Postprint of: Extremophiles (4): 853-861 (2015)

Isolation and Characterization of a *Thermotoga* sp. Strain PD524, a Hyperthermophile Isolated from a Hot Spring in Thailand

Wirojne Kanoksilapatham[1*], Porranee Keawram[1], Juan M. Gonzalez[2] and Frank T. Robb[3#]

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

[2]IRNAS-CSIC, Avda. Reina Mercedes 10, 41012 Sevilla, Spain

[3]Institute of Marine and Environmental Technology, 701 E. Pratt Street, and Department of Microbiology and Immunology, University of Maryland, Baltimore, MD 21202, USA

- * Address correspondence to: wirojne@su.ac.th
- and[#] : frobb@som.umaryland.edu

Keywords: Thermotogales, hyperthermophile, thermal sensitivity, SDS sensitivity, hot spring

Running title: *Thermotoga* sp. Strain PD524

Abstract

A hyperthermophilic *Thermotoga* sp. strain PD524^T was isolated from a hot spring in Northern Thailand. Cells were slender rod shaped (0.5-0.6x2.5-10 μm) surrounded by a typical outer membranous toga. Strain PD524^T is aero-tolerant at 4ºC but aero-sensitive at 80ºC. A heat resistant subpopulation was observed in late stationary growth phase. Cells from late stationary growth phase were revealed substantially more resistant to 0.001% SDS treatment than cells from exponential growth phase. The temperature range for growth was 70-85ºC (opt. temp. 80ºC), pH range was 6-8.5 (opt. pH 7.5-8.0) and NaCl conc. range of 0-<10 g/L (opt. conc. 0.5 g/L). Glucose, sucrose, maltose, D-fructose, xylose, D-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^T revealed the 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 includes many thermophilic and hyperthermophilic species that thrive in geothermal environments including continental hot springs, high temperature oil reservoirs and undersea hydrothermal vents. Members of this order all possess a characteristic outer sheath-like membranous structure, the so-called toga. Most of them have optimal temperatures ranging from 50-90°C that include five hyperthermophilic species of *Thermotoga maritima* MSB8^T, *T. neapolitana* NES^T, *T. petrophila* RKU-1^T, *T. naphthophila* RKU-10^T and *Fervidobacterium changbaicum* (Huber et al. 1986; Jannasch et al. 1988; Takahata et al. 2001; Cai et al. 2007). To date, 11 genera have been classified 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) and *Defluviitoga* (Ben Hania et al. 2012). 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. Species assigned to the genus *Thermotoga* 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 *T. subterranea* strain SL1^T growing in late exponential growth phase are sensitive to dissolved oxygen at growth temperature however, cell viability was retained at 4 °C for 1 day, and approximately 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 on morphology, phylogenetic analysis of *16S rRNA* gene, growth kinetics and physiological properties. Resistance to oxygen (at permissive and nonpermissive growth temperatures), SDS concentrations and thermal sensitivity of strain PD524^T in exponential and stationary growth phases were compared.

Materials and methods

Media

The 480GM5 medium (an isolation medium) was composed of (per L) 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 (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 $Na_2S.9H_2O$ solution [25% (w/v), pH 7]. The pH was adjusted to 7.2-7.5 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. Sterilization was performed at 121°C for 20 min. The A5 stock 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) per L.

The 480G medium is as described for the 480GM5 medium except that pancreatic digestion of casein (1 g/L) was employed. YE5 medium has similar composition to the 480GM5 medium except that the pancreatic

digestion of casein (5g/L) was replaced by 5 g/L of yeast extract. CT basal medium is similar to the 480GM5 medium except that concentrations of pancreatic digestion of casein (0.1 g/L) and yeast extract (0.05 g/L) were reduced, respectively. CT5 basal medium contains similar composition to the 480GM5 medium except that pancreatic digestion of casein (0.5 g/L) was employed as major organic nitrogen source. This medium was used to test for utilization of carbohydrates.

Isolation of strain PD524^T

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

Morphology

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

Specimen preparation for scanning electron microscopy

Strain PD524^T was grown in 480GM5 and YE5 media for 24 h. Cell pellets were harvested by centrifugation. The pellet was 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^{o}C. The cover slide was rinsed (30 sec.) 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 gold sputter coated 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 1N NaOH or 1N 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 growth 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, D-fructose, lactose, D-galactose, trehalose, arabinose, D-mannose, xylose, L-sorbose, xylitol, D-sorbitol, mannitol, cellobiose, carboxymethyl cellulose and starch were tested at 0.1 % w/v final concentration in CT5 basal medium (triplicate tubes). Cell numbers at 48 h were counted under a phase contrast microscope 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 ≥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 was tested in a 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 80ºC 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. Controls were diluted with equal volume of sterile distilled water. All were further incubated for an additional 24 h period. The effect of S^o on growth was tested in 2%S^o 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^T growing at 80ºC in mid exponential (15-16 h) and late stationary growth (>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 sterile air through 0.45 µm membrane filters (one inlet and one outlet filter) until the resazurin containing medium turned pink (few minutes). One set of aerated samples was exposed to air at 4ºC, and the others were further incubated at 80ºC (with the inserted filters). Survival over time was determined using the MPN method (5 replicates) and YE5 medium as diluent. Turbid cultures were recorded after 2 days to determine MPN values.

SDS sensitivity test

Strain PD524^T growing at 80ºC in mid exponential (16 h) and late stationary (64 h) growth phases were subjected to SDS treatments. Briefly, 5 ml of 16 h and 64 h cultures (approx. total of 10^7-10^8 MPN) were employed as load and treated for 5 min with equal volume of SDS (to obtained final conc. 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 scored after 2 days. Decrease in survival numbers relative to recovery in controls were recorded as follows: not sensitive (≤1 log cycle), slightly sensitive (≤3 log cycle) and sensitive (>3-7 log cycle), respectively.

Thermal sensitivity test

Strain PD524^T growing at 80ºC in mid exponential and late stationary growth phases were subjected to heat shock at 98ºC. Briefly, cells from 16 h and 66 h cultures were transferred into 100 ml of prewarmed YE medium (98ºC) to obtain approx. final concentrations of 10^7 and 10^5 MPN/ml, respectively. Bottles were immediately

incubated up-side-down at 98ºC. For the exponential growth phase, three sets of experiment were conducted. For the late stationary growth 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 to appropriate dilutions using YE5 medium as diluent at room temperature. Survival was enumerated using the MPN technique. Turbid cultures were scored 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 0-4 min treatment to compare vegetative cells to dormant cultures.

Phylogenetic analysis of *16S rRNA* gene sequences

THER3F primer (5' AGGGTTTGATCMTGG 3') was designed in this study. Nucleotide sequence of UA1406R (5' ACGGGCGGTGWGTRCAA 3') was as previously described (Baker and Cowan 2004). The primer pairs were employed to amplify a 16S rDNA fragment (1380 bp). The fragment was cloned into a PCR cloning vector, pTG19-T. Plasmid DNAs were sequenced twice 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.

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^T was 45 mol% (Table 1). A 1380 bp stretch of *16S rRNA* gene (KF164213) was sequenced. 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 *Thermotoga thermarum* DSM 5069^T; and 90-92% identity to *Thermotoga elfii* G1^T, *T. subterranea* SL1^T, *T. lettingae* TMO^T, *T. profunda* AZM34c06^T, *T. hypogea* SEBR 7054^T, and *T. caldifontis* AZM44c09^T. Phylogenetic analysis has separated the genus *Thermotoga* into two clades (Fig. 1). Strain PD524^T was classified as the deepest branch 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). The isolated strain is *Thermotoga* sp. strain PD524^T, DSM 28089^T.

Morphology

Morphology was examined after growth in 480GM5 and YE5 media. Typical cells of strain PD524^T are encapsulated by a "toga", a characteristic membranous structure ballooning over the ends. Cells appeared as

long, curved slender rods with a small terminal bleb at both termini (Fig. 2), and were stained Gram-negative. A size range of 0.5-0.6x2.5-10 µm was determined. Filamentous cells sized of up to 40 µm were easily detected (Fig. 2a, e). Spheroid bodies (diameter range of 2-4 µm) were common (Fig. 2b). Strain PD524^T produced a novel spherical protuberance at a subterminal position, named in this study a "golf club structure" (Fig. 2c, d, e).

Growth kinetics

A temperature range of 70-<90ºC (opt. temp 80ºC), a pH range of >5.5-8.5 (opt. pH 7.5-8.0) and a NaCl range of 0-<10 g/L (opt. conc. 0.5 g/L) were determined (Fig. 3a, b, c). At optimal condition (80ºC, pH 7.5, 0.5 g/L NaCl), viable cell densities of $4.6x10^7 \pm 1.5x10^7$ MPN/ml was detected in mid exponential growth phase (16 h) however, remarkably decrease in viable cell density ($1.4x10^5 \pm 3.6x10^4$ MPN/ml) was observed in late stationary growth phase (64 h).

Carbohydrate utilization

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

Effect of inorganic compounds and elemental sulfur on growth

Growth in 480G medium ($3.0x10^7 \pm 2.1x10^6$ cells/ml) was inhibited by S^o and no growth was detected in S^o containing 480G medium. Growth yield after addition of $SO_4^=$ ($3.5x10^7 \pm 2.5x10^6$ cells/ml) was enhanced significantly (p-value <0.05). However, growth was not stimulated by addition of $S_2O_3^=$ ($2.9x10^7 \pm 3.1x10^5$ cells/ml) and NO_3^- ($2.6x10^7 \pm 1.2x10^6$ cells/ml).

Air sensitivity test

Strain PD524^T tested negative for catalase and did not grow in aerobic medium, as indicated by a pink colour of the redox indicator resazurin. Cells were highly sensitive to aerobic conditions at 80ºC. Initial active cell density of the 15 h culture age ($1.7x10^8$ MPN/ml) declined sharply within 3 h and no survival was detected after 6 h aeration at 80ºC. A specific death rate constant (a negative µ of -1.56 h^{-1}) was determined. Under these conditions, an initial cultivatable cell density ($4.0x10^6$ 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 4ºC for >221 h and 96 h, respectively (Supplementary Fig. S1).

SDS sensitivity

Strain PD524^T growing in exponential (16 h) and late stationary (64 h) phases were revealed sensitive to SDS in a dose dependent manner compared with their controls (Supplementary Fig. S2). Approx. $3.1x10^7 \pm 1.3x10^7$ (16%

of the total load) and $6.5x10^5 \pm 5.2x10^5$ (6% of the total load) MPN/ml 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). Repeated experiment performed at 0.001% SDS confirmed that actively growing cells (survival of $2.2x10^4 \pm 7.9x10^3$ MPN/ml) were substantially more sensitive than those dormant cells (survival of $2.1x10^5 \pm 2.7x10^4$ MPN/ml), respectively (Supplementary Table S2 and Fig. S3).

Thermal sensitivity of cells in exponential and late stationary

Cells in exponential growth phase (16 h) proved to be 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 recorded (Fig. 4a). However, approx.10% of the population survived after 60 min treatment (Fig. 4b). A D-value (at 98 °C) of 60 min was predicted for this subpopulation. In contrast, cells in late stationary growth phase were more resistant to heat. A D-value (at 98°C) of 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^T was isolated and identified as a hyperthermophilic clade of the genus *Thermotoga* (Fig. 1). The G+C content of strain PD524^T (45 mol%) was determined. Typical cells of strain PD524^T appeared as pleomorphic, flexible rods (0.5-0.6x2.5-10 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. Strain PD524^T developed a novel structure named in this study a "golf club structure" (Fig. 2). An optimal growth condition (80°C, pH 7.5, 0.5 g/L NaCl) was revealed (Fig. 3). Differential characteristics of strain PD524^T and other *Thermotoga* species are summarized in Table 1. Similar to most microorganisms, strain PD524^T developed a dormant subpopulation (approx. 0.1-10% of active growing cells) to cope with unfavorable environmental conditions (Roszak and Colwell 1987; González et al. 1996). The dormant subpopulation of strain PD524^T was substantially more resistant to air at optimal growth temperature than actively growing cells. However, both cell types were resistant to air at the non-permissive 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 highly sensitive to high concentrations of SDS (≥0.003%), a strong detergent, dormant cells in late stationary phase were remarkably more resistant to 0.001% SDS than exponential phase (Supplementary Fig. S3). Heat shock experiments revealed that approx. 90% of active cells were sensitive to heat at 98°C (D-value of 3.9 min). However, a dormant subpopulation survived heat treatment at 98°C for >60 min (Fig. 4b, c) indicating heat resistant variants in late stationary growth phase.

Acknowledgements

This work was supported by the following grants: the Scientific Promotion and Development Fund, Faculty of Science, Silpakorn University (SFR-SRG-2558-01) and the Silpakorn University Research and Development

Institution from Thailand; and the Ministry of Economy and Productivity (Consolider CSD2009-00006) and the Andalusian Government (BIO288) from Spain with participation of FEDER funds. FTR acknowledges support from the US National Science Foundation. Special thanks to Witoon Wattananit, Scientific and Technological Equipment Centre, Silpakorn University for the SEM work and to Juergen Wiegel for helpful comments and discussion.

References

Baker GC, Cowan DA (2004) 16S rDNA primers and the unbiased assessment of thermophile diversity. Biochem Soc Trans 32:218–221

Balk M, Weijma J, Stams, AJ (2002) *Thermotoga lettingae* sp. nov., a novel thermophilic, methanol-degrading bacterium isolated from a thermophilic anaerobic reactor. Int J Syst Evol Microbiol 52:1361–1368

Ben Hania W, Godbane R, Postec A, Hamdi M, Ollivier B, Fardeau, ML (2012) *Defluviitoga tunisiensis* gen. nov., sp. nov., a thermophilic bacterium isolated from a mesothermic and anaerobic whey digester. Int J Syst Evol Microbiol 62:1377–1382

Cai J, Wang Y, Liu D, Zeng Y, Xue Y (2007) *Fervidobacterium changbaicum* sp. nov., a novel thermophilic anaerobic bacterium isolated from a hot spring of the Changbai Mountains, China. Int J Syst Evol Microbiol 57:2333–2336

González JM, Kato C, Horikoshi K (1996) Culturability and survival of an extreme thermophile isolated from deep-sea hydrothermal vents. Arch Microbiol 166:64–67

Davey ME, Wood WA, Key R, Nakamura K, Stahl DA (1993) Isolation of three species of *Geotoga* and *Petrotoga*: two new genera, representing a new lineage in the bacterial line of descent distantly related to the "Thermotogales". Syst Appl Microbiol 16:191–200

DiPippo JL, Nesbø CL, Dahle H, Doolittle WF, Birkland NK, Noll KM (2009) *Kosmotoga olearia* gen. nov., sp. nov., a thermophilic, anaerobic heterotroph isolated from an oil production fluid. Int J Syst Evol Microbiol 59:2991–3000

Duckworth AW, Grant WD, Jones BE, Van Steenbergen RP (1996) Phylogenetic diversity of soda lake alkaliphiles. FEMS Microbiol Ecol 19:181–191

Fardeau ML, Ollivier B, Patel BKC, Magot M, Thomas P, Rimbault A, Rocchiccioli F, Garcia JL (1997) *Thermotoga hypogea* sp. nov., a xylanolytic, thermophilic bacterium from an oil-producing well. Int J Syst Bacteriol 47:1013–1019

Feng Y, Cheng L, Zhang X, Li X, Deng Y, Zhang H (2010) *Thermococcoides shengliensis* gen. nov., sp. nov., a new member of the order Thermotogales isolated from oil-production fluid. Int J Syst Evol Microbiol 60:932–937

Jannasch HW, Huber R, Belkin S, Stetter KO (1988) *Thermotoga neapolitana* sp. nov. of the extremely thermophilic, eubacterial genus *Thermotoga*. Arch Microbiol 150:103–104

Jayasinghearachchi HS, Lal B (2011) *Oceanotoga teriensis* gen. nov., sp. nov., a thermophilic bacterium isolated from offshore oil-producing wells. Int J Syst Evol Microbiol 61:554–560

Jeanthon C, Reysenbach AL, L'Haridon S, Gambacorta A, Pace NR, Glénat P, Prieur D (1995) *Thermotoga subterranea* sp. nov., a new thermophilic bacterium isolated from a continental oil reservoir. Arch Microbiol 164:91–97

Huber R, Langworthy TA, König H, Thomm M, Woese CR, Sleytr UB, Stetter KO (1986) *Thermotoga maritima* sp. nov. represents a new genus of unique extremely thermophilic eubacteria growing up to 90°C. Arch Microbiol 144:324–333

Huber R, Woese CR, Langworthy TA, Fricke H, Stetter KO (1989) *Thermosipho africanus* gen. nov., represents a new genus of thermophilic eubacteria within the "Thermotogales". Syst Appl Microbiol 12:32–37

Marmur J, Doty P (1962) Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J Mol Biol 5:109–118

Nesbø CL, Bradnan DM, Adebusuyi A, Dlutek M, Petrus AK, Foght J, Doolittle WF, Noll KM (2012) *Mesotoga prima* gen. nov., sp. nov., the first described mesophilic species of the Thermotogales. Extremophiles 16:387-393

Nunoura T, Hirai M, Imachi H, Miyazaki M, Makita H, Hirayama H, Furushima Y, Yamamoto H and Takai K (2010) *Kosmotoga arenicorallina* sp. nov. a thermophilic and obligately anaerobic heterotroph isolated from a shallow hydrothermal system occurring within a coral reef, southern part of the Yaeyama Archipelago, Japan, reclassification of *Thermococcoides shengliensis* as *Kosmotoga shengliensis* comb. nov., and emended description of the genus *Kosmotoga*. Arch Microbiol 192:811–819

Patel BKC, Morgan HW, Daniel RM (1985) *Fervidobacterium nodosum* gen. nov. and spec. nov., a new chemoorganotrophic, caldoactive, anaerobic bacterium. Arch Microbiol 141:63-69

Ravot G, Magot M, Fardeau ML, Patel BKC, Prensier G, Egan A, Garcia JL, Ollivier B (1995) *Thermotoga elfii* sp. nov., a novel thermophilic bacterium from an African oil-producing well. Int J Syst Bacteriol 45:308–314

Roszak DB, Colwell RR (1987) Survival strategies of bacteria in natural environment. Microbiol Rev 51:365–379

Takahata Y, Nishijima M, Hoaki T, Maruyama T (2001) *Thermotoga petrophila* sp. nov. and *Thermotoga naphthophila* sp. nov., two hyperthermophilic bacteria from the Kubiki oil reservoir in Niigata, Japan. Int J Syst Evol Microbiol 51:1901–1909

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Thompson JD, Higgins DG, Gibson TJ (1994) The CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Wery N, Lesongeur F, Pignet P, Derennes V, Cambon-Bonavita MA, Godfroy A, Barbier G (2001) *Marinitoga camini* gen. nov., sp. nov., a rod-shaped bacterium belonging to the order Thermotogales, isolated from a deep-sea hydrothermal vent. Int J Syst Evol Microbiol 51:495–504

Windberger E, Huber R, Trincone A, Fricke H, Stetter KO (1989) *Thermotoga thermarum* sp. nov. and *Thermotoga neapolitana* occurring in African continental solfataric springs. Arch Microbiol 151:506-512

Figure legends

Fig. 1 A Neighbor joining tree of *16S rRNA* gene sequences of species belonging to genus *Thermotoga.* A bootstrap value of 1000 is presented as percentage. The *16S rRNA* gene sequence of *Carboxydothermus pertinax* (AB573433.1) was employed as outgroup.

Fig. 2 SEM (**a, d-e**) and phase contrast (**b-c**) images of strain PD524^{T}. (**a**) Typical rod shaped and filamentous cells. (**b**) A spheroid. (**c**) A golf club shaped cell. (**d**) A typical rod shaped and a golf club shaped cells. (**e**) Cluster of typical cells and a cell harboring a subterminal club head. Scale bars are as indicated.

Fig. 3 Growth kinetics of strain PD524^{T} determined on 480GM5 medium (**a**) Growth rates *versus* temperature. (**b**) Growth rates *versus* pH. (**c**) Growth rates *versus* concentrations of NaCl.

Fig. 4 Thermal sensitivity of strain PD524^{T} tested at 98ºC on cells from exponential (16 h) and late stationary growth phases (66 h). (**a**) Two series of experiment were conducting on 16 h culture age. Doses of the treatment were as indicated. Symbols: White diamond represents survivors during the first 4 min treatment. Black circle and line represents survivor after 5 min treatment. Dotted line represents exponential trend line (μ = - 0.59 min^{-1}). (**b**) Experiment was conducted on resistant subpopulation of the 16 h culture age for 60 min treatment. Symbol: Dotted line represents exponential trend (μ = - 0.04 min^{-1}). (**c**) Experiment was conducted on dormant population (66 h) for 70 min treatment. Symbol: Dotted line represents exponential trend line (μ = - 0.02 min^{-1}).

Figure 1

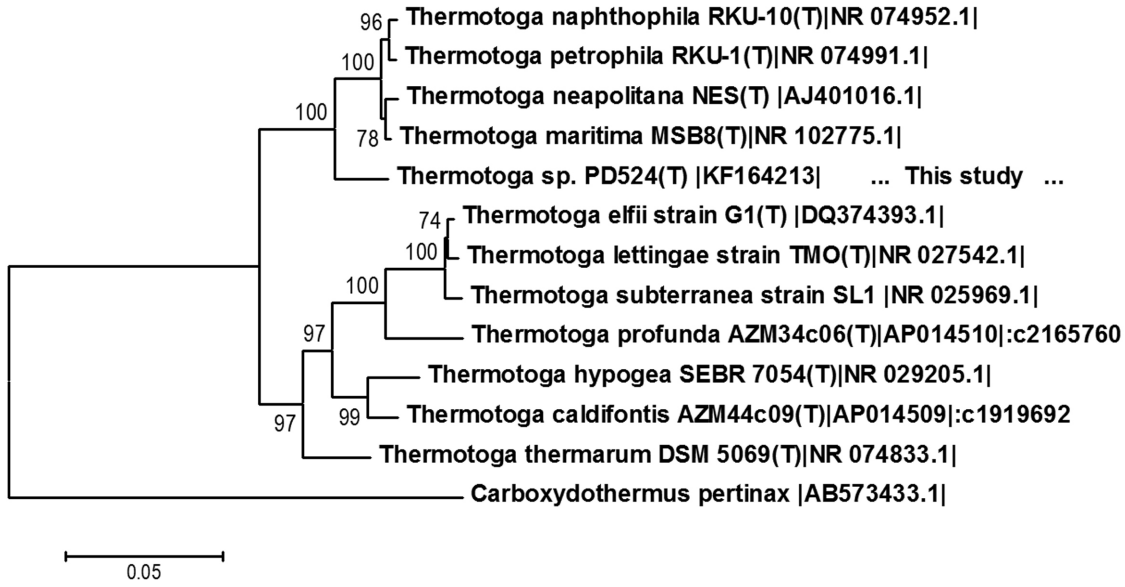
96
Thermotoga naphthophila RKU-10(T)|NR 074952.1|
100
Thermotoga petrophila RKU-1(T)|NR 074991.1|
Thermotoga neapolitana NES(T) |AJ401016.1|
100
78
Thermotoga maritima MSB8(T)|NR 102775.1|
Thermotoga sp. PD524(T) |KF164213| ... This study ...
74
Thermotoga elfii strain G1(T) |DQ374393.1|
100
Thermotoga lettingae strain TMO(T)|NR 027542.1|
100
Thermotoga subterranea strain SL1 |NR 025969.1|
97
Thermotoga profunda AZM34c06(T)|AP014510|:c2165760
Thermotoga hypogea SEBR 7054(T)|NR 029205.1|
97
99
Thermotoga caldifontis AZM44c09(T)|AP014509|:c1919692
Thermotoga thermarum DSM 5069(T)|NR 074833.1|
Carboxydothermus pertinax |AB573433.1|
0.05

Figure 2

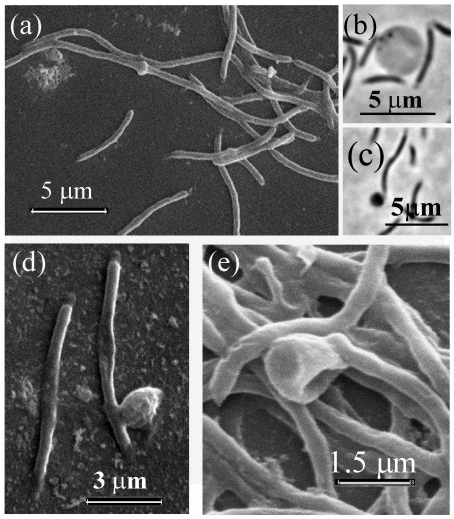

Figure 3

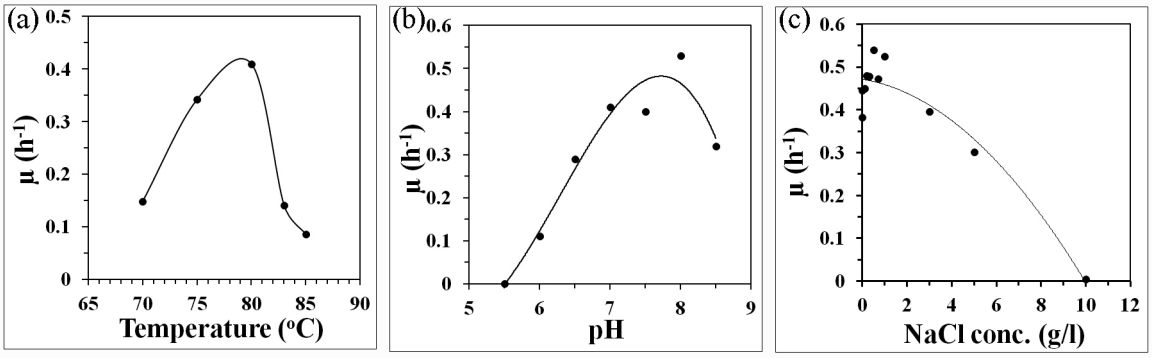

Figure 4

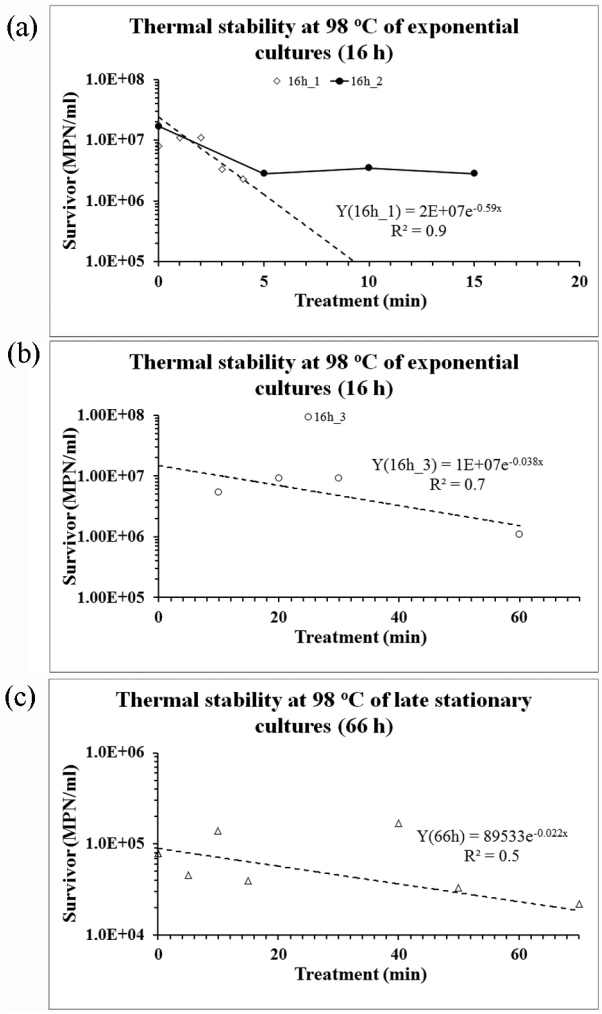
(a)
Thermal stability at 98 ºC of exponential cultures (16 h)
16h_1
16h_2
Survivor (MPN/ml)
1.0E+08
1.0E+07
1.0E+06
1.0E+05
Y(16h_1) = 2E+07e-0.59x
R² = 0.9
0
5
10
15
20
Treatment (min)
(b)
Thermal stability at 98 ºC of exponential cultures (16 h)
16h_3
Survivor (MPN/ml)
1.00E+08
1.00E+07
1.00E+06
1.00E+05
Y(16h_3) = 1E+07e-0.038x
R² = 0.7
0
20
40
60
Treatment (min)
(c)
Thermal stability at 98 ºC of late stationary cultures (66 h)
Survivor (MPN/ml)
1.0E+06
1.0E+05
1.0E+04
Y(66h) = 89533e-0.022x
R² = 0.5
0
20
40
60
Treatment (min)

Table 1. Characteristics of strain PD524^T and type strains of known species of the genus *Thermotoga*.

Taxa 1, *Thermotoga* sp. PD524^T (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, *Thermotoga subterranean* SL1^T (Jeanthon et al. 1995); 7, *Thermotoga elfii* SEBR 6459^T (Ravot et al. 1995); 8, *Thermotoga lettingae* TMO^T (Balk et al., 2002). +, Positive; ±, slightly positive; -, negative; nr, not reported; Yes, stimulate; No, not stimulate.

Characteristic	1 (PD524^T)	2 (MSB8^T)	3 (NES^T)	4 (RKU-1^T)	5 (RKU10^T)	6 (SL1^T)	7 (SEBR 6459^T)	8 (TMO^T)
Habitats	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)	0.5-0.6 x 2.5-10	0.6 x 1.5-11	0.6 x 1.5-11	0.7-1.0 x 2-7	0.8-1.2 x 2-7	0.5 x 3-10	0.5-1 x 2-3	0.5-1 x 2-3
Temp. range (opt.), ºC	70-85 (80)	55-90 (80)	55-90 (80)	47-88 (80)	48-86 (80)	50-75 (70)	50-72 (66)	50-75 (65)
pH range (opt.)	6-8.5 (8.0)	5.5-9 (6.5-7)	5.5-9 (7.0)	5.2 – 9.0 (7.0)	5.2 – 9.0 (7.0)	6 – 8 (7.0)	5.5 – <9 (7.5)	6 – 8.5 (7.0)
NaCl range (opt.), g/L	0-<10 (0.5)	2.5-37.5 (27)	nr	1 – 55 (10)	1 – 60 (10)	0 – 24 (12)	0 – 28 (10)	0 – 28 (10)
Generation time, min	78	75	45	55-60	50-60	285	168	240
Utilization of:								
Glucose	+	+	+	+	+	±	+	+
Sucrose	+	+	+	+	+	nr	+	+
Maltose	+	+	+	+	+	+	+	+
Lactose	-	+	+	+	+	nr	+	+
D-fructose	+	+	nr	+	+	nr	+	+
Xylose	+	+	+	-	-	nr	-	+
Arabinose	±	nr	nr	+	+	nr	+	+
D-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^o	inhibition	NO	nr	inhibition	inhibition	inhibition	inhibition	YES
$SO_4^=$	YES	nr	nr	nr	nr	NO	NO	NO
$S_2O_3^=$	NO	nr	nr	inhibition	NO	YES	YES	YES
NO_3^-	NO	nr	nr	nr	nr	nr	nr	NO