Thermus caliditerrae sp. nov., a novel thermophilic species isolated from a geothermal area

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Two thermophilic bacterial strains, designated YIM 77925T and YIM 77777, were isolated from two hot springs, one in the Hydrothermal Explosion (Shuirebaozhaqu) area and Frog Mouth Spring in Tengchong county, Yunnan province, south-western China. The taxonomic positions of the two isolates were investigated by a polyphasic approach. Cells of the two strains were Gram-stain-negative, aerobic and rod-shaped. They were able to grow at 50–70 °C, pH 6.0–8.0 and with a NaCl tolerance up to 0.5 % (w/v). Colonies are circular, convex, non-transparent and produce yellow pigment. Phylogenetic analyses based on 16S rRNA gene sequences comparison clearly demonstrated that strains YIM 77925T and YIM 77777 represent members of the genus Thermus, and they also detected low-level similarities of 16S rRNA gene sequences (below 97 %) compared with all other species in this genus. Their predominant menaquinone was MK-8. The genomic DNA G + C contents of strains YIM 77925T and YIM 77777 were 65.6 mol% and 67.2 mol%, respectively. Based on the results of physiological and biochemical tests and phylogenetic analyses, strains YIM 77925T and YIM 77777 could not be classified as representing any species of the genus Thermus with a validly published name. Thus the two strains are considered to represent a novel species of the genus Thermus, for which the name Thermus caliditerrae sp. nov. is proposed. The type strain is YIM 77925T (=DSM 25901T =CCTCC 2012061T).

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The genus Thermus, which was first proposed by Brock & Freeze (1969), was considered to belong to the family Thermaceae described by da Costa & Rainey (2001). After Freeze (1969), was considered to belong to the family Thermus thermophilus (Brock & Freeze, 1969), Thermus arciformis (Zhang et al., 2010), Thermus brockianus (Williams et al., 1995), Thermus composti (Vajna et al., 2012), Thermus filiformis (Hudson et al., 1987), Thermus islandicus (Bjornsdottir et al., 2010), Thermus oshimai (Williams et al., 1996), Thermus scotoductus (Kristjansson et al., 1994), Thermus thermophilus (Oshima & Imahori, 1974; Williams et al., 1995) and Thermus tengchongensis (Yu et al., 2013).

In the course of screening the thermostable amylases produced by thermophiles, strains YIM 77925T and YIM
77777 with amylase activity were isolated from the Hydrothermal Explosion area (Shuirebaozhaqu, 24.95014° N 98.43743° E) and Frog Mouth Spring (24.95006° N 98.43830° E), respectively. The two hot springs lies in Tengchong county, Yunnan province, south-western China. Tengchong county is the largest and most intensively geological field in China and famous for the wide physico-chemical diversity of the hot springs (temperatures from 70 to 97 °C; pH values from 1.8 to 9.3) which provide a multitude of niches for extremophilic micro-organisms (Hedlund et al., 2012). The aim of the present study is to determine the taxonomic status of the two isolates, by using a polyphasic approach. The results of phenotypic, chemotaxonomic and phylogenetic analyses indicated that strains YIM 77925T and YIM 77777 represent a novel species of the genus Thermus.

Geothermally heated sediment with some water mixture samples were collected from the Hydrothermal Explosion (Shuirebaozhaqu) area and Frog Mouth Spring. The sediment sample from the Hydrothermal Explosion (Shuirebaozhaqu) area was sandy, khaki coloured and the temperature and pH were 79.8 °C and 7.5, respectively. The sediment sample from Frog Mouth Spring was sandy, dark grey and the temperature and pH were 84.0 °C and 8.0, respectively. Mixture samples (10 ml) were injected into tubes with condensed sterilized modified T5 medium (tryptone 0.5 g, yeast extract 2 g, glucose 1 g, lotus root starch 1 g, CaSO4 0.04 g, 0.01 M iron citrate 0.5 ml, KH2PO4 0.01 g, K2HPO4 0.01 g, NaHCO3 0.1 g, NaCl 0.2 g, CaCl2 0.02 g, MgSO4 0.02 g, water 1000 ml, agar 20 g, pH 7.5), and the final concentrations in the medium were halved when the sample was injected into the tube. The treated tubes were enriched in situ for 10 days. A 1 ml sample of the enriched mixture was diluted 10- and 100-fold with sterile water and then 0.2 ml of the diluted sample was spread on 50 % T5 medium. After incubation at 70 °C for 7 days, several colonies were isolated and 14 Thermus-like isolates among them were obtained. On the basis of 16S rRNA gene sequence comparison, ten strains were affiliated with the type strain T. scotoductus (99.0–100 % similarity) and two strains were affiliated with the type strain T. brockianus (99.2–100 % similarity). Strains YIM 77925T and YIM 77777 had a low level of 16S rRNA gene similarity (90.7–96.2 %). An apparently distinct branch with the type strains of the genus Thermus was formed on modified T5 medium at 65 °C for 3 days. Biomass for chemical and molecular studies was obtained by cultivation on modified T5 medium at 65 °C for 3 days. Cells were checked for purity, harvested and washed twice with distilled water and then freeze-dried.

Genomic DNA was extracted, and the 16S rRNA gene was PCR-amplified and sequenced as described by Li et al. (2007). The amplicons were purified by using a PCR purification kit (Sangon Biotech). The full-length 16S rRNA gene sequences were compiled by using the SeqMan program (DNASTAR software) and then compared with the corresponding sequences of cultured species by using BLAST search (Altschul et al., 1990) and the EzTaxon server 2.1 database (Kim et al., 2012). To determine the phylogenetic relationships of strains YIM 77925T and YIM 77777, multiple alignments of their sequences with those of type strains of species of the genus Thermus were performed using the CLUSTAL_W software package (Thompson et al., 1997). The Kimura two-parameter model was used to calculate the evolutionary distances (Kimura, 1980, 1985). Phylogenetic and molecular evolutionary analyses were performed using the software package MEGA version 5.0 (Tamura et al., 2011) and the PHYML package (Guindon & Gascuel, 2003). Phylogenetic trees were reconstructed with the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) algorithms. Bootstrap analysis was used to evaluate the topology of each tree with 1000 replications (Felsenstein, 1985).

Meiothermus hypogaeus AZM34c1T (AB586707) was used as outgroup.

The almost-complete 16S rRNA gene sequences of strains YIM 77925T (1545 nt) and YIM 77777 (1450 nt) were determined. Comparison of their sequences with the corresponding 16S rRNA gene sequences in the GenBank/EMBL/DDBJ databases clearly demonstrated that strains YIM 77925T and YIM 77777 were members of the genus Thermus. Phylogenetic analyses showed that strains YIM 77925T and YIM 77777 formed a monophyletic lineage (99.0 % similarity) by themselves and they formed a well separated branch with the type strains of T. scotoductus SE-1T (96.8 %), T. antranikianii HN3-7T (95.9 %) and T. tengchongensis YIM 77924T (96.2 %) in the neighbour-joining phylogenetic tree (Fig. 1). Sequence similarities between the two isolates and all other members of the genus Thermus ranged from 90.7 to 96.2 %. An apparently distinct subclade was also supported by the maximum-parsimony phylogenetic tree and maximum-likelihood phylogenetic tree (Figs. S2 and S3, available in IJSEM Online). The subclades identified in all three phylogenetic trees are marked with asterisks in each branch in Fig. 1.

Cell motility was studied by the development of turbidity throughout a tube of semi-solid medium (Leifson, 1960). Gram staining was carried out according to the standard Gram reaction with 3 % KOH (Buck, 1982). Microscopic observation was performed after growing the bacteria at
65 °C for 3 days on modified T5 medium. Morphological characteristics were observed under a light microscope and a scanning electron microscope (XL30 ESEM-TMP, Philips). For scanning electron microscopy, harvested cells of strains YIM 77925T and YIM 77777 were suspended with sterilized water and then were fixed with glutaraldehyde (2 %) for 2 h. Subsequently the fixed cells were dehydrated through a gradient series of alcohol (30, 50, 70, 90 and 100 %, respectively). The cell specimens were sputter coated with gold powder for 200 s and then were examined. The pH (4.0–10.5, at intervals of 0.5 pH units) and temperature (45, 50, 55, 60, 65, 70, 75 and 80 °C) ranges for growth were tested as described by Chung et al. (2000) and Bjornsdottir et al. (2009) except that the modified T5 medium agar was used. The buffer systems used for pH range examination included 30 mM acetate for pH 4.0–5.5, 30 mM MES for pH 6.0–6.5, 30 mM Tris for pH 7.0–8.5 and 30 mM CAPSO for pH 9.0–10.5, respectively. Salt tolerance tests with 0–5 % (w/v) NaCl (0, 0.5, 1.0, 1.5 2, 2.5, 3.0, 4.0 and 5.0 % w/v, respectively) were carried out on modified T5 agar at 65 °C for 3 days. Single-carbon-source assimilation were tested in a minimal modified T5 medium composed of basal salts with lotus root starch (0.1 g l⁻¹), supplemented with 2 g filter-sterilized (0.22 μm, Millipore) test compound sources l⁻¹ (Vajna et al., 2012). Cultures were incubated at 65 °C for 7 days as recommended by Santos et al. (1989). API galleries (API 20NE and API ZYM) were used to determine some enzyme activities and other phenotypic tests. Firstly, sterile water was injected into the bottom of incubation boxes then the API strips were put in the cells. The API strips with incubation boxes were put into a container with sterile water to prevent the strips drying out and then were incubated in a bacteriological incubator. The API strips were observed after incubation at 65 °C for 4, 8, 16 and 24 h. Each time the sterile water at the bottom of the cell was replenished when necessary. The strips did not crinkle until incubation was finished. Further handling was according to the instructions of the manufacturer (bioMérieux). Other physiological and biochemical characteristics were examined in modified T5 agar or an incubator at 65 °C for 3 days as described by Chung et al. (2000), Manaia & da Costa (1991) and Hudson et al. (1986).

Cells of strains YIM 77925T and YIM 77777 were Gram-reaction-negative, aerobic and rod-shaped. The cells were 0.2–0.5 μm in diameter and 2.0–6.0 μm in length. The bacteria were non-motile and did not form spores (Fig. S1). The colonies were circular, convex, non-transparent and yellow-pigmented when grown on modified T5 and R2A media. Growth occurred at temperatures ranging from 50 to 70 °C (optimum 65 °C), no growth occurred below 50 °C. Strains YIM 77925T and YIM 77777 were able to grow at pH 6.0–8.0 (optimum pH 7.0) and with low NaCl concentrations (up to 0.5 % w/v), and the strains grew well with no NaCl. Both strains were positive for

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**Fig. 1.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strains YIM 77925T and YIM 77777 among members of the genus *Thermus*. Numbers at branch points refer to bootstrap percentages. Only bootstrap values above 50 % are shown at branch points. Asterisks indicate branches that were also recovered using maximum-parsimony and maximum-likelihood trees. Bar, 0.02 substitutions per nucleotide position. *Meiothermus hypogaeus* AZM34c11T (AB586707) was used as an outgroup.
catalase, oxidase and urease activities and could degrade Tween 20, but not Tweens 40, 60 and 80. Positive results were detected for reduction of nitrate and starch, aesculin and gelatin hydrolysis. Results were negative for casein, chitin, CM-cellulose, xylan and tyrosine hydrolysis. Phenotypic properties useful for distinguishing strains YIM 77925\(^T\) and YIM 77777 from \(T.\) tengchongensis YIM 77924\(^T\), \(T.\) scotoductus SE-1\(^T\) and \(T.\) antranikianii HN3-7\(^T\) are shown in Table 1. The detailed physiological characteristics of strains YIM 77925\(^T\) and YIM 77777 are given in the species description. The results in Table 1 showed that there were a few differences between YIM 77925\(^T\) and YIM 77777 with respect to cellobiose and inositol utilization, casein hydrolysis and leucine arylamidase activity.

Chemotaxonomic characteristics of strains YIM 77925\(^T\), YIM 77777 and the reference strains \(T.\) scotoductus SE-1\(^T\), \(T.\) antranikianii HN3-7\(^T\) and \(T.\) tengchongensis YIM 77924\(^T\) were observed using several standard methods under identical conditions. Menaquinones were extracted from lyophilized cells as described by Collins et al. (1977) and Minnikin et al. (1984), and the extracts were purified and analysed by HPLC (Kroppenstedt, 1982; Tamaoka et al., 1983). For analysis of fatty acids, strains YIM 77925\(^T\), YIM 77777, \(T.\) scotoductus SE-1\(^T\), \(T.\) antranikianii HN3-7\(^T\) and \(T.\) tengchongensis YIM 77924\(^T\), were grown on modified T5 medium at 65 °C for 24 h. The cellular fatty acids were extracted, methylated and analysed following the instructions of Microbial Identification System (MIDI) (Sherlock Version 6.1; MIDI database: TSBA6) (Sasser, 1990). Polar lipids were extracted and the individual polar lipids were separated by two-dimensional TLC on silica gel G 60 plates (Merck), and the profile was identified using the described procedures (Collins & Jones, 1980; Minnikin et al., 1979, 1984). The genomic DNA G+C contents of strains YIM 77925\(^T\) and YIM 77777 were determined by HPLC.

<table>
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Strains: 1, YIM 77925\(^T\); 2, YIM 77777; 3, \(T.\) scotoductus SE-1\(^T\); 4, \(T.\) antranikianii HN3-7\(^T\); 5, \(T.\) tengchongensis YIM 77924\(^T\). All data were obtained in the present study. +, Positive/utilized; −, negative, not utilized; w, weakly positive.
(Mesbah et al., 1989), with *Escherichia coli* JM-109 as the reference strain. DNA–DNA relatedness was tested to determine the relationship between strains YIM 77925T and YIM 77777 according to the fluorometric micro-well method (Ezaki et al., 1989; Christensen et al., 2000; He et al., 2005). The hybridizations were performed by using DNA probes labelled (strains YIM 77925 and YIM 77777) with photobiotin (A1935; Sigma) and 96-well microdilution plates (Greiner BioOne). Each sample was tested with eight replications.

The predominant menaquinone of strains YIM 77925T and YIM 77777 was MK-8, which is common in the genus *Thermus*. Therefore, based on the phenotypic, phylogenetic and chemotaxonomic results, strains YIM 77925T and YIM 77777 represent a novel species of the genus *Thermus* for which the name *Thermus caliditerrae* sp. nov. is proposed.

**Description of Thermus caliditerrae** sp. nov.

*Thermus caliditerrae* (ca.li.di.ter’ræ. L. adj. calidus, hot; L. n. terra, soil, earth; N.L. gen. n. caliditerrae, of a hot soil, referring to where the strain was isolated).

Cells are Gram-stain-negative, aerobic and rod-shaped. The rods are 0.2–0.5 μm in diameter and 2.0–6.0 μm in length. Colonies are circular, convex, non-transparent and yellow-pigmented. Growth occurs at 50–70 °C, pH 6.0–8.0 and in the presence of 0–0.5 % (w/v) NaCl. Utilizes L-alanine, L-arabinose, L-arginine, L-asparagine, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, raffinose, L-rhamnose, D-ribose, sodium malate, succinic acid, sucrose, L-threonic, L-trehalose, L-valine, but not glycerol, sorbitol, trehalose, D-xylitol or D-xylose. Activities of catalase, oxidase and urease are positive. Positive for Tween 20 degradation, starch, ascelin and gelatin hydrolysis, negative for degradation of Tewens 40, 60 and 80, hydrolysis of chitin, CM-cellulose and tyrosine. Arginine dihydrolase was positive and nitrate was reduced. Indole production from tryptophan and glucose fermentation were negative. Activites of alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase, β-galactosidase and naphthol-AS-BI-phosphohydrolase were present; activities of *N*-acetyl-β-glucosaminidase, *z*-chymotrypsin, cystine arylamidase, β-fucosidase, *z*-galactosidase, *z*-glucosidase, *β*-glucosidase, *β*-glucuronidase, lipase (C14), *z*-mannosidase, trypsin and valine arylamidase were absent. The predominant menaquinone is MK-8. The major fatty acids are iso-C17 : 0, iso-C16 : 0 and iso-C15 : 0. Please keep them. The polar lipid profile consists of an aminophospholipid, one phospholipid and two glycolipids (GL1 and GL2) (Fig. S4). The genomic DNA G + C contents of strains YIM 77925T and YIM 77777 were 65.6 mol% and 67.2 %, respectively. DNA–DNA relatedness between strains YIM 77925T and YIM 77777 was 89.8±2.8 % and supported them belonging to the same species, which is consistent with their homogeneous phylogenetic analysis results, physiological and biochemical characteristics.

The combination of phylogenetic, chemotaxonomic and phenotypic data indicated that strains YIM 77925T and YIM 77777 were members of the genus *Thermus*. Although strains YIM 77925T and YIM 77777 had many properties that overlapped with those of most of species of the genus *Thermus*, the two isolates also displayed a few physiological and biochemical properties that distinguished them from their closest relatives *T. tengchongensis* YIM 77924T, *T. scotoductus* SE-1T and *T. antranikianii* HN3-7T (see Table 1 and the species description). Strains YIM 77925T and YIM 77777 possessed a low level of 16S rRNA gene sequence similarity to and formed a distinct lineage from the species of the genus *Thermus* with validly published names. However, the compositions of cellular fatty acids of strains YIM 77925T and YIM 77777 are variable even though they shared high 16S rRNA sequence similarity and DNA–DNA hybridization values. This phenomenon has also been verified for other species in the genus *Thermus* (Nobre et al., 1996). da Costa et al. (2006) pointed out that variation in the fatty acid compositions in the same species

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References


