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^T, was isolated from a deep-sea hydrothermal field at the Yonaguni Knoll IV, Southern Okinawa Trough. Cells of strain ABI70S6^T were motile rods, 0.9–2.0 µm in length and 0.4–0.8 µm in width. Strain ABI70S6^T was an obligately anaerobic chemolithotroph, exhibiting hydrogen oxidation coupled with sulfur reduction. Growth was observed 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 were utilized as electron donor and acceptor, respectively. The major fatty acids were C_{16:0} (40.0 %) and C_{20:1} (60.0 %). The G + C content of genomic DNA was 44.2 mol%. The physiological attributes of strain ABI70S6^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^T (=JCM 13301^T=DSM 17441^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 *Aquificaceae* 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 & Jeanthon, 2001; Reysenbach, 2001; Takai *et al.*, 2003b) compared with members of the *Aquificaceae* 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

Abbreviation: STR-ISCS, self-temperature-recording *in situ* colonization system.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of ABI70S6^T is AB282756.

Graphs showing the effects of temperature, pH and NaCl on growth of strain ABI70S6^T and a phylogenetic tree of representative deeply branching members of the *Bacteria* including environmental phylotypes based on 16S rRNA gene sequences are available with the online version of this paper.

'*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 ABI70S6^T (=JCM 13301^T=DSM 17441^T). 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 YK03-05 (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 [$<4.0 \times 10^3$ cells (g pumice)⁻¹]. A pure culture of strain ABI70S6^T 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 ABI70S6^T 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 2.0×10^8 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;

Fig. 1. Electron micrographs of a negatively stained cell (a) and a thin section (b) of strain ABI70S6^T. Bars, 500 nm (a); 200 nm (b).

the optimum NaCl concentration was 3.0 % (see Supplementary Fig. S1, available in IJSEM Online).

Utilization of various combinations of electron donors (H_2 , 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 % H_2 /20 % CO_2 and 80 % N_2 /20 % CO_2 (both at 200 kPa) were used as the headspace gas for hydrogen and sulfur oxidation conditions, respectively. In the O_2 utilization test, O_2 (1 and 5 %) was added to the headspace gases (200 kPa). The isolate utilized H_2 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 NH_4Cl , and under 80 % H_2 /20 % CO_2 (200 kPa), except when nitrogen gas was tested. NH_4Cl , $NaNO_2$ and $NaNO_3$ were used at 0.02 % (w/v). Nitrogen gas utilization was examined under 65 % H_2 /15 % CO_2 /20 % N_2 (200 kPa). Strain ABI70S6^T grew with ammonium as a nitrogen source, but not with nitrogen gas, nitrate or nitrite.

Utilization of organic carbon sources was tested in the absence of $NaHCO_3$ in MMJS medium, using H_2 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, cellobiose, mannose, rhamnose, xylose, mannitol, glycerol, ethanol, methanol, fumarate, tartrate, acetate, formate, citrate, pyruvate, malate, propionate, succinate, mannitol, glutamate, glycine and alanine. Strain ABI70S6^T 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 ABI70S6^T 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 $C_{16:0}$ (40.0 %) and $C_{20:0}$ (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 ABI70S6^T 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 ABI70S6^T 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 ABI70S6^T and/or environmental phylotypes that were obtained from hydrothermal sediments, the family *Thermodesulfobacteriaceae* clustered with the *Desulfurobacteriaceae* and strain ABI70S6^T 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 ABI70S6^T 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).

Fig. 2. Phylogenetic tree of representative deeply branching members of the class *Bacteria* based on 16S rRNA gene sequences. The tree was constructed by the neighbour-joining method, using 1038 sequence positions for each organism. Numbers at nodes indicate bootstrap values from 100 trials. GenBank/EMBL/DBJ accession numbers are given in parentheses. *Thermococcus celer* (M21529), *Methanocaldococcus jannaschii* (M59126), *Desulfurococcus mobilis* (X06188) and *Pyrobaculum oguniense* (AB029339) were used as the outgroup. Bar, two substitutions per 100 nt.

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 ABI70S6^T 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 ABI70S6^T represents a novel species in a novel genus, for which the name *Thermosulfidibacter takaii* gen. nov., sp. nov. is proposed; the type strain is ABI70S6^T (=JCM 13301^T=DSM 17441^T). 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 & Pivovarov, 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 T_m method.

Character	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
DNA G + C content (mol%)	44.2 ^{HPLC}	35 ^{TM}	37 ^{HPLC}	42 ^{HPLC}	41 ^{HPLC}	45 ^{TM}	54.6 ^{HPLC}	46 ^{HPLC}	34.6 ^{HPLC}	34.4 ^{TM}	38 ^{TM}	40 ^{HPLC}	28 ^{TM}	46 ^{HPLC}	ND
Temperature range (°C)	55–78	40–75	50–70	55–85	50–80	50–80	60–80	50–88	45–80	45–82	45–85	55–74	50–80	55–80	65–100
Optimal temperature (°C)	70	70	60–65	75	70–75	75	75	75–80	70–75	70	65	70–74	75	70	85–90
NaCl range (%)	0.5–4.5	1.5–7.0*	2.0–4.0*	1.5–5.0	1.5–5.0	2.0–4.7	0.5–4.5	1.5–5.0	0.8–5.6	<2.0	<2.0	ND	0.5–5.5	1.0–3.5	0.0–0.75
Optimal NaCl (%)	3	3.5*	3	3	3	3	2	3	3.2	0	0.05–1.0	ND	3	2.5	0.0–0.05
pH range	5.0–7.5	4.4–8.0	5.0–7.5	5.5–7.5	5.5–7.0	5.0–6.5	5.0–7.0	5.5–7.5	5.0–7.0	6.0–8.0	ND	ND	6.3–6.8	6.0–6.7	ND
Optimal pH	5.5–6.0	6.0–6.5	6.0–6.5	6.0–6.2	5.8–6.0	6.0	5.5	6.0–6.2	5.4	7.0	ND	ND	6.5	6.25	6.8–7.0
Electron donors:															
Pyruvate	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−
Lactate	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−
Fermentation	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−
Electron acceptors:															
Polysulfide	ND	+	−	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	−
Sulfur	+	+	+	+	−	+	+	+	+	ND	ND	−	−	−	−
Thiosulfate	−	+	−	+	+	−	−	−	−	+	+	+	−	−	−
Sulfite	−	+	−	−	−	−	−	−	−	−	+	+	−	−	−
Sulfate	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−
Nitrate	−	−	+	+	−	+	+	+	−	−	ND	−	−	−	−
Fe(III)	−	ND	−	ND	ND	ND	ND	ND	−	ND	ND	ND	ND	ND	+
Nitrogen sources:															
Ammonium	+	−	ND	−	−	ND	ND	−	+	ND	ND	ND	ND	+	ND
Nitrate	−	+	ND	+	+	+	ND	+	−	ND	ND	ND	ND	−	ND
Cell size (µm)	0.4–0.8 × 0.9–2.0	0.4–0.5 × 1.0–2.0	0.4–0.7 × 0.9–3.5	0.4–0.5 × 1.0–2.0	0.4–0.5 × 2.5–3.5	0.5–0.8 × 1.5–3.0	0.6 × 1.0	1.0–2.0 × 1.0–2.0	0.7–0.9 × 2.5–3.5	0.3 × 0.9	0.6 × 2.0	0.5 × 2.8	0.4–0.5 × 0.5–0.8	0.4–0.5 × 0.8–1.0	0.5 × 1.0–1.2

*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 ABI70S6^T 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.fi.di.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 C_{16:0} (40.0 %) and C_{20:1} (60.0 %). Ammonium is required as a nitrogen source.

The type strain is ABI70S6^T (=JCM 13301^T=DSM 17441^T), 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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