBR 6459^T (Ravot et al. 1995); 8, *Pseudothermotoga lettingae* TMO^T (Balk et al. 2002)

+, Positive; ±, slightly positive; −, negative; nr, not reported; yes, stimulate; no, not stimulate
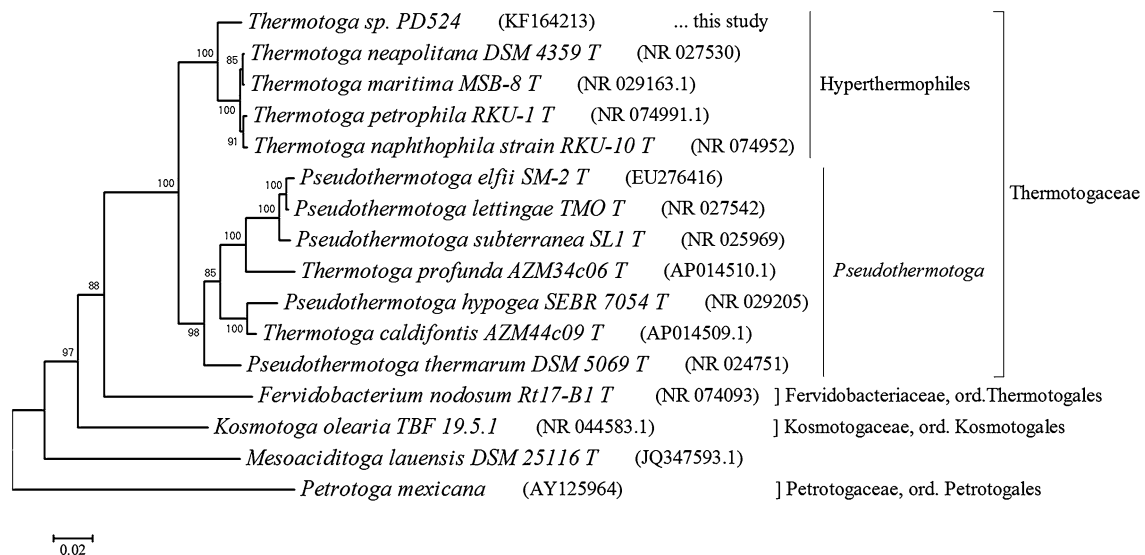
Fig. 1 A neighbor joining tree of *16S rRNA* gene sequences of species belonging to genera *Thermotoga* and *Pseudothermotoga* (fam. Thermotogaceae, ord. Thermotogales). The *16S rRNA* gene sequences of *Fervidobacterium nodosum* Rt17-B1[T] (NR_074093.1) (fam. Fervidobacteriaceae, ord. Thermotogales), *Petrotoga mexicana* (AY125964) (fam. Petrotogaceae, ord. Petrotogales), *Kosmotoga olearia* TBF 19.5.1[T] (NR_044583.1) (fam. Kosmotogaceae, ord. Kosmotogales), and *Mesoaciditoga lauensis* DSM 25116[T] (JQ347593.1) were employed as representatives for the other families and orders belonging to phylum Thermotogae. A bootstrap value of 1000 is presented as percentage

cultivatable cell density (4.0×10^6 MPN/mL) of the 66 h culture age appeared to be more resistant (a negative μ of -1.08 h^{-1}), and about 20 MPN/mL survival was detected at 6 h after the air treatment. In contrast, both active and dormant populations survived aerobic conditions at 4 °C for >221 h and 96 h, respectively (Supplementary Fig. S1).

SDS sensitivity

Cells of strain PD524 from mid-exponential (16 h) and late-stationary phases (64 h) were revealed sensitive to SDS in a dose-dependent manner compared with their controls (Supplementary Fig. S2). Approx. $3.1 \times 10^7 \pm 1.3 \times 10^7$ (16 % of the initial load) and $6.5 \times 10^5 \pm 5.2 \times 10^5$ (6 % of the initial load) MPN of controls were recovered, respectively. Survival of 0–1000 MPN/mL were obtained after the 0.003 and 0.005 % SDS treatments. However, cells from both phases were slightly sensitive to 0.001 % SDS treatment (Supplementary Table S1). To compare resistant properties of the actively growing and dormant cells, the experiment was repeated at 0.001 % SDS. The results confirmed that actively growing cells (survival of $2.2 \times 10^4 \pm 7.9 \times 10^3$ MPN/mL) were substantially more sensitive to the membrane active detergent than those dormant cells (survival of $2.1 \times 10^5 \pm 2.7 \times 10^4$ MPN/mL), respectively (Supplementary Table S2 and Fig. S3).

Thermal sensitivity of cells in exponential and late-stationary phases

Cells in exponential phase (16 h) proved to be extremely sensitive to lethal heat shock at 98 °C, and decimation of viable cells was detected with the decimal reduction time (*D* value) value of 3.9 min (Fig. 4a). However, approx. 10 % of the population was detected and survived after 60 min treatment. A D value at 98 °C (60 min) was predicted for this subpopulation (Fig. 4b). In contrast, cells in late-stationary phase were more resistant to heat. A longer *D* value at 98 °C (100 min) was calculated, and no substantial reduction of the culture viability was observed after 70 min treatment (Fig. 4c).

Discussion

A hyperthermophilic strain PD524 was isolated from a fresh water hot spring in Thailand. Based on *16S rRNA* gene sequence (KF164213), strain PD524 was identified as belonging to the hyperthermophilic clade of the genus *Thermotoga* (Fig. 1). The G+C content of strain PD524 (45 mol %) was determined. Typical cells appeared as flexible rods (0.5–0.6 × 2.5–10 μm), completely surrounded by an outer membrane, with extrusions at both ends, a structural characteristic shared with most bacterial lineages belonging to the deep branching order Thermotogales.

Fig. 2 SEM (**a, d, e**) and phase-contrast (**b, c**) images of strain PD524. **a** Typical rod-shaped and filamentous cells. **b** A spheroid. **c** A golf club-shaped cell. **d** Typical rod-shaped and golf club-shaped cells. **e** Cluster of typical cells and a cell harboring, a subterminally dark spherical form or a club head. *Scale bars* are indicated
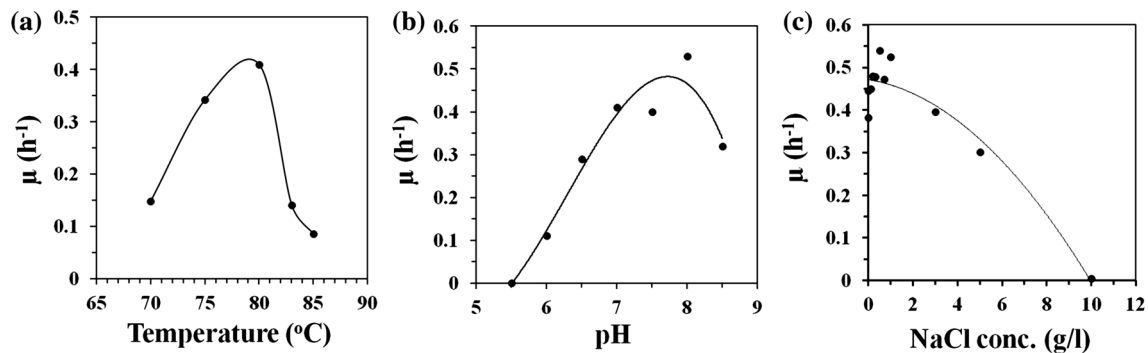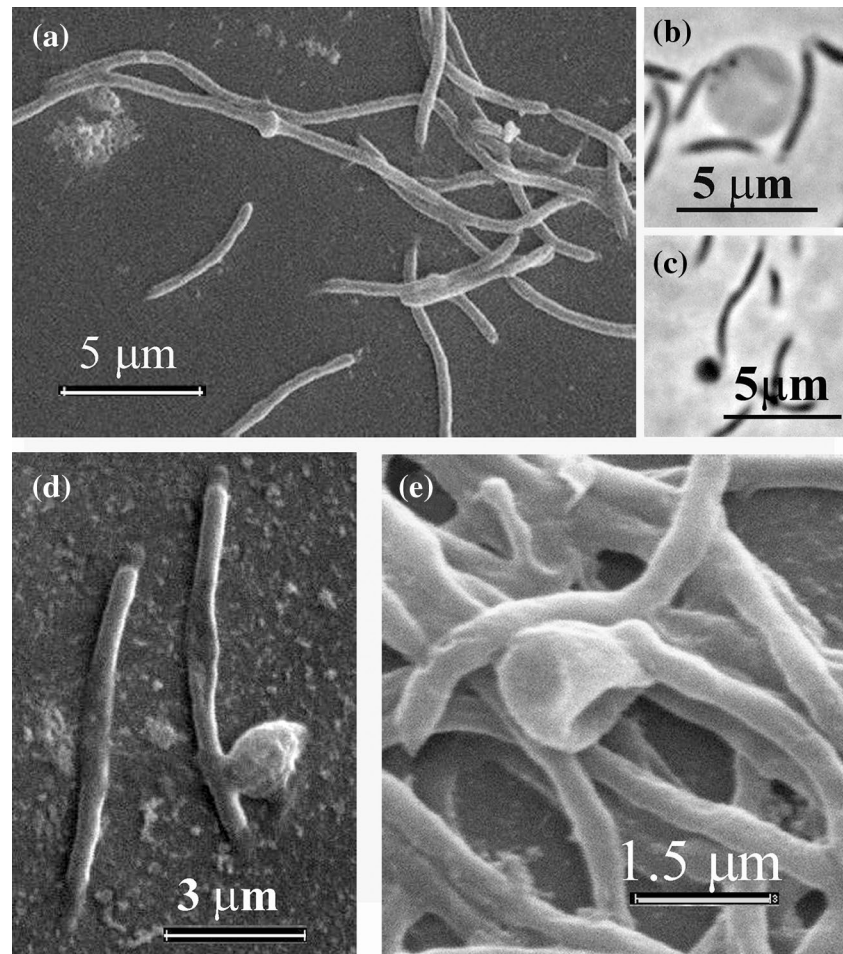
Fig. 3 Growth kinetics of strain PD524 determined on 480GM5 medium **a** Growth rates versus temperature. **b** Growth rates versus pH. **c** Growth rates versus concentrations of NaCl

Similar to *Pseudothermotoga subterranea* strain SL1[T], cells of strain PD524 were observed pleomorphic in a stationary phase. Strain PD524 developed a novel protuberance as named in this study a "golf club structure" in both 480GM5 and YE5 media (Fig. 2). An optimal growth condition (80 °C, pH 7.5, 0.5 g/l NaCl) was identified (Fig. 3). Differential characteristics of strain PD524

and other *Thermotoga* species are shown in Table 1. In Table 1, strain PD524 is sensitive to the lowest NaCl concentration (max. conc. <10 g/L), unlike the marine species *T. maritima* MSB8[T] (max. conc. <37.5 g/L), *T. petrophila* RKU-1[T] (max. conc. 55 g/L), and *T. naphthophila* RKU-10[T] (max. conc. 60 g/L). Similar to *T. petrophila* RKU-1[T] and *T. naphthophila* RKU-10[T], growth of strain PD524 was

(a)

(b)

(c)
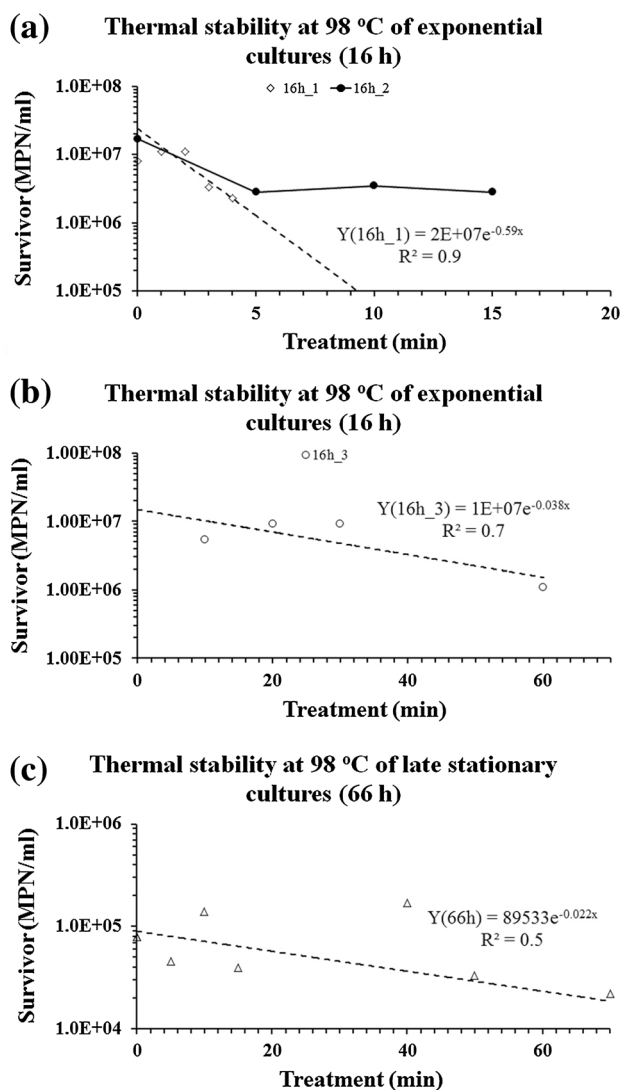
Fig. 4 Thermal sensitivity of strain PD524 tested at 98 °C on cells from exponential (16 h) and late-stationary phases (66 h). **a** Two series of experiment were conducted on 16 h culture age, 0–4 and 0–15 min treatments. Sampling intervals (dosed) were as indicated. *Symbols*: *White diamond* represents survivors during the first 4 min treatment. *Black circle and line* represents survivor during the first 15 min treatment. *Dotted line* represents exponential trend line ($\mu = -0.59$ min^{-1}). **b** A 60 min treatment was conducted on resistant subpopulation of the 16 h culture age. *Dotted line* represents exponential trend ($\mu = -0.04$ min^{-1}). **c** A 70 min treatment experiment was conducted on dormant population (66 h). *Dotted line* represents exponential trend line ($\mu = -0.02$ min^{-1})

inhibited by S°. Growth yield of strain PD524 was stimulated by adding $SO_4^=$, but not by adding $S_2O_3^=$ and NO_3^-.

In general, most microorganisms developed resistant cell stage(s) to cope with unfavorable environmental conditions (Roszak and Colwell 1987; González et al. 1996). In order to understand survival mechanism of this obligately anaerobic, hyperthermophilic strain PD524, its sensitivities to air at 4 and 80 °C, SDS concentrations and thermal sensitivity

of cells from the exponential and stationary phases were compared. In this study, viable cells of strain PD524 growing in the late-stationary phase sharply decreased and developed a dormant subpopulation (approx. 0.1–10 %) relative to cells in the exponential phase ($4.6 \times 10^7 \pm 1.5 \times 10^7$ MPN/mL). The dormant subpopulation of strain PD524 was substantially more resistant to air at optimal growth temperature than actively growing cells. However, both cell types were resistant to air at the nonpermissive growth temperature (Supplementary Fig. S1). It is likely that this is a survival strategy of this strictly anaerobic, hyperthermophilic bacterium in coping with aerobic conditions downstream of its anaerobic geothermal isolation locale. Although active growing and dormant cells were very sensitive to high concentrations of SDS (≥ 0.003 %), strong detergent, dormant cells in late-stationary phase were remarkably more resistant to 0.001 % SDS than exponential phase (Supplementary Fig. S3), suggesting differential cell surface compositions of both subpopulations belonging to this membrane encapsulated bacteria. Heat shock experiments revealed that approx. 90 % of active cells were highly sensitive to heat (*D* value of 3.9 min at 98 °C). However, a dormant subpopulation survived heat treatment at 98 °C for >60 min (Fig. 4b, c) indicating heat resistant variants in late-stationary phase. Although no known morphologically distinguishable resistant stage could be demonstrated using microscopic methods, an unknown functional golf club structure mentioned above as well as other related unusually shaped cells was observed in the early stationary phase. Whether or not they play a role in survival mechanism remains to be investigated.

In conclusion, this study presents a hyperthermophilic bacterium, *Thermotoga* sp. strain PD524, isolated from an unexplored hot spring in Thailand and its characterization including morphological, physiological, and phylogenetical perspectives. It highlights the survival strategies of this microorganism against potential environmental changing conditions of oxygenation, denaturing agents, and temperature pointing to the presence of differential survival in distinctive subpopulations within this hyperthermophile. The results suggest that strain PD524 employed a number of strategies to thrive within an unstable range of conditions, at or around their typical hot spring environments.

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