Thermus arciformis sp. nov., a thermophilic species from a geothermal area

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Two aerobic, Gram-negative, non-motile, non-sporulating, yellow-pigmented bacteria, strains TH92^T and TH91, were isolated from a hot spring located in Laibin, Guangxi, in the south-eastern geothermal area of China. The isolates grew at 40–77 °C (optimally at 70 °C) and at pH 6.0–9.5 (optimally at pH 7.5–8.0). Phylogenetic analysis of 16S rRNA gene sequences and levels of DNA–DNA relatedness together indicated that the new isolates represented a novel species of the genus *Thermus* with closest affinity to *Thermus aquaticus*, *Thermus igniterrae* and *Thermus thermophilus*. Compared with their closest relatives, strains TH92^T and TH91 were able to assimilate a wider range of carbohydrates, amino acids and organic acids as sole carbon sources for growth, such as lactose and melibiose. The new isolates had lower combined levels of $C_{16:0}$ and iso-$C_{16:0}$ compared with their closest relatives. On the basis of polyphasic taxonomic characterization, strains TH92^T and TH91 are considered to represent a single novel species of the genus *Thermus*, for which the name *Thermus arciformis* sp. nov. is proposed. The type strain is TH92^T (=CGMCC 1.6992^T =JCM 15153^T).

The genus *Thermus* was originally proposed by Brock & Freeze (1969) with the description of *Thermus aquaticus*. Since then, *Thermus* strains have been isolated worldwide, generally from terrestrial hydrothermal areas where the temperature and pH of the water range from 55 to 70 °C and pH 5.0 to 10.5, but also from shallow marine hot springs, abyssal geothermal areas and even artificial thermal environments (da Costa *et al.*, 2006). At the time of writing, the genus comprised eight recognized species, namely *T. aquaticus* (Brock & Freeze, 1969), *T. thermophilus* (Oshima & Imahori, 1974; Manaia *et al.*, 1995; Williams *et al.*, 1995), *T. filiformis* (Hudson *et al.*, 1987), *T. scotoductus* (Kristjansson *et al.*, 1994), *T. brockianus* (Williams *et al.*, 1995), *T. oshimai* (Williams *et al.*, 1996), *T. igniterrae* and *T. antranikianii* (Chung *et al.*, 2000).

Due to intraspecific phenotypic variability, such as extremely variable morphology, physiological and biochemical characteristics, and fatty acid composition, it is difficult to define *Thermus* species with distinct phenotypes (da Costa *et al.*, 2001, 2006). Only a few species of the genus have phenotypic characteristics that may be used to distinguish them. *T. aquaticus* is characterized by containing moderate levels of iso-branched 3-OH fatty acids. Strains of *T. thermophilus* have the distinct ability to grow in media containing 3 % NaCl. *T. igniterrae* has the highest combined levels of iso-$C_{15:0}$ and iso-$C_{17:0}$ of all recognized *Thermus* species (Chung *et al.*, 2000). In the present paper, a novel *Thermus* species is proposed based on the description of two strains, TH92^T and TH91. Although distinct phenotypes have not been found, the novel species, as with most species of the genus *Thermus*, represents a distinct genotype that has a number of phenotypic differences from other *Thermus* species.

Water samples were collected from a hot spring located in Laibin, Guangxi, in the south-eastern geothermal area of China (23° 58′ 16″ N 109° 45′ 17″ E), and were transported without temperature control. For enrichment, water samples were filtered through membrane filters (0.22 μm). The samples were then used to inoculate the medium, which consisted of autoclaved spring water supplemented with tryptone (1.0 g l^{-1}) and yeast extract (1.0 g l^{-1}). The preparations were cultivated at 70 °C in a rotary water-bath (shaking at 120 r.p.m.) for 3 days. Turbid cultures were serially diluted and spread onto

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TH92^T and TH91 are EU247889 and EU247888, respectively.

Thermus agar plates (da Costa *et al.*, 2006), which were subsequently incubated at 70 °C until obvious colonies formed. Distinct colonies were picked out and purified by subculturing at least five times before being preserved by freeze-drying.

Cell morphology and motility were examined by optical microscopy (BX40; Olympus) and transmission electron microscopy (JEM-1230; JEOL). Temperature and pH ranges for growth were tested as specified by Chung *et al.* (2000) except that different buffering agents were used, including MES (pH 5.0–6.5), MOPSO (pH 6.5–7.5), Tricine (pH 7.5–9.0) and CAPSO (pH 9.0–10.5). Salt tolerance was examined at 70 °C in *Thermus* medium containing 1–4 % NaCl.

Single-carbon-source assimilation tests were performed as described by Chung *et al.* (2000), except that vitamin solution was added to the minimal medium and L-lysine (0.02 g l^{-1}) was added for tests with *T. thermophilus* DSM 579^T (Santos *et al.*, 1989). Cultures were incubated at 65 °C for 7 days as recommended by Santos *et al.* (1989). Other physiological and biochemical characteristics were examined as described by Hudson *et al.* (1986), Manaia & da Costa (1991) and Chung *et al.* (2000) in *Thermus* medium or on *Thermus* agar at 70 °C for 4 days.

Sensitivity to antibiotics was assayed with a two-layer plate consisting of a lower layer of solid *Thermus* agar and an upper layer of semisolid *Thermus* agar. Cultures incubated overnight were inoculated into semisolid *Thermus* agar just before the plates were poured. The prepared two-layer plates were pre-incubated at 70 °C for 4 h. Filter paper discs containing measured amounts of antibiotics were then placed onto the surface of the plates. Growth on each plate was checked after further incubation at 70 °C for 24 h.

Cultures for polar lipid and lipoquinone analysis were grown in 300-ml flasks containing 100 ml *Thermus* medium at 70 °C in a rotary water-bath (shaking at 100 r.p.m.) until the exponential phase. For polar lipid analysis, lipids were extracted as described by Chung *et al.* (2000). Individual polar lipids were separated by two-dimensional TLC on silica gel 60 F_{254} plates (Merck) as described by Ferraz *et al.* (1994). For lipoquinone analysis, lipoquinones were extracted from freeze-dried cells and were purified as described by Tindall (1989). Prepared lipoquinones were then separated with an Agilent 1200 HPLC system equipped with a ZORBAX Eclipse XDB-C18 column (150 × 4.6 mm, particle size 5 μm; Agilent) (Chung *et al.*, 1997).

Cultures for fatty acid analysis were prepared as specified by Chung *et al.* (2000). Fatty acid methyl esters were obtained from freeze-dried cells as described by Kuykendall *et al.* (1988) except that several drops of saturated NaCl solution were added together with 0.3 M NaOH at the final extraction step. Identification and quantification of fatty acid methyl esters, as well as numerical analysis of the fatty acid profiles, were performed automatically by using the Sherlock Microbial Identification System (MIDI Inc.) with the standard MIS Library Generation Software (Microbial ID Inc.).

Genomic DNA used in PCR amplification was extracted as specified by Rainey *et al.* (1996). The 16S rRNA gene was amplified with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACGGYTACCTTGTTACGACTT-3′). Sequence similarity analysis and multiple sequence alignment were performed via the EzTaxon service (Chun *et al.*, 2007) and with CLUSTAL W version 1.8 (Thompson *et al.*, 1994), respectively. The method of Jukes & Cantor (1969) was used to calculate evolutionary distances. Phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987) with the MEGA4 package (Tamura *et al.*, 2007) and by the maximum-parsimony method (Fitch, 1971) with the PHYLIP3.6 package (Felsenstein, 1993).

The G+C content of the genomic DNA of strains TH92^T and TH91 was determined by HPLC as described by Mesbah *et al.* (1989). DNA–DNA hybridization experiments were performed with the thermal denaturation and renaturation method described by De Ley *et al.* (1970) as modified by Huß *et al.* (1983) by using a Beckman DU800 spectrophotometer.

Following enrichment, 12 *Thermus*-like strains were isolated from the hot spring (water temperature 80 °C, around pH 6.7). On the basis of 16S rRNA gene sequence comparison, ten of these strains were found to be affiliated with the type strains of *T. oshimai*, *T. scotoductus* or *T. brockianus* (98.9–100 % sequence similarity), while strains TH92^T and TH91 were considered to represent a potentially novel *Thermus* species and were thus selected for further taxonomic study.

Strains TH92^T and TH91 were yellow-pigmented, non-motile and non-sporulating. Rod-shaped cells were predominant, but arc-shaped and filamentous cells were also observed (Fig. 1). Cells stained Gram-negative. Thin-section electron micrographs also showed a typical Gram-negative cell wall, which consisted of an inner, electron-dense thin layer representing the peptidoglycan and a thick outer layer that connected with the former (not shown). The two strains showed optimum growth at 70 °C and pH 7.5–8.0. The pH range for growth was 6.0–9.5. No growth occurred below 40 °C. The maximum growth temperature of strain TH92^T was 78 °C, whereas that of strain TH91 was 77 °C. Although the new isolates grew in *Thermus* medium containing 1 % (w/v) NaCl, higher growth rates were observed in the same medium without added NaCl.

Detailed results of physiological and biochemical examination are summarized in Table 1, which showed that strain TH91 could only be distinguished from strain TH92^T based on the ability to hydrolyse Tween 80. Nevertheless, a number of characteristics could be used

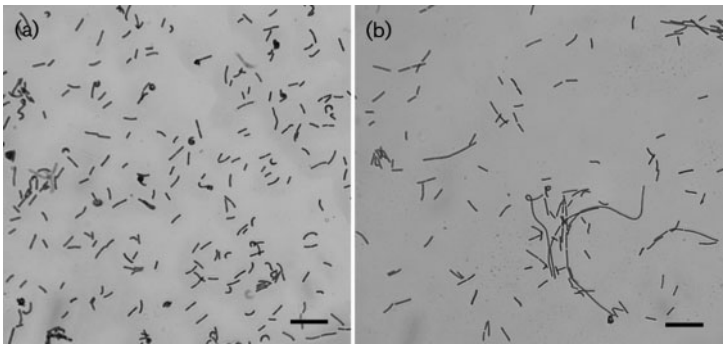

Fig. 1. Cell morphology of strains TH92^T (a) and TH91 (b) grown in *Thermus* medium at 70 °C and pH 7.5. Bars, 10 μm.

to differentiate the new isolates from their closest relatives, namely *T. aquaticus* DSM 625^T, *T. igniterrae* DSM 12459^T and *T. thermophilus* DSM 579^T. For example, strains TH92^T and TH91 used a wider variety of carbohydrates, amino acids and organic acids as sole carbon sources than the three former strains. As shown in Table 1, the number of carbon sources utilized by strains TH92^T or TH91 is 21, whereas that for the reference species is only 14 or 15. Moreover, compared with the new isolates, *T. igniterrae* DSM 12459^T and *T. thermophilus* DSM 579^T were resistant to more antibiotics, whereas *T. aquaticus* DSM 625^T was sensitive to more antibiotics.

Polar lipids of strains TH92^T and TH91 consisted of one major glycolipid (GL-1) and one major phospholipid (PL-2), along with one prominent glycolipid (GL-2) and one prominent phospholipid (PL-1) (not shown). The predominant respiratory lipoquinone of the new isolates was menaquinone 8 (MK-8). These two chemotaxonomic characteristics are common to the genus *Thermus* (da Costa *et al.*, 2001).

The fatty acid composition of the new isolates and related *Thermus* species is shown in Table 2. The predominant fatty acids of all tested strains were iso-$C_{15:0}$ and iso-$C_{17:0}$. The next most prominent fatty acids of strains TH92^T and TH91 were anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$, compared with $C_{16:0}$ and iso-$C_{16:0}$ in *T. aquaticus* DSM 625^T, *T. igniterrae* DSM 12459^T and *T. thermophilus* DSM 579^T. Strains TH92^T and TH91 had the lowest levels of $C_{16:0}$ of the strains tested.

Almost-complete 16S rRNA gene sequences of 1480 nt were determined for the new isolates. Comparison of these sequences with those referenced in the GenBank/EMBL/DDBJ databases indicated that strains TH92^T and TH91 were members of the genus *Thermus*. The 16S rRNA gene sequences of the new isolates, which were identical, shared highest similarity (97.3 %) with that of the type strain of *T. aquaticus*, followed by the type strains of *T. igniterrae* and *T. thermophilus* (96.7 %). Levels of 16S rRNA gene sequence similarity between the new isolates and the type strains of other recognized *Thermus* species were in the range 92.1–95.8 %. Phylogenetic analysis based on 16S rRNA gene sequences consisting of unambiguous nucleotides between positions 28 and 1491 (Brosius *et al.*, 1978) also showed that strains TH92^T and TH91 represented a distinctive lineage within the radiation of the genus *Thermus* which diverged between the branches of *T. thermophilus* and *T. aquaticus* (Fig. 2).

The DNA G+C content of strains TH92^T and TH91 was 68.3 and 67.2 mol%, respectively. Levels of DNA–DNA relatedness between strains TH92^T, TH91, *T. aquaticus* DSM 625^T, *T. igniterrae* DSM 12459^T and *T. thermophilus* DSM 579^T are given in Table 1. DNA–DNA relatedness between strains TH92^T and TH91 was 91 %, which is consistent with their homogeneous physiological and biochemical characteristics. Levels of DNA–DNA relatedness between strains TH92^T and TH91 and the reference strains ranged from 43.2 to 50.6 %.

Strains TH92^T and TH91 were highly homogeneous, especially as regards physiological and biochemical characteristics, possibly due to the consistent nature of the habitat from which they were isolated. However, some differences in morphology were noted. For example, strain TH92^T formed deep-yellow, compact colonies, whereas those of strain TH91 were yellow and spreading. Moreover, strain TH92^T was more apt to form arc-shaped cells, whereas filaments longer than 20 μm were observed more commonly with strain TH91.

Phylogenetic analysis based on 16S rRNA gene sequences indicated that strains TH92^T and TH91 were affiliated with the genus *Thermus* but represented a distinct genotype that was closely related to *T. aquaticus*, *T. igniterrae* and *T. thermophilus*. Although the new isolates shared around 97 % 16S rRNA gene sequence similarity with the type strains of these three *Thermus* species, levels of DNA–DNA relatedness demonstrated the interspecific relationship among those strains. Some phenotypic differences were also found between the new isolates and their closest relatives. Strains TH92^T and TH91 were able to assimilate more and different carbon sources compared with recognized *Thermus* species. Strains TH92^T and TH91 had the lowest combined levels of $C_{16:0}$ and iso-$C_{16:0}$ as well as the highest combined levels of anteiso-$C_{15:0}$ and $C_{17:0}$ compared with reference *Thermus* species.

On the basis of data from the present polyphasic taxonomic study, strains TH92^T and TH91 are considered

Table 1. Differential characteristics between strains TH92^T and TH91 and the type strains of related *Thermus* species

Strains: 1, TH92^T; 2, TH91; 3, *T. aquaticus* DSM 625^T; 4, *T. igniterrae* DSM 12459^T; 5, *T. thermophilus* DSM 579^T. +, Positive; w, weakly positive; −, negative. All data were from the present study. All strains were catalase- and oxidase-positive and hydrolysed casein, DNA, gelatin, starch and Tweens 20, 40 and 60. Acetate, L-asparagine, L-glutamate, L-glutamine, maltose, D-mannose, L-proline and pyruvate were utilized by all strains. None of the strains hydrolysed xylan or utilized acetamide, L-arabinose, erythritol, formate, *myo*-inositol, mannitol, propionate, raffinose, L-rhamnose, ribitol, D-ribose, sorbitol, L-sorbose, xylitol or D-xylose. All strains were sensitive to azithromycin (15 μg), bacitracin (0.04 U), chloramphenicol (30 μg), kanamycin (30 μg), novobiocin (30 μg) and polymixin B (300 IU) and resistant to tetracycline (30 μg).

Characteristic	1	2	3	4	5
Growth at/on:					
2 % NaCl	−	−	−	−	+
80 °C	−	−	−	−	+
2.5 % Trypticase peptone	+	+	+	−	+
Reduction of:					
Nitrate	+	+	−	+	−
Methylene blue	−	−	+	−	+
β-Galactosidase	+	+	−	+	+
Hydrolysis of:					
Aesculin	+	+	−	+	+
Tween 80	−	+	+	+	−
Utilization of:					
L-Arginine	w	+	w	−	−
Cellobiose	+	+	−	−	+
Citrate	−	−	−	−	+
D-Fructose	+	+	w	+	−
D-Galactose	+	+	−	+	−
D-Glucose	+	+	+	+	−
Glycerol	+	+	−	+	w
Lactose	+	+	−	−	−
Malate	+	+	w	−	−
Melibiose	+	+	−	−	−
L-Serine	+	+	+	−	+
Succinate	+	+	w	+	−
Sucrose	+	+	−	+	+
Trehalose	+	+	−	+	+
Sensitive to:					
Amoxicillin (10 μg)	+	+	+	−	−
Ampicillin (10 μg)	+	+	+	−	−
Neomycin (30 μg)	+	+	w	+	−
Oxacillin (1 μg)	−	−	+	−	−
Penicillin (10 IU)	+	+	+	−	−
Phosphomycin (200 μg)	−	−	+	−	−
Streptomycin (10 μg)	+	+	w	−	−
Vancomycin (30 μg)	+	+	+	−	+
DNA G+C content (mol%)	68.3	67.2	65.5	68.0	68.5
DNA–DNA relatedness (%) with:					
TH92^T	(100)	91.0	50.2	50.6	45.1
TH91	91.0	(100)	43.2	47.8	45.7

Table 2. Fatty acid composition of strains TH92^T and TH91 and the type strains of related *Thermus* species

Strains: 1, TH92^T; 2, TH91; 3, *T. aquaticus* DSM 625^T; 4, *T. igniterrae* DSM 12459^T; 5, *T. thermophilus* DSM 579^T. Data are from the present study. −, Not detected (<0.5 %). Values are percentages of total fatty acids. Fatty acids present at <0.5 % in all tested strains are not shown.

Fatty acid	1	2	3	4	5
iso-$C_{13:0}$	1.0	0.8	−	0.8	0.5
Unknown ECL 13.565	−	−	1.6	−	−
iso-$C_{14:0}$	−	−	−	−	1.1
$C_{14:0}$	−	−	0.6	−	−
iso-$C_{15:0}$	41.2	37.0	20.4	46.9	36.7
anteiso-$C_{15:0}$	7.1	6.5	1.9	2.6	5.3
$C_{15:0}$	1.9	1.5	−	0.9	−
iso-$C_{16:0}$	1.7	2.2	6.2	3.6	6.9
$C_{16:0}$	3.8	3.9	9.2	7.8	6.2
iso-$C_{15:0}$ 3-OH	−	−	3.3	−	−
iso-$C_{17:1}\omega 10c$	−	−	1.9	−	−
iso-$C_{17:0}$	34.6	38.5	36.8	33.6	37.9
anteiso-$C_{17:0}$	5.5	6.5	4.5	2.2	5.2
$C_{17:0}$	0.7	0.7	−	−	−
iso-$C_{16:0}$ 3-OH	−	−	0.9	−	−
$C_{16:0}$ 3-OH	−	−	0.7	−	−
iso-$C_{18:0}$	−	−	0.7	−	−
$C_{18:0}$	−	−	0.7	−	−
iso-$C_{17:0}$ 3-OH	−	−	8.4	−	−
$C_{17:0}$ 2-OH	−	−	0.6	−	−

to represent a single novel species of the genus *Thermus*, for which the name *Thermus arciformis* sp. nov. is proposed.

Description of *Thermus arciformis* sp. nov.

Thermus arciformis [ar.ci.for′mis. L. n. *arcus* a bow or a building arch; L. masc. suff. *-formis* (from L. n. *forma* shape, appearance) in the shape of; N.L. masc. adj. *arciformis* bow-shaped, indicating one particular cell morphology].

Cells are non-motile, non-sporulating rods, about 0.6 μm wide and variable in length (mainly 1.5–6 μm). Arc-shaped cells and filaments (>20 μm) are also found. Gram-staining is negative. After growth on *Thermus* agar at 70 °C for 72 h, colonies of strain TH92^T are deep-yellow and compact whereas those of strain TH91 are yellow and spreading. Optimal growth occurs at 70 °C and pH 7.5–8.0. Growth occurs at 40–77 °C and pH 6.0–9.5, as well as in the presence of 2.5 % (w/v) trypticase peptone (BBI), but not in the presence of 2 % (w/v) NaCl. Strain TH92^T grows slowly at 78 °C. Aerobic and heterotrophic. Catalase-, oxidase- and β-galactosidase-positive. Aesculin, casein, DNA, gelatin, starch and Tweens 20, 40 and 60 are hydrolysed, whereas xylan is not. Nitrate and methylene blue are reduced. Utilizes acetate, L-arginine, L-asparagine, cellobiose, D-fructose, D-galactose, D-glucose, L-glutamate, L-glutamine, glycerol, lactose, malate, maltose, D-mannose, melibiose, L-proline, pyruvate, L-serine, sucrose and

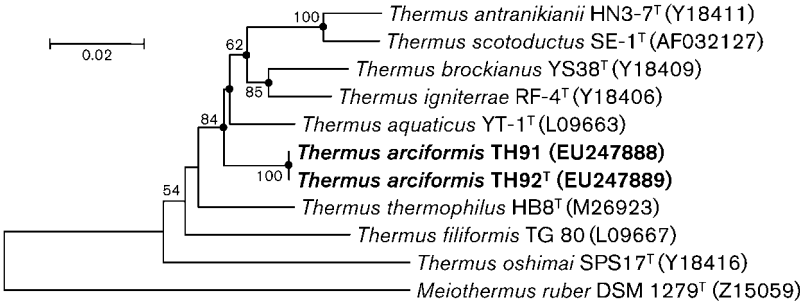

Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships between strains TH92^T and TH91 and related taxa. Bootstrap values as percentages of 1000 replicates are given at branch points; only values >50 % are shown. Dots indicate branches that were also recovered in the maximum-parsimony tree. Bar, 0.02 substitutions per nucleotide position.

trehalose, but not acetamide, L-arabinose, citrate, erythritol, formate, *myo*-inositol, mannitol, propionate, raffinose, L-rhamnose, ribitol, D-ribose, sorbitol, L-sorbose, xylitol or D-xylose. Resistant to phosphomycin, oxacillin and tetracycline. The major fatty acids are iso-$C_{15:0}$, iso-$C_{17:0}$, anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$. The DNA G+C content of strains TH92^T and TH91 is 68.3 and 67.2 mol%, respectively.

The type strain, TH92^T (=CGMCC 1.6992^T =JCM 15153^T), was isolated from a hot spring located in Laibin, Guangxi, in the south-eastern geothermal area of China. Strain TH91 (=CGMCC 1.6991 =JCM 15152), isolated from the same hot spring, is a second strain of the species.

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