Thermus islandicus sp. nov., a mixotrophic sulfur-oxidizing bacterium isolated from the Torfajokull geothermal area

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Strains PRI 2268 and PRI 3838T were isolated from two separate hot springs in the Torfajokull geothermal area of South Iceland. The cells were non-motile rods, approximately 0.3 \( \mu \text{m} \) in width and 1.5–2.5 \( \mu \text{m} \) in length. Electron microscopy revealed a Gram-negative cell-wall structure. The strains grew at 45–79 °C (optimum, 65 °C) and pH 5.5–10.5 (optimum, pH 6.0–7.0). 16S rRNA gene sequence analysis indicated that they formed a separate branch within the genus Thermus with ‘Thermus kawarayensis’ KW11 as their closest cultured relative (96.5 % similarity). The gene sequence similarities of both new isolates to Thermus aquaticus YT-1T and Thermus igniterrae RF-4T were 96.1 % and 95.5 %, respectively. DNA–DNA relatedness between strain PRI 3838T and ‘T. kawarayensis’ was 46.1 %. The DNA G+C content of strain PRI 3838T was 69.0 mol%. The predominant menaquinones, pigmentation, fatty acid profiles and phospholipid profiles of the novel strains were similar to those of other members of the genus Thermus. However, the new strains could be differentiated from the type strains of all other species of the genus Thermus by their lack of catalase activity and their utilization of only a few carbon sources. Furthermore, the novel strains exhibited mixotrophic growth with sulfur oxidation. On the basis of 16S rRNA gene sequence comparisons, DNA–DNA hybridization and physiological and biochemical characteristics, the new isolates represent a novel species. Since the species appears to be ubiquitous in Icelandic hot springs, the name Thermus islandicus sp. nov. is proposed. The type strain is PRI 3838T (=DSM 21543T=ATCC BAA-1677T).

Bacteria of the genus Thermus have been prominent in the history of thermophile research since the discovery of Thermus aquaticus by Brock & Freeze in 1969. Since then, several additional Thermus species have been recognized. As a group, strains of the genus Thermus are both biochemically and metabolically quite homogeneous. Most strains are yellow-pigmented, non-sporulating rods that grow heterotrophically on various carbon sources (da Costa et al., 2006). Optimal and maximal temperatures for growth are usually about 65–70 °C and below 80 °C, respectively, and optimal pH for growth is around neutrality. Most Thermus strains have been described as obligate aerobes but some are known to use nitrate, ferric ion, elemental sulfur or arsenate as terminal electron acceptors (Kieft et al., 1999; Gihring & Banfield, 2001; Skirnisdottir et al., 2001). Mixotrophic growth with the oxidation of sulfur compounds has been reported as a common trait in members of the genus Thermus (Hreggvidsson et al., 2006).

Species of the genus Thermus have been isolated from natural and artificial thermal environments around the world but exhibit different patterns of distribution. Thermus aquaticus and Thermus filiformis have so far only been encountered in the USA and New Zealand, respectively (Brock & Freeze, 1969; Hudson et al., 1987). Thermus brockianus, Thermus oshimai and Thermus scotoductus...
show global distributions (Kristjansson et al., 1994; Williams et al., 1995, 1996), while Thermus igniterae and Thermus antranikianii have only been isolated from hot springs in Iceland (Chung et al., 2000). Thermus thermophilus has been isolated from marine and coastal hot springs all over the world (Williams et al., 1995). T. thermophilus was the only Thermus species isolated from Japanese hot springs until recently, when the discovery of ‘Thermus kawarayensis’ was reported (Kurosawa et al., 2000). During an environmental assessment of the Torfajokull geothermal area commissioned by the National Energy Authority of Iceland, we encountered a hot spring, HS-605, harbouring 16S rRNA gene sequences showing 96% similarity to ‘T. kawarayensis’ (unpublished results). Nearly identical sequences had previously been identified in two hot springs in south-western Iceland (Skirnisdottir et al., 2000; Hjorleifsdottir et al., 2001; Hobel et al., 2004). The aim of this work was to isolate and describe this unknown species of the genus Thermus.

Samples were collected from hot spring HS-605, which is located 603 m above sea level in the Torfajokull geothermal area in southern Iceland. The temperature was 74 °C at the inlet of the opening and 60 °C at the borders. The pH was around 6.0 and the conductivity was 234 μS cm⁻¹. The concentrations of chloride, magnesium and sulfur were estimated as 0.6 mg l⁻¹, 2 mg l⁻¹ and 10 mg l⁻¹, respectively, using inductively coupled plasma mass spectrometry (ICP-MS). The concentration of hydrogen sulfide was determined to be 1.3 mg l⁻¹ using the methylene blue method (Eaton et al., 2005). The hot spring was characterized by a conspicuous grey microbial mat that was at approximately 66–74 °C when sampled. Biomass was inoculated into R2A liquid medium (Becton Dickinson) and incubated for 24 h at 65 °C without shaking. Turbid cultures were streaked on agar plates containing R2A, which were then incubated at 65 °C for up to 7 days. Different colony types appeared and were picked at random with an emphasis on yellow-pigmented strains. Selected strains were purified by streaking on to the same medium. For phylogenetic characterization, DNA was isolated from cultured strains using Dynabeads DNA Direct (Invitrogen) and partial 16S rRNA gene sequences were determined as described below. Strain PRI 3838T, which showed 99% gene sequence similarity to the previously detected clone sequence SRI-248 (Skirnisdottir et al., 2000), was chosen for phenotypic characterization.

During this work, we also examined yellow-pigmented strains that had been deposited uncharacterized in the Matis-Prokaria strain collection. This resulted in the identification of strain PRI 2268, which also exhibited 99% sequence similarity to the clone sequence SRI-248. This strain was isolated from a hot spring at 65–70 °C and pH 5.5 in Hrafntinnusker in the Torfajokull area. It was isolated on an R2A plate at 65 °C. The reference strains used in this study, ‘T. kawarayensis’ (DSM 16200), T. aquaticus (DSM 625T) and T. igniterae (DSM 12459T) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

Isolates PRI 2268 and PRI 3838T stained Gram-negative. They produced irregular, umbonate and undulate colonies of 1–1.5 mm after incubation at 65 °C on medium 166 (Hjorleifsdottir et al., 2001) for 4 days. Both strains produced yellow pigments in contrast to the colourless ‘T. kawarayensis’ and the absorbance spectra of acetone extracts showed a peak at 450 nm. Both novel strains exhibited oxidase activities but were catalase-negative and did not reduce nitrate, in contrast to ‘T. kawarayensis’ (Table 1). The sensitivity of strains PRI 2268 and PRI 3838T and the reference strains to antibiotics was examined. Plates containing medium 166 and standard (6 mm) antibiotic discs (Oxoid) were incubated at 65 °C. The inhibition zone was measured and the strains scored as sensitive to the antibiotic if the zone was >20 mm in diameter (Kristjansson et al., 1994). All strains tested as sensitive to ampicillin (10 μg), gentamicin (10 μg), novobiocin (30 μg), kanamycin (30 μg), penicillin G (10 μg) and streptomycin (10 μg). They were resistant to nalidixic acid (30 μg), polymyxin B (300 U) and rifampicin (2 μg). Cells of the novel strains were non-motile and a capsule was not

**Table 1. Characteristics that distinguish the new Thermus isolates from their closest relatives**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tbody>
<tr>
<td>Pigmentation</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
<td>Yellow</td>
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<tr>
<td>Catalase</td>
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<td>–</td>
<td>+</td>
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<td>Nitrate reduction</td>
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<td>–</td>
<td>+</td>
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<td>Thiosulfate oxidation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Growth at 75 °C</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>DNA G+C content</td>
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<td>69.0</td>
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<td>64</td>
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<tr>
<td>d-Arabinose</td>
<td>w</td>
<td>–</td>
<td>w</td>
<td>–</td>
<td>+</td>
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<tr>
<td>l-Arginine</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>+</td>
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<tr>
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<td>myo-Inositol</td>
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<td>–</td>
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<td>d-Mannose</td>
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<td>w</td>
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<tr>
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<td>+</td>
<td>–</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>L-Serine</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Starch</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Sucrose</td>
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<td>Trehalose</td>
<td>–</td>
<td>–</td>
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detected by crystal violet and copper sulfate or by India ink staining. Scanning electron micrographs of strain PRI 3838\textsuperscript{T} showed straight rod-shaped cells, approximately 0.3 μm in diameter and 1.5–2.5 μm in length (see Supplementary Fig. S1, available in IJSEM Online). Transmission electron micrographs showed a thick envelope and a typical Gram-negative cell-wall profile (see Supplementary Figs S2 and S3).

Strains PRI 2268 and PRI 3838\textsuperscript{T} and the reference strains were routinely grown at 65 °C in medium 166 (Hjorleifsdottir et al., 2001). The new isolates were aerobic and did not grow anaerobically with nitrate. The pH ranges for growth were examined at 65 °C in medium 166 buffered with 30 mM acetate to pH 4.0–5.5, 30 mM MES to pH 6.0, 30 mM phosphate to pH 6.5–8.0, 30 mM Tricine to pH 9.0 and by 30 mM CAPS to pH 10.0–11.0. Strains PRI 2268 and PRI 3838\textsuperscript{T} grew optimally at pH 6.0–7.0 and pH 6.5–7.0, respectively. The maximum and minimum pH values for growth were pH 5.5 and pH 10.5 for both strains. Their growth temperature ranges were examined by measuring the turbidity (OD\textsubscript{600}) of liquid cultures. Both strains PRI 2268 and PRI 3838\textsuperscript{T} grew optimally at about 65 °C and their maximum specific growth rates (\( \mu \)) were determined as 0.37 h\textsuperscript{−1} and 0.29 h\textsuperscript{−1}, respectively (see Supplementary Fig. S4, available in IJSEM Online). The minimum and maximum growth temperatures were about 44 °C and 79 °C, respectively, for both strains. Salt tolerance tests were performed on plates with medium 166 at 65 °C and in liquid cultures and neither novel strain grew in the presence of 1% (w/v) NaCl. Growth on 27 different single and complex carbon sources was tested on agar plates and compared with that of 'T. kawarayensis' DSM 16200, T. aquaticus DSM 625\textsuperscript{T} and T. igniterrae DSM 12459\textsuperscript{T}. Modified minimal medium 162 (Degryse et al., 1978) containing EGTA instead of Tris was used. It also contained 0.05 % NH\textsubscript{4}Cl and 0.4 % vitamin solution (Degryse et al., 1978) and was prepared either with or without 0.2 % yeast extract. Carbon sources were added in concentrations of 0.2 % for l-amino acids and 0.4 % for other substrates. Plates were scored as positive if growth was clearly visible after 7 days. A negative control without an added organic compound was also included. Strains PRI 2268 and PRI 3838\textsuperscript{T} assimilated very few carbon sources when compared with the reference strains. However, by lowering the growth temperature to 60 °C, several more carbon sources were utilized and both strains grew well on pyruvate, starch and glycerol, when supplemented with yeast extract. Strain PRI 2268 also grew well on l-glutamine, D-arabinose and ribitol (Table 1). Both novel strains showed some growth on cellobiose, lactose and myo-inositol. They also grew on minimal medium supplemented with yeast extract and thiosulfate (16 mM), both on plates and in liquid medium. Furthermore, when thiosulfate was added to medium 166, the maximum growth rate increased, but only at high aeration. This indicated mixotrophic growth and energy harnessing oxidation of thiosulfate. This was confirmed by the production of sulfate from thiosulfate in the presence of yeast extract as determined by using BaCl\textsubscript{2} as described by Tabatabai (1974). Strains PRI 2268 and PRI 3838\textsuperscript{T} produced 4.3 and 4.5 mM sulfate from 16 mM thiosulfate after growth for 48 h at 65 °C with a concomitant decrease in pH from 7.7 to 5.3 and 5.0, respectively. The strains were also capable of sulfur oxidation in the presence of yeast extract, but less efficiently than with thiosulfate. Both produced 0.6 mM sulfate when incubated with sulfur (2 g l\textsuperscript{−1}) at 65 °C for 48 h. Similar results were obtained for both ‘T. kawarayensis’ DSM 16200 and T. igniterrae DSM 12459\textsuperscript{T} (Table 1). Previously, T. scotoductus IT-7254 has been shown to oxidize thiosulfate and elemental sulfur (Skirnisdottir et al., 2001). Furthermore, the growth of this strain in nutrient medium was enhanced by thiosulfate as shown here for strains PRI 2268 and PRI 3838\textsuperscript{T}.

Cellular fatty acids were extracted from bacteria grown in medium 166 at 65 °C according to the protocol of the MIDI system. Analysis by GC was controlled by the MIS software and the peaks were automatically integrated and identified by the Microbial Identification software package (Sasser, 1990). The predominant fatty acids of strain PRI 3838\textsuperscript{T} were iso-17:0 (26.2 %), iso-15:0 (23.7 %), anteiso-15:0 (20.3 %) and anteiso-17:0 (15.2 %). Other fatty acids detected were mainly 16:0 (5.5 %), iso-16:0 (4.7 %), iso-14:0 (1.2 %) and 17:0 (1.1 %). The ratio of fatty acids anteiso-15:0 and anteiso-17:0 for strain PRI 3838\textsuperscript{T} was relatively high compared with many other species of the genus Thermus (Chung et al., 2000). The predominant fatty acids of strain PRI 3838\textsuperscript{T} were also detected in high ratios in the reference strain, ‘T. kawarayensis’. This strain contained mainly iso-15:0 (32.8 %), iso-17:0 (25.5 %), anteiso-15:0 (13.4 %) and anteiso-17:0 (9.0 %). Analyses of polar lipids and respiratory quinones were carried out by the DSMZ (B. J. Tindall). Menaquinones MK8 (96 %) and MK7 (2 %) were detected in strain PRI 3838\textsuperscript{T}. Two major phospholipids (PL1 and PN) and one major glycolipid (GL5) were identified along with a minor phospholipid (PL2) and several minor glycolipids (GL1–GL4 and GL6).

The species status of strains PRI 2268 and PRI 3838\textsuperscript{T} was demonstrated by a DNA–DNA hybridization value of 46.1 % between strain PRI 3838\textsuperscript{T} and ‘T. kawarayensis’ DSM 16200. DNA–DNA hybridization was performed by the DSMZ as described by De Ley et al. (1970) with the modifications of Huß et al. (1983). The G+C content of DNA was determined by the DSMZ according to Mesbah et al. (1989). DNA was purified as described by Cashon et al. (1977). The DNA G+C composition of strain PRI 3838\textsuperscript{T} was determined as 69.0 mol%. This was similar to the value for ‘T. kawarayensis’, which was determined as 68.0 mol% and to that of T. igniterrae (Chung et al., 2000). However, this value was higher than that obtained for other species of the genus Thermus (63–65 mol% G+C) (da Costa et al., 2001).

The 16S rRNA genes of strains PRI 3838\textsuperscript{T} and PRI 2268 were amplified using Darnazyme (Finzymes) and primers

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gene sequence similarity and DNA–DNA relatedness values of 96.5% and 46.1%, respectively, between strain PRI 3838T and the closest relative ‘T. kawarayensis’, a novel species of the genus *Thermus*, namely *Thermus islandicus* sp. nov., is proposed to accommodate strains PRI 2268 and PRI 3838T (=DSM 21543T=ATCC BAA-1677T).

**Description of Thermus islandicus** sp. nov.

*Thermus islandicus* (is.lan’di.cus. N.L. masc. adj. islandicus from Iceland, pertaining to the location of its first isolation).

Cells are non-spore-forming, non-motile rods measuring 0.3 μm in width and 1.5–2.5 μm in length. Cells stain Gram-negative and contain a thick envelope and a typical Gram-negative cell-wall profile. Aerobic, oxidase-positive, catalase-negative and does not reduce nitrate. Colonies are light yellow, slightly irregular, umbonate, undulate and 1–1.5 mm after 4 days at 65 °C on medium 166. Grows between 45 and 79 °C (optimum, 65 °C) and from pH 5.5–10.5 (optimum, pH 6.0–7.0). Grows well in medium 166 and R2A. Pyruvate, glycerol and starch support growth of the type strain at 60 °C when supplemented with yeast extract. Weak growth is exhibited on cellobiose, lactose and inositol. Does not utilize arabinose, fructose, galactose, glucose, mannose, melibiose, raffinose, rhamnose, ribose, sucrose, trehalose, xylose, ribitol, mannitol, sorbitol, citrate, malate, l-arginine, l-glutamine, l-serine or Casamino acids. Capable of mixotrophic growth with sulfur-oxidation in medium 166 supplemented with thiosulfate and in minimal medium supplemented with thiosulfate and yeast extract. Susceptible to ampicillin, gentamicin, novobiocin, kanamycin, penicillin and streptomycin. Resistant to nalidixic acid, polymyxin B and rifampicin. The predominant menaquinone is MK8. The

F9 (5′-GAGGTTTGATCCTGGCTCAG-3′) and R1544 (5′-AGAAAGGAGGTGATCCA-3′) (Skirnisdottir et al., 2000).

Reactions were subjected to 5 min at 94°C, 30 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1.5 min and finally to 7 min at 72°C. Amplification products were purified using ExoSapIt (Amersham Biosciences) and their sequences determined using ABI 3730 DNA Analyzer and a BigDye Terminator Cycle Sequencing kit (Applied Biosystems). The primers F9 and R1544 were used for sequencing as well as R805 (5′-GACTACCCGGGTATCTAATCC-3′), F338 (5′-ACICCTACGGGIGGCAGCAG-3′), R357 (5′-CTGCTGCCICTTAGG-3′) and R1195 (5′-GACGTCITCCCCICTTCTCC-3′) (Skirnisdottir et al., 2000). The sequences were assembled and analysed using Sequencher version 4.8 (GeneCodes). They were aligned with those of other species of the genus *Thermus* (1442 bp) by using CLUSTAL_X software (Thompson et al., 1997). Evolutionary distance matrices were calculated using the Kimura two-parameter model (Kimura, 1980). A phylogenetic tree was constructed according to the neighbour-joining method of Saitou & Nei (1987). The tree was displayed with NjPlot (Perrière & Gouy, 1996) and its topology was evaluated by a bootstrap analysis with 1000 trial replications. The 16S rRNA gene sequences of strain PRI 2268 and PRI 3838T were found to be 99.7% and 98.8% similar to the previously identified sequence SRI-248 (Skirnisdottir et al., 2000). The phylogenetic tree (Fig. 1) revealed that ‘*T. kawarayensis*’ KW11 was the closest relative of the new isolates (96.5% sequence similarity). Gene sequence similarity values of 96.1% and 95.9% were found to *T. aquaticus* YT-1T and *T. igniterrae* RF-4T, respectively.

On the basis of physiological differences between the novel strains and related organisms (Table 1) and on 16S rRNA gene sequence similarity and DNA–DNA relatedness values of 96.5% and 46.1%, respectively, between strain PRI 3838T and the closest relative ‘*T. kawarayensis*’, a novel species of the genus *Thermus*, namely *Thermus islandicus* sp. nov., is proposed to accommodate strains PRI 2268 and PRI 3838T (=DSM 21543T=ATCC BAA-1677T).

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major fatty acids are iso-17:0, iso 15:0, anteiso 15:0 and anteiso 17:0.

The type strain, PRI 3838T (=DSM 21543T=ATCC BAA-1677T), was isolated from a hot spring in the Torfajökull geothermal area in Iceland. Strain PRI 2268 is a reference strain. The DNA G+C content of the type strain is 69 mol%.

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References


