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

Hisaya Kojima*, Kazuhiro Umezawa, and Manabu Fukui

The Institute of Low Temperature Science, Hokkaido University. Kita-19, Nishi-8, Kita-ku, Sapporo 060-0819, Japan

*Corresponding author.

E-mail: kojimah@pop.lowtem.hokudai.ac.jp

Phone: +81-11-706-5460

Fax: +81-11-706-5460

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence and genome of strain TF1^T are LC055107 and AP014945, respectively.

Summary

A novel autotrophic thermophilic bacterium, strain TF1^T was isolated from a hot spring in Japan. Cells of strain TF1^T 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 $C_{16:0}$, $C_{18:0}$, and anteiso-$C_{17:0}$. The temperature range for growth was 40ºC–77ºC, and optimum temperature was 75ºC. The pH range for growth was 5.9–9.5, and the optimum pH was 7.5–8.8. Strain TF1^T 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^T, with the highest sequence similarity of 96%. The genome of strain TF1^T 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^T (= DSM 29380^T = NBRC 110713^T) 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 $TF1^T$ 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 $MgCl_2$. $6H_2O$, 0.1 g $CaCl_2$. $2H_2O$, 0.1 g NH_4Cl, 0.1 g KH_2PO_4, 0.1 g KCl, 1 ml trace element solution, 1 ml selenite-tungstate solution, 1 ml vitamin mixture solution, 1 ml vitamin B_{12} solution, 1 ml thiamine solution, 30 ml $NaHCO_3$ solution, and 1.5 ml

54 $Na_2S_2O_3$ solution. All stock solutions were prepared as described previously (Widdel &
55 Bak, 1992). To establish the first enrichment, a piece of microbial streamer was
56 inoculated in to 70 ml of the medium supplemented with 10 mM $Na_2S_2O_3$. Into the
57 culture, anoxic stock slurry of poorly crystalline Fe(III) oxide was added to obtain final
58 concentrations of 10 mM. The slurry was prepared by neutralizing $FeCl_3$ solution and
59 washing with water (Lovley, 2013). The head space of the bottle was filled with N_2/CO_2
60 (80:20 v/v), and incubation was performed at 70°C. After progression of
61 disproportionation was confirmed by change in color, a small portion of culture (1–2%
62 volume of fresh medium) was transferred to the medium of same composition. Finally,
63 strain was isolated in pure culture by repeated serial dilution. Purity of the isolate was
64 checked by microscopy and sequencing of the 16S rRNA gene fragments. For further
65 characterizations, strain $TF1^T$ was cultured at 70°C with the same medium
66 supplemented with 20 mM $Na_2S_2O_3$ and 20 mM Fe(III) oxide, unless otherwise
67 specified.
68 Fatty acid profile was analyzed at the Techno Suruga Co. Ltd (Shizuoka, Japan), by
69 using the Sherlock Microbial Identification System (Version 6.0; database, MOORE6;
70 MIDI). Gram-stain test was conducted with a kit (Fluka). Presence of desulfoviridin
71 was tested by its fluorescence as described previously (Watanabe *et al*., 2015). Effect of

72 the temperature on growth was examined by culturing at various temperatures (37, 40,
73 42, 45, 48, 50, 52, 55, 70, 72, 75, 76, 77, 78, and 80°C). Effects of the pH and salt
74 concentration on growth were examined by culturing in modified media of various pH
75 (adjusted with HCl or NaOH) and NaCl concentration (0, 1, 2, 3, and 4% w/v).
76 Utilization of electron donors and acceptors was tested in media supplemented with
77 substances in varied combinations. The poorly crystalline Fe(III) oxide and soluble
78 substances were tested at concentration of 20 mM, unless otherwise specified.
79 Hydrogen utilization as an electron donor was tested by filling the headspace with gas
80 mixture (H_2 : N_2 : CO_2, 20 : 64 : 16; *ca.* 1.25 atm total pressure). Disproportion of
81 thiosulfate (20 mM), sulfite (5 mM) and elemental sulfur (ca. 0.5 g l^{-1}) was tested in the
82 presence of poorly crystalline Fe(III) oxide and confirmed by production of sulfate
83 quantified with ion chromatography. Fermentation was tested in thiosulfate-free media,
84 which were supplemented with sulfide or sulfate as sole sulfur source (1 mM).
85 To find out phylogenetic position, the 16S rRNA gene of strain TF1^T amplified with
86 the primers 27F and 1492R (Lane, 1991) was directly sequenced, for phylogenetic
87 analysis using the program MEGA version 5.1 (Tamura *et al*., 2011). After its novelty
88 was confirmed, whole genome of was strain TF1^T sequenced. Genomic DNA was
89 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 TF1^T 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; H_2, 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; H_2/SO_4^{2-}, lactate/SO_4^{2-}, formate/SO_4^{2-}, H_2/poorly crystalline Fe(III) oxide, H_2/NO_3^-, $S_2O_3^{2-}/NO_3^-$, pyruvate/NO_3^-, and $S_2O_3^{2-}/O_2$ (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 TF1^T was observed at temperature range of 40ºC–77ºC, 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^T, $C_{16:0}$, $C_{18:0}$, and anteiso-$C_{17:0}$ were major components, accounting for 28.6%, 28.1% and 18.4% of total, respectively. The other fatty acids detected were iso-$C_{16:0}$ (5.9%), summed feature 9 (iso-$C_{16:0}$ 3-OH and/or unknown 17.157 DMA; 3.1%), $C_{14:0}$ (3.0%), iso-$C_{17:0}$ (2.1%), $C_{17:0}$ (1.8%), anteiso-$C_{15:0}$ (1.8%), iso-$C_{18:0}$ (1.7%), $C_{18:1}\omega9c$ (1.0%), $C_{16:1}\omega7c$ DMA (0.9%), summed feature 10 ($C_{18:1}\omega7c$ and/or unknown 17.843; 0.7%), anteiso-$C_{19:0}$ (0.6%), summed feature 6 (anteiso-$C_{15:0}$ 3-OH and/or $C_{16:1}\omega9c$ DMA; 0.6%), $C_{15:0}$ (0.6%), iso-$C_{15:0}$ 3-OH (0.6%), iso-$C_{15:0}$ (0.4%), anteiso-$C_{17:0}$ 3-OH (0.4%).

The 16S rRNA gene sequence of strain TF1^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^T was *Caldimicrobium rimae* DS^T (96%).

126 The genome of strain TF1^T consists of one circular chromosome, with a size of
127 1,814,952 bp and G+C content of 38.30%. In the genome, 1,755 protein coding
128 sequences, 46 tRNA-encoding genes, and 1 rRNA operon were predicted. In the 23S
129 rRNA gene, an insert region was identified. The insertion was exhibited homology to
130 self-splicing group I introns in the 23S rRNA gene of *Coxielle burnetii*, including a
131 region encoding a homing endnuclease (Raghavan *et al*., 2007).
132
133 Among existing genera with validly published names, only the genus *Caldimicrobium*
134 can accommodate the novel strain (Fig. 1, Fig. S2). It has been shown that
135 *Caldimicrobium rimae* DS^T was not capable of disproportionation of elemental sulfur or
136 thiosulfate (Slobodkin *et al*., 2012), in contrast to strain TF1^T. On the other hand,
137 heterotrophic growth of strain TF1^T was not observed with the substrates which support
138 growth of *Caldimicrobium rimae* DS^T (Table 1). These physiological differences and
139 relatively low sequence similarity of 16S rRNA gene (96%) indicate that these two
140 strains should not be placed in the same species. Therefore, *Caldimicrobium*
141 *thiodismutans* sp. nov. is proposed with type strain of TF1^T (= DSM 29380^T = NBRC
142 110713^T).
143

144 Description of *Caldimicrobium thiodismutans* sp. nov.

145 *Caldimicrobium thiodismutans* (thi.o.dis'mu.tans. Gr. n. theion sulfur; L. particle. dis, in
146 two; L. pres. part. mutans, altering; N.L. part. adj. thiodismutans, dismutating sulfur).
147 Cells are rod-shaped, 1.0–2.0 µm in length and 0.5–0.6 µm in width. Growth occurs at
148 40–77°C with the optimum growth temperature of 75°C. The pH range for growth is
149 5.9–9.5, and optimum growth occurs at pH 7.5–8.8. Grows in the presence of 0–1%
150 (w/v) NaCl in medium. Major cellular fatty acids are $C_{16:0}$, $C_{18:0}$, and anteiso-$C_{17:0}$.
151 The G+C content of genomic DNA is 38 mol%. Autotrophic growth occurs with
152 disproportionation of thiosulfate, sulfite and elemental sulfur. Lactate, fumarate,
153 pyruvate, acetate, propionate, formate, succinate, malate, ethanol, glucose, sucrose,
154 galactose and peptone do not support growth under thiosulfate-reducing conditions.
155 Sulfate, thiosulfate, nitrate and poorly crystalline Fe(III) oxide do not serve as electron
156 acceptor for H_2 oxidation. The type strain TF1^T (= DSM 29380^T = NBRC 110713^T) was
157 isolated from a hot spring in Japan.

158

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

160 Extremely thermophilic and strictly anaerobic. Cells are Gram-stain-negative. Able to
161 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 rimae*.

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245 Table 1. Differential properties of strain TF1^T and the most closely related strain.

246 Strains: 1, TF1^T; 2, *Caldimicrobium rimae* DS^T (Miroshnichenko *et al*., 2009).

247

Characteristics	1	2
DNA G+C content (%)	38.3	35.2
Growth temperature range	40–77	52–82
Electron donors		
H_2	-	+
Fumarate	-	+
Malate	-	+
Succinate	-	+
Ethanol	-	+
Disproportionation of		
Thiosulfate	+	-
Elemental sulfur	+	-

248

249

Figure legends

Fig. 1 Maximum-likelihood tree showing the phylogenetic position of strain TF1^{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.