Thermosulfidibacter takaii gen. nov., sp. nov., a thermophilic, hydrogen-oxidizing, sulfur-reducing chemolithoautotroph isolated from a deep-sea hydrothermal field in the Southern Okinawa Trough

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A novel thermophilic, sulfur-reducing chemolithoautotroph, strain ABI70S6\textsuperscript{T}, was isolated from a deep-sea hydrothermal field at the Yonaguni Knoll IV, Southern Okinawa Trough. Cells of strain ABI70S6\textsuperscript{T} were motile rods, 0.9–2.0 m in length and 0.4–0.8 m in width. Strain ABI70S6\textsuperscript{T} was an obligately anaerobic chemolithotroph, exhibiting hydrogen oxidation coupled with sulfur reduction. Growth was observed at 56–78 °C (optimum, 70 °C), pH 5.0–7.5 (optimum, pH 5.5–6.0) and 0.5–4.5 % NaCl (optimum, 3.0 % NaCl). H\textsubscript{2} and elemental sulfur were utilized as electron donor and acceptor, respectively. The major fatty acids were C\textsubscript{16:0} (40.0 %) and C\textsubscript{20:1} (60.0 %). The G+C content of genomic DNA was 44.2 mol%. The physiological attributes of strain ABI70S6\textsuperscript{T} are similar to those of species of genera within the family Desulfurobacteriaceae, most of which are thermophilic and chemolithoautotrophic sulfur reducers. However, 16S rRNA gene sequence similarities between the novel isolate and type strains of all species within the family Desulfurobacteriaceae were <87 %, which is close to the similarities found between the novel isolate and members of the family Thermodesulfobacteriaceae (<85 %). Based on physiological and phylogenetic features of the novel isolate, it is proposed that it represents a novel species in a novel genus, Thermosulfidibacter takaii gen. nov., sp. nov., within the phylum Aquificae. The type strain of T. takaii is ABI70S6\textsuperscript{T} (\textsuperscript{=}JCM 13301\textsuperscript{T}=DSM 17441\textsuperscript{T}).

Recent studies on microbial ecosystems in deep-sea hydrothermal environments have indicated that deeply branching, thermophilic, hydrogenotrophic and sulfidogenic bacteria in the families Desulfurobacteriaceae and Thermodesulfobacteriaceae play important roles as potential primary producers (L’Haridon et al., 2006; Nakagawa et al., 2005; Reysenbach et al., 2000a, b; Takai et al., 2004; Van Dover et al., 2001). The family Desulfurobacteriaceae, which comprises the genera Desulfurobacterium, Thermovibrio and Balnearium (L’Haridon et al., 2006; Takai et al., 2003b), is proposed to belong to the order Aquificales (L’Haridon et al., 2006), although the family can be differentiated physiologically and phylogenetically from the families Aquificae and Hydrogenothermaceae and the possibility of establishing a new phylum for this family has been discussed (L’Haridon et al., 1998; Reysenbach, 2001). All species of the family Desulfurobacteriaceae are obligately anaerobic hydrogenotrophs that utilize elemental sulfur, sulfite and nitrate, but not oxygen or sulfate, as electron acceptors and grow optimally at relatively low temperatures (<80 °C) (L’Haridon et al., 1998, 2006; L’Haridon & Jeantoni, 2001; Reysenbach, 2001; Takai et al., 2003b) compared with members of the Aquificae and Hydrogenothermaceae (Eder & Huber, 2002).

Thermodesulfobacteriaceae is the only family in the phylum Thermodesulfobacteria, which originally comprised thermophilic, strictly anaerobic and chemoheterotrophic sulfate reducers isolated from terrestrial geothermal environments (Garrity & Holt, 2001; Hatchikian et al., 2001). On the other hand, recent isolates that have been categorized in the family Thermodesulfobacteriaceae have come from submarine and terrestrial geothermal hydrothermal systems and are hydrogenotrophic and obligate chemolithoautotrophic organisms, such as Thermodesulfobacterium hydrogenophilum, Thermodesulfatator indicus and
'Geothermobacterium ferrireducens' (Jeanthon et al., 2002; Kashefi et al., 2002; Moussard et al., 2004). In addition, a number of rRNA gene phylotypes detected from both terrestrial and deep-sea hydrothermal environments have been identified and these are related phylogenetically to members of the family Thermodesulfobacteriaceae (Hugenholtz et al., 1998; Nakagawa et al., 2005; Skirnisdottir et al., 2000; Van Dover et al., 2001). Thus, it seems likely that members of the family Thermodesulfobacteriaceae play important roles as primary producers in the terrestrial and deep-sea hydrothermal ecosystems in which they can be found.

Here, isolation of a novel strictly anaerobic, thermophilic chemolithoautotroph from a deep-sea hydrothermal field at the Yonaguni Knoll IV in the Southern Okinawa Trough is reported. This bacterium has a simple metabolism of hydrogen oxidation coupled with reduction of elemental sulfur, similar to that observed for members of the family Desulfurobacteriaceae. However, the newly isolated strain appears to hold a novel phylogenetic position among the deeply branching members of the class Bacteria. It is proposed that this strain represents a novel species in a novel genus, Thermosulfidibacter takaii gen. nov., sp. nov.; the type strain is ABI70S6T (=JCM 13301T=DSM 17441T). Moreover, phylogenetic analysis and comparison of physiological properties among the novel isolate and the members of the families Desulfurobacteriaceae and Thermodesulfobacteriaceae present the possibility of novel taxonomic relationships among deeply branching members of the class Bacteria.

A self-temperature-recording in situ colonization system (STR-ISCS), described by Takai et al. (2003a), was deployed for 4 days in a hydrothermal vent site named the Abyss Vent that is present in soft sediments at the Yonaguni Knoll IV hydrothermal field in the Southern Okinawa Trough (24°50.769′N 122°42.036′E) during cruise YKO3-05 (July 2003) using the R/V Yokosuka and the manned submersible Shinkai 6500. The temperature of the hydrothermal fluid was 80 °C. For cultivation analyses, the pumice stuffed in the recovered ISCS was stored anaerobically with MJ synthetic seawater (Sako et al., 1996) supplemented with 0.05 % neutralized Na₂S in a glass bottle under 100 % N₂ (200 kPa) and sealed with butyl rubber stoppers. The samples were used in serial-dilution cultivation tests using various media. After culturing in MMJS medium (pH 6.0), which is a modified version of MMJ medium (Takai et al., 2002) supplemented with elemental sulfur (3 % w/v) and with a headspace gas of 80 % H₂/20 % CO₂ (200 kPa) at 70 °C, the presence of short, motile rods was observed in the most diluted series (<4.0 × 10³ cells (g pumice)⁻¹). A pure culture of strain ABI70S6T was obtained at 70 °C by using the 'serial dilution to extinction' technique (Takai et al., 2000). The purity of the isolate was tested by microscopic observation and partial sequencing of the 16S rRNA gene.

Cells were observed routinely by using an Olympus BX51 microscope. Transmission electron micrographs of negatively stained and thin section cells grown in MMJS medium at 70 °C in the late-exponential phase were obtained as described by Zillig et al. (1990). The cells were straight rods, approximately 0.9–2.0 μm in length and 0.4–0.8 μm in width (Fig. 1a, b), motile with a polar flagellum (Fig. 1a) and with both outer and inner membrane structure (Fig. 1b).

Growth of isolate was determined by direct cell counting under epifluorescence after staining with 4',6-diamidino-2-phenylindole (Porter & Feig, 1980) using an Olympus BX51 microscope. To determine the temperature, pH and NaCl concentration ranges for growth, cultures were grown in 20 ml test tubes containing 3 ml MMJS medium with shaking (100 r.p.m.) in a temperature-controlled drying oven. Strain ABI70S6T grew at 55–78 °C, with an optimum growth temperature of 70 °C. The doubling time at the optimum temperature was 7.2 h and the maximum cell density observed was 2.0 × 10⁸ cells ml⁻¹. No growth was observed at 50 or 80 °C. The effect of initial pH on growth was examined at 70 °C by using MMJS medium under a variety of pH conditions adjusted as described previously (Takai et al., 2005). The pH range for growth at 70 °C was 5.0–7.5; optimum growth was at pH 5.5–6.0. The range of NaCl concentrations in MMJS medium that supported growth at 70 °C and pH 6.0 was 0.5–4.5 % (w/v) NaCl;
the optimum NaCl concentration was 3.0 % (see Supplementary Fig. S1, available in IJSEM Online).

Utilization of various combinations of electron donors (H₂, elemental sulfur and thiosulfate) and acceptors (elemental sulfur, thiosulfate, sulfite, sulfate, ferrihydrite, goethite, ferric citrate, nitrate, nitrite and oxygen) were tested by using MMJ medium. The concentrations of thiosulfate, sulfite and sulfate added as sodium salts, elemental sulfur and Fe(III) were 0.1, 3 and 0.1 % (w/v), respectively. Gas mixtures of 80 % H₂/20 % CO₂ and 80 % N₂/20 % CO₂ (both at 200 kPa) were used as the headspace gas for hydrogen and sulfur oxidation conditions, respectively. In the O₂ utilization test, O₂ (1 and 5 %) was added to the headspace gases (200 kPa). The isolate utilized H₂ as the sole energy source and elemental sulfur as the sole electron acceptor.

Nitrogen sources for growth (ammonium, nitrogen gas, nitrite and nitrate) were determined by using MMJS medium without nitrogen compounds such as NH₄Cl, and under 80 % H₂/20 % CO₂ (200 kPa), except when nitrogen gas was tested. NH₄Cl, NaNO₂ and NaN₃O were used at 0.02 % (w/v). Nitrogen gas utilization was examined under 65 % H₂/15 % CO₂/20 % N₂ (200 kPa). Strain ABI70S6ᵀ grew with ammonium as a nitrogen source, but not with nitrogen gas, nitrite or nitrate.

Utilization of organic carbon sources was tested in the absence of NaHCO₃ in MMJS medium, using H₂ as the headspace gas. The following substrates were added at 0.05 % (w/v): yeast extract (Difco), peptone, tryptone peptone (Difco), Casamino acids (Difco), glucose, maltose, fructose, sucrose, lactose, galactose, cellulbiose, mannose, rhamnose, xylose, mannitol, glycerol, ethanol, methanol, fumarate, tartrate, acetate, formate, citrate, pyruvate, malate, propionate, succinate, mannitol, glutamate, glycine and alanine. Strain ABI70S6ᵀ could not utilize any of the organic compounds tested in this study as a sole carbon source. The results suggest that this strain is a chemolithoautotroph.

The cellular fatty acid composition of strain ABI70S6ᵀ was analysed with cells grown in MMJS medium at 70 °C and harvested at late-exponential phase using methods described previously (Takai et al., 2003b). The fatty acids detected were C₁₆:₀ (40.0 %) and C₂₀:₀ (60.0 %).

Genomic DNA was prepared as described by Lauerer et al. (1986). The G+C content was determined by direct analysis of deoxyribonucleotides by HPLC (Tamaoka & Komagata, 1984). The G+C content of genomic DNA from strain ABI70S6ᵀ was 44.2 mol%. The 16S rRNA gene was amplified by PCR using primers Bac27F and 1492R (DeLong, 1992; Lane, 1985). The sequence of approximately 1.5 kb of amplified fragment was determined directly by using the deoxynucleotide chain-termination method with a DNA sequencer model 3100 (Applied Biosystems). This near-complete rRNA gene sequence (1450 bp) was analysed by using the FASTA algorithm (http://fasta.ddbj.nig.ac.jp/top-j.html). The results revealed clearly that the isolate is related only distantly to strains in known phylogenetic groups of the class Bacteria. The most closely related group was the family Desulfurobacteriaceae, and sequence similarity to rRNA genes from type strains of members of the three genera in this family (Desulfurobacterium, Balnearium and Thermovibrio) ranged from 85.0 to 86.3 %. The next most similar group was the family Thermodesulfobacteriaceae, which includes the genera ‘Geothermobacterium’, Thermodesulfobacterium and Thermodesulfatator (sequence similarities of 83.3–84.6 %).

For phylogenetic analysis, 16S rRNA gene sequences from deeply branching members of the class Bacteria and environmental phylotypes were aligned by using ARB version 20030822 (Ludwig et al., 2004) and CLUSTAL_X (Thompson et al., 1997). The environmental 16S rRNA gene phylotypes (GenBank accession numbers AB295463–AB295469), except for PS-B30 and SRI-27, were retrieved from hydrothermal sediments around the Abyss Vent by PCR amplification using primers Bac27F and Bac927R (T. Nunoura and others, unpublished results). Phylogenetic topology was calculated by using unambiguously aligned nucleotide positions. Neighbour-joining analysis was performed by using CLUSTAL_X and phylogenetic topology was confirmed by maximum-likelihood and maximum-parsimony analyses using PAUP version 4 (Sinauer Associates, Sunderland, MA, USA). The results of phylogenetic analyses that included strain ABI70S6ᵀ reveal a novel phylogenetic relationship among deeply branching members of the class Bacteria. The isolate grouped phylogenetically with members of the families Desulfurobacteriaceae and Thermodesulfobacteriaceae in the cluster of the phylum Aquificae (Fig. 2). In addition, in the phylogenetic tree that includes strain ABI70S6ᵀ and/or environmental phylotypes that were obtained from hydrothermal sediments, the family Thermodesulfobacteriaceae clustered with the Desulfurobacteriaceae and strain ABI70S6ᵀ within the phylum Aquificae (see Supplementary Fig. S2, available in IJSEM Online). In phylogenetic trees in previous studies, the family Desulfurobacteriaceae always appeared to be a potential sister group of the order Aquificales (L’Haridon et al., 1998, 2006; Reysenbach, 2001) and Thermodesulfobacteriaceae was the only family in the phylum Thermodesulfobacteria (Garrity & Holt, 2001; Hatchikian et al., 2001). However, bootstrap values in previous phylogenetic topologies were always lower than those in the novel phylogenetic topology presented in this study including 16S rRNA gene sequences from novel isolates and environmental phylotypes. Therefore, we adopt the novel phylogenetic topology in this study.

Strain ABI70S6ᵀ is similar to members of the families Desulfurobacteriaceae and Thermodesulfobacteriaceae with respect to many growth characteristics, including temperature and pH ranges for growth, morphology and obligately anaerobic hydrogenotrophy (Table 1).
Regarding utilization of electron acceptors, the isolate is able to use only elemental sulfur. This is similar to the electron acceptor-utilization profiles of members of the family Desulfurobacteriaceae, most of which use elemental sulfur as their primary electron acceptor, but differs from members of the family Thermodesulfobacteriaceae, which use sulfate or Fe(III) as the primary electron acceptor (Table 1). However, 16S rRNA gene sequence similarity between strain ABI70S6T and any previously described species in the family Desulfurobacteriaceae is <87%, which is lower than the index value of the genus level of differentiation (Gillis et al., 2001). Therefore, strain ABI70S6T represents a novel species in a novel genus, for which the name Thermosulfidibacter takaii gen. nov., sp. nov. is proposed; the type strain is ABI70S6T (=JCM 1330T=DSM 17441T). This species is physiologically and morphologically similar to members of the family Desulfurobacteriaceae, as described above, and formed a sister cluster in the phylogenetic tree (Fig. 2). However, phylogenetic distances between Thermosulfidibacter takaii and members of the families Desulfurobacteriaceae and Thermodesulfobacteriaceae are nearly equivalent, the
Table 1. Characteristics of *Thermosulfidibacter takaii* and members of the families *Desulfurobacteriaceae* and *Thermodesulfobacteriaceae*

Species and references: 1, *Thermosulfidibacter takaii* sp. nov. (this study); 2, *Desulfurobacterium thermolithotrophum* (L’Haridon et al., 1998); 3, *Desulfurobacterium crinifex* (Alain et al., 2003); 4, *Desulfurobacterium pacificum* (L’Haridon et al., 2006); 5, *Desulfurobacterium atlanticum* (L’Haridon et al., 2006); 6, *Thermovibrio ruber* (Huber et al., 2002); 7, *Thermovibrio ammonificans* (Vetriani et al., 2004); 8, *Thermovibrio guaymasensis* (L’Haridon et al., 2006); 9, *Balnearium lithotrophicum* (Takai et al., 2003b); 10, *Thermodesulfobacterium commune* (Zeikus et al., 1983); 11, *Thermodesulfobacterium mobile* (Rozanova & Pivovarova, 1988); 12, *Thermodesulfobacterium hveragerdense* (Sonne-Hansen & Ahring, 1999); 13, *Thermodesulfobacterium hydrogeniphilum* (Jeanthon et al., 2002); 14, *Thermodesulfatator indicus* (Moussard et al., 2004); 15, *Geothermobacterium ferrireducens* (Kashefi et al., 2002). All species utilize H₂ as an electron donor.

+Positive; −, negative; ND, not determined; HPLC, determined by HPLC method; TM, determined by Tₘ method.

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<td>42 HPLC</td>
<td>41 HPLC</td>
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<td>46 HPLC</td>
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</table>

*Sea salts concentration.
topology between Thermosulfidibacter takaii and the families Desulfurobacteriaceae and Thermodesulfobacteriaceae is not stable (Fig. 2; Supplementary Fig. S2, available in IJSEM Online), and the phylogenetic tree including environmental phylotypes presents a novel cluster comprising strain ABI70S6T and environmental phylotypes (Supplementary Fig. S2). Accordingly, the genus Thermosulfidibacter does not seem to belong to either of the families Desulfurobacteriaceae or Thermodesulfobacteriaceae, but probably represents a novel family; the genus Thermosulfidibacter and the families Desulfurobacteriaceae and Thermodesulfobacteriaceae have the potential of forming a novel order in the phylum Aquificae.

**Description of Thermosulfidibacter gen. nov.**

Thermosulfidibacter (Ther.mo.sul.fid.i.bac’ter. Gr. fem. n. therme heat; N.L. n. sulfidum sulfide; L. masc. n. bacter a rod; N.L. masc. n. Thermosulfidibacter a thermophilic, sulfide-producing, rod-shaped bacterium). Gram-negative rods. Spores are not formed. Anaerobic, thermophilic and neutrophilic. Strictly chemolithoautotrophic. The type species is Thermosulfidibacter takaii.

**Description of Thermosulfidibacter takaii sp. nov.**

Thermosulfidibacter takaii (ta.kai’i. N.L. gen. n. takaii of Takai, named after Dr Ken Takai, a microbiologist who has devoted himself to the study of terrestrial and deep-sea hydrothermal microbial ecosystems and chemolithoautotrophs present in those environments).

Motile rods, 0.9–2.0 × 0.4–0.8 µm, with a polar flagellum. Strictly anaerobic. Growth occurs at 55–78 °C (optimum, 70 °C), pH 5.0–7.5 (optimum, pH 5.5–6.0) and 0.5–4.5 % NaCl (optimum, 3.0 % NaCl). H2 and elemental sulfur are utilized as electron donor and acceptor, respectively, and hydrogen sulfide is produced. Major fatty acids are C16:0 (40.0 %) and C20:1 (60.0 %). Ammonium is required as a nitrogen source.

The type strain is ABI70S6T (=JCM 13301T = DSM 17441T), isolated from an STR-ISCS deployed in a hydrothermal vent site at the Yonaguni Knoll IV field, Southern Okinawa Trough. The DNA G+C content of the type strain is 44.2 mol%.

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**References**


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Thermosulfidibacter takaii gen. nov., sp. nov.


