Caldimicrobium thiodismutans sp. nov., a sulfur-disproportionating bacterium isolated from a hot spring

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Subject category: New taxa: Other Bacteria

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence and genome of strain TF1T are LC055107 and AP014945, respectively.
A novel autotrophic thermophilic bacterium, strain TF1<sup>T</sup> was isolated from a hot spring in Japan. Cells of strain TF1<sup>T</sup> were motile, Gram-stain-negative, rod-shaped, 1.0–2.0 µm in length and 0.5–0.6 µm in width. Major components in the cellular fatty acid profile were C<sub>16:0</sub>, C<sub>18:0</sub>, and anteiso-C<sub>17:0</sub>. The temperature range for growth was 40°C–77°C, and optimum temperature was 75°C. The pH range for growth was 5.9–9.5, and the optimum pH was 7.5–8.8. Strain TF1<sup>T</sup> grew chemolithoautotrophically by disproportionation of sulfur, thiosulfate, and sulfite. Phylogenetic analysis based on 16S rRNA gene indicated that the strain belongs to the family *Thermodesulfobacteriaceae*. The closest cultivated relative was *Caldimicrobium rimae* DS<sup>T</sup>, with the highest sequence similarity of 96%. The genome of strain TF1<sup>T</sup> consists of one circular chromosome, with a size of 1.8 Mbp and G+C contents of 38.30 mol%. On the basis of its phylogenetic and phenotypic properties, strain TF1<sup>T</sup> (= DSM 29380<sup>T</sup> = NBRC 110713<sup>T</sup>) is proposed as type strain of a new species *Caldimicrobium thiodismutans* sp. nov.
All known members of the family *Thermodesulfobacteriaceae* are thermophilic anaerobes, including sulfate reducers of the genera *Thermodesulfobacterium* (Zeikus et al., 1983) and *Thermodesulfatator* (Moussard et al., 2004), sulfur reducer of the genus *Caldimicrobium* (Miroshnichenko et al., 2009) and sulfur disproportionater of the genus *Thermosulfurimonas* (Slobodkin et al., 2012). In addition, an iron reducer ‘*Geothermobacterium ferrireducens*’ FW-1a also belongs to this lineage (Kashefi et al., 2002). In this study, a thermophilic sulfur-disproportionating bacterium was isolated as representative of a new species in the family *Thermodesulfobacteriaceae*.

Strain TF1T was isolated from a neutral to slightly alkaline hot spring in central Japan, Nakabusa hot spring (36˚23’ N, 137˚45’ E), where microbial community structure has been investigate by culture-independent methods (Nakagawa & Fukui, 2003; Kubo et al., 2011; Everroad et al., 2012). A sample of submerged microbial streamer was obtained from a pool of spring water (around 75°C, pH 8.6–8.7). The basal medium used in this study was a bicarbonate-buffered defined medium previously described (Kojima & Fukui, 2011). The composition of the medium was as follows (l⁻¹): 0.2 g MgCl₂ . 6H₂O, 0.1 g CaCl₂ . 2H₂O, 0.1 g NH₄Cl, 0.1 g KH₂PO₄, 0.1 g KCl, 1 ml trace element solution, 1 ml selenite-tungstate solution, 1 ml vitamin mixture solution, 1 ml vitamin B₁₂ solution, 1 ml thiamine solution, 30 ml NaHCO₃ solution, and 1.5 ml
Na$_2$S$_2$O$_3$ solution. All stock solutions were prepared as described previously (Widdel & Bak, 1992). To establish the first enrichment, a piece of microbial streamer was inoculated in to 70 ml of the medium supplemented with 10 mM Na$_2$S$_2$O$_3$. Into the culture, anoxic stock slurry of poorly crystalline Fe(III) oxide was added to obtain final concentrations of 10 mM. The slurry was prepared by neutralizing FeCl$_3$ solution and washing with water (Lovley, 2013). The head space of the bottle was filled with N$_2$/CO$_2$ (80:20 v/v), and incubation was performed at 70°C. After progression of disproportionation was confirmed by change in color, a small portion of culture (1–2% volume of fresh medium) was transferred to the medium of same composition. Finally, strain was isolated in pure culture by repeated serial dilution. Purity of the isolate was checked by microscopy and sequencing of the 16S rRNA gene fragments. For further characterizations, strain TF1$^T$ was cultured at 70°C with the same medium supplemented with 20 mM Na$_2$S$_2$O$_3$ and 20 mM Fe(III) oxide, unless otherwise specified.

Fatty acid profile was analyzed at the Techno Suruga Co. Ltd (Shizuoka, Japan), by using the Sherlock Microbial Identification System (Version 6.0; database, MOORE6; MIDI). Gram-stain test was conducted with a kit (Fluka). Presence of desulfoviridin was tested by its fluorescence as described previously (Watanabe et al., 2015). Effect of
the temperature on growth was examined by culturing at various temperatures (37, 40, 42, 45, 48, 50, 52, 55, 70, 72, 75, 76, 77, 78, and 80°C). Effects of the pH and salt concentration on growth were examined by culturing in modified media of various pH (adjusted with HCl or NaOH) and NaCl concentration (0, 1, 2, 3, and 4% w/v).

Utilization of electron donors and acceptors was tested in media supplemented with substances in varied combinations. The poorly crystalline Fe(III) oxide and soluble substances were tested at concentration of 20 mM, unless otherwise specified. Hydrogen utilization as an electron donor was tested by filling the headspace with gas mixture (H₂ : N₂ : CO₂, 20 : 64 : 16; ca. 1.25 atm total pressure). Disproportion of thiosulfate (20 mM), sulfite (5 mM) and elemental sulfur (ca. 0.5 g l⁻¹) was tested in the presence of poorly crystalline Fe(III) oxide and confirmed by production of sulfate quantified with ion chromatography. Fermentation was tested in thiosulfate-free media, which were supplemented with sulfide or sulfate as sole sulfur source (1 mM).

To find out phylogenetic position, the 16S rRNA gene of strain TF1ᵀ amplified with the primers 27F and 1492R (Lane, 1991) was directly sequenced, for phylogenetic analysis using the program MEGA version 5.1 (Tamura et al., 2011). After its novelty was confirmed, whole genome of was strain TF1ᵀ sequenced. Genomic DNA was subjected to paired-end sequencing by Illumina HiSeq. After assembly using Velvet
ver1.2.08 (11,027,898 reads were used), all gaps were closed by PCR and Sanger sequencing. As a result, a single circular contig was obtained. Annotation of the genome sequence was performed as described previously (Kojima et al., 2015).

Cells of TF1ᵀ were motile, rod-shaped, Gram-stain-negative, 1.0–2.0 μm in length and 0.5–0.6 μm in width (Fig. S1). The novel strain was capable of autotrophic growth with disproportionation of elemental sulfur, thiosulfate, and sulfite. Desulfoviridin was not detected in cells grown with thiosulfate disproportionation. None of the following electron donors supported growth of the strain by thiosulfate reduction: H₂, lactate, fumarate, pyruvate, acetate, propionate, formate, succinate, malate, ethanol, glucose (10 mM), sucrose (4 mM), galactose (4 mM) and peptone (Difco, 1%). No growth was observed with the following combinations of electron donor/acceptor: H₂/SO₄²⁻, lactate/SO₄²⁻, formate/SO₄²⁻, H₂/poorly crystalline Fe(III) oxide, H₂/NO₃⁻, S₂O₃²⁻/NO₃⁻, pyruvate/NO₃⁻, and S₂O₃²⁻/O₂ (1% and 10% v/v in the headspace). Fermentative growth was not observed with lactate, fumarate and pyruvate in the presence of sulfide or sulfate as sulfur source.

The growth of the strain TF1ᵀ was observed at temperature range of 40°C–77°C, and the optimum growth was observed at 75°C. Range of initial pH for growth was 5.9–9.5, and the optimum pH was 7.5–8.8. Negative effect of 1% NaCl on growth was observed,
and no growth occurred in the media containing 2% or more NaCl.

In the cellular fatty acid profile of strain TF1\textsuperscript{T}, C\textsubscript{16} : 0, C\textsubscript{18} : 0, and anteiso-C\textsubscript{17} : 0 were major components, accounting for 28.6%, 28.1% and 18.4% of total, respectively. The other fatty acids detected were iso-C\textsubscript{16} : 0 (5.9%), summed feature 9 (iso-C\textsubscript{16} : 0 3-OH and/or unknown 17.157 DMA; 3.1%), C\textsubscript{14} : 0 (3.0%), iso-C\textsubscript{17} : 0 (2.1%), C\textsubscript{17} : 0 (1.8%), anteiso-C\textsubscript{15} : 0 (1.8%), iso-C\textsubscript{18} : 0 (1.7%), C\textsubscript{18} : 1\omega9c (1.0%), C\textsubscript{16} : 1\omega7c DMA (0.9%), summed feature 10 (C\textsubscript{18} : 1\omega7c and/or unknown 17.843; 0.7%), anteiso-C\textsubscript{19} : 0 (0.6%), summed feature 6 (anteiso-C\textsubscript{15} : 0 3-OH and/or C\textsubscript{16} : 1\omega9c DMA; 0.6%), C\textsubscript{15} : 0 (0.6%), iso-C\textsubscript{15} : 0 3-OH (0.6%), iso-C\textsubscript{15} : 0 (0.4%), anteiso-C\textsubscript{17} : 0 3-OH (0.4%).

The 16S rRNA gene sequence of strain TF1\textsuperscript{T} was identical or very closely related to partial sequences of the 16S rRNA gene which had been detected in Nakabusa hot spring (Nakagawa & Fukui, 2003; Kubo et al., 2011; Everroad et al., 2012). In the maximum-likelihood phylogenetic tree, it was shown that the novel isolate is a member of the family Thermodesulfobacteriaceae (Fig. 1). Affiliation of the novel isolate within this family was consistently observed in trees constructed with the methods of minimum-evolution and neighbor-joining (Fig. S2). The characterized bacterium which showed highest sequence similarity to strain TF1\textsuperscript{T} was Caldimicrobium rimae DS\textsuperscript{T} (96%).
The genome of strain TF1\(^T\) consists of one circular chromosome, with a size of 1,814,952 bp and G+C content of 38.30%. In the genome, 1,755 protein coding sequences, 46 tRNA-encoding genes, and 1 rRNA operon were predicted. In the 23S rRNA gene, an insert region was identified. The insertion was exhibited homology to self-splicing group I introns in the 23S rRNA gene of Coxiella burnetii, including a region encoding a homing endonuclease (Raghavan et al., 2007).

Among existing genera with validly published names, only the genus Caldimicrobium can accommodate the novel strain (Fig. 1, Fig. S2). It has been shown that Caldimicrobium rimae DS\(^T\) was not capable of disproportionation of elemental sulfur or thiosulfate (Slobodkin et al., 2012), in contrast to strain TF1\(^T\). On the other hand, heterotrophic growth of strain TF1\(^T\) was not observed with the substrates which support growth of Caldimicrobium rimae DS\(^T\) (Table 1). These physiological differences and relatively low sequence similarity of 16S rRNA gene (96%) indicate that these two strains should not be placed in the same species. Therefore, Caldimicrobium thiodismutans sp. nov. is proposed with type strain of TF1\(^T\) (= DSM 29380\(^T\) = NBRC 110713\(^T\)).
Description of *Caldimicrobium thiodismutans* sp. nov.

*Caldimicrobium thiodismutans* (thi.o.dis’mu.tans. Gr. n. theion sulfur; L. particle. dis, in two; L. pres. part. mutans, altering; N.L. part. adj. thiodismutans, dismutating sulfur).

Cells are rod-shaped, 1.0–2.0 µm in length and 0.5–0.6 µm in width. Growth occurs at 40–77°C with the optimum growth temperature of 75°C. The pH range for growth is 5.9–9.5, and optimum growth occurs at pH 7.5–8.8. Grows in the presence of 0–1% (w/v) NaCl in medium. Major cellular fatty acids are C_{16:0}, C_{18:0}, and anteiso-C_{17:0}.

The G+C content of genomic DNA is 38 mol%. Autotrophic growth occurs with disproportionation of thiosulfate, sulfite and elemental sulfur. Lactate, fumarate, pyruvate, acetate, propionate, formate, succinate, malate, ethanol, glucose, sucrose, galactose and peptone do not support growth under thiosulfate-reducing conditions.

Sulfate, thiosulfate, nitrate and poorly crystalline Fe(III) oxide do not serve as electron acceptor for H₂ oxidation. The type strain TF1<sup>T</sup> (= DSM 29380<sup>T</sup> = NBRC 110713<sup>T</sup>) was isolated from a hot spring in Japan.

Emended description of the genus *Caldimicrobium* Miroshnichenko et al., 2009

Extremely thermophilic and strictly anaerobic. Cells are Gram-stain-negative. Able to grow chemolithoautotrophically with hydrogen oxidation or disproportionation of sulfur.
compounds. The DNA G+C content of the type strain of the type species is 35.2 mol%.

16S rRNA gene sequence analysis places the genus in the family *Thermodesulfobacteriaceae*. The type species is *Caldimicrobium rima*.

**ACKNOWLEDGMENTS**

We thank R. Tokizawa and A. Shinohara for technical assistance. This study was supported by JSPS KAKENHI Grant Number 15K07209 to H. Kojima.

**REFERENCES**


Lovley, D. R. (2013). Dissimilatory Fe(III)- and Mn(IV)-reducing prokaryotes. In


Table 1. Differential properties of strain TF1<sup>T</sup> and the most closely related strain.

Strains: 1, TF1<sup>T</sup>; 2, *Caldimicrobium rimae* DS<sup>T</sup> (Miroshnichenko et al., 2009).

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<tr>
<th>Characteristics</th>
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<tr>
<td>DNA G+C content (%)</td>
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<td>Growth temperature range</td>
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<td>52–82</td>
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Fig. 1 Maximum-likelihood tree showing the phylogenetic position of strain TF1\textsuperscript{T} within the family *Thermodesulfobacteriaceae*. The tree was constructed by using 1079 sites in the 16S rRNA gene sequences. Numbers on nodes represent percentage values of 1000 bootstrap resampling.