Thiofaba tepidiphila gen. nov., sp. nov., a novel obligately chemolithoautotrophic, sulfur-oxidizing bacterium of the Gammaproteobacteria isolated from a hot spring

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A novel obligately chemolithoautotrophic, sulfur-oxidizing bacterium designated strain BDA453T was isolated from a hot spring in Fukushima prefecture, Japan. The cells were short-rod-shaped and possessed an inclusion, a Gram-negative type cell wall and a single polar flagellum. Strain BDA453T grew by sulfur-oxidizing respiration with thiosulfate, elemental sulfur, sulfide and tetrathionate as electron donors and used only carbon dioxide as a carbon source. The optimum growth conditions were 45 °C, pH 6.5 and the absence of NaCl. Analysis of the 16S rRNA gene revealed that the isolate was a member of the Gammaproteobacteria and related to the genera Halothiobacillus and Thiiovirga in the family Halothiobacillaceae. However, the phylogenetic tree constructed using 16S rRNA gene sequences showed that strain BDA453T was distant from any other known bacteria with sequence similarities of less than 90%. On the basis of phenotypic features and phylogenetic analysis, strain BDA453T is considered to represent a novel species of a new genus within the family Halothiobacillaceae, for which the name Thiofaba tepidiphila gen. nov., sp. nov. is proposed. The type strain of Thiofaba tepidiphila is BDA453T (=NBRC 103218T=DSM 19618T).

In natural and artificial environments, many sulfide/oxygen interface layers exist (e.g. hot springs, hydrothermal vents, cold seep sediments, wastewater biofilms and anaerobic zones of lakes), which are inhabited by various chemolithoautotrophic, sulfur-oxidizing micro-organisms. These chemolithoautotrophic, sulfur-oxidizing micro-organisms play important roles in the sulfur cycle, as well as in the production of organic materials as primary producers. Various kinds of chemolithoautotrophic, sulfur-oxidizing micro-organisms have been detected at different sulfide concentrations, pH values, temperatures and under other chemical and physical conditions and they are phylogenetically widespread among the prokaryotes. Most of those identified to date belong to the phylum Proteobacteria, with other species belonging to a deep lineage in the Bacteria and the order Sulfolobales in the Archaea (Huber & Prangishvili, 2006; La Rivière & Schmidt, 2006; Robertson & Kuenen, 2006; Sorokin et al., 2006; Takai et al., 2003a).

Hot springs and hydrothermal vents are major sites with a constant supply of sulfide, where large biomasses of sulfur-oxidizing bacteria, called microbial mats, often develop (Elshahed et al., 2003; Reysenbach et al., 1994; Schulz et al., 1999; Skirnisdottir et al., 2000; Yamamoto et al., 1998). The temperature of hot springs is very important for the community structures of microbial mats (Hiraishi et al., 1999; Skirnisdottir et al., 2000) and various thermophilic, chemolithoautotrophic, sulfur-oxidizing bacteria have been isolated from them depending on the in situ temperature, e.g. Sulfurihydrogenibium subterraneum (Takai et al., 2003b), Thermocrinis ruber (Huber et al., 1998), Thio bacter subterraneus (Hirayama et al., 2005) and Thiobacillus aquaesulis (Wood & Kelly, 1988). Most thermophilic, chemolithoautotrophic, sulfur-oxidizing isolates belong to the orders Aquificales (Götz et al., 2002; Huber et al., 1992, 1998; Nakagawa et al., 2004; Takai et al., 2003b) and Sulfolobales (Huber & Stetter, 1991; Segerer et al., 1986, 1991), although some strains have been reported to belong to the class Gammaproteobacteria. The only species of thermophilic (or thermotolerant), chemolithoautotrophic, sulfur-oxidizing bacteria that are known to belong to the Gammaproteobacteria are Acidithiobacillus caldus (Hallberg & Lindström, 1994; Kelly & Wood, 2000), Thermithiobacillus tepidarius (Kelly & Wood, 2000; Wood & Kelly, 1985), Thiomicrospira thermophila (Takai et al., 2004) and Sulfuririgiva caldicularii (Takai et al., 2006).

Recently, a novel moderately thermophilic, obligately chemolithoautotrophic, sulfur-oxidizing bacterium, designated...
strain BDA453\textsuperscript{T}, was isolated from a hot spring in Fukushima prefecture, Japan. Based on phylogenetic analysis, this isolate has been classified in a new genus in the class \textit{Gammaproteobacteria}. In this paper, a novel taxon is proposed for strain BDA453\textsuperscript{T}: \textit{Thiofaba tepidiphila} gen. nov., sp. nov.

Akayu hot spring is a sulfur spring located in Fukushima prefecture, Japan. The temperature and pH of the water in this hot spring are 45 \degree C and 7.0, respectively. In order to enrich and isolate sulfur-oxidizing bacteria, AP96SO1 medium, i.e. basal medium supplemented with 5 mM Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3} under an N\textsubscript{2}/CO\textsubscript{2}/O\textsubscript{2} [60:20:20 (v/v); 150 kPa] atmosphere, was used. The basal medium was composed of the following salts and solutions (I\textsuperscript{-1}): 1.2 g KH\textsubscript{2}PO\textsubscript{4}, 0.2 g K\textsubscript{3}HPO\textsubscript{4}, 0.75 g MgCl\textsubscript{2}·6H\textsubscript{2}O, 0.15 g CaCl\textsubscript{2}, 2H\textsubscript{2}O, 0.5 g NH\textsubscript{4}Cl, 0.5 g Na\textsubscript{2}CO\textsubscript{3}, 2 ml trace element solution of NBRC medium 377 (NBRC, 2005) and 2 ml vitamin solution of NBRC medium 377 (NBRC, 2005). The medium was prepared in vials and bottles sealed with butyl-rubber stoppers and aluminium caps under N\textsubscript{2}/CO\textsubscript{2}/O\textsubscript{2}. For primary enrichment of sulfur-oxidizing bacteria, 2 ml hot spring water was inoculated into 20 ml AP96SO1 medium and cultivated at 45 \degree C. After incubation for 1 week, bacterial growth was observed in the culture microscopically and then the enrichment culture was transferred to fresh AP96SO1 medium several times. Because growth of the enriched bacteria could not be achieved successfully on solid AP96SO1 medium, an attempt was made to isolate bacteria by serial dilution using AP96SO1 medium. After repeating the maximum dilution several times, a sulfur-oxidizing bacterium, designated strain BDA453\textsuperscript{T}, was obtained. The purity of the isolate was verified by microscopic observation, inoculation into the basal medium containing various heterotrophic substrates and determination of the 16S rRNA gene sequence amplified using the following primer sets: universal primer set 530f and 1392r, bacterial primer set 27f and 1492r (Lane, 1991) and archaeal primer set A109f (Grosskopf \textit{et al.}, 1998) and Arch915 (Stahl & Amann, 1991).

Cells of strain BDA453\textsuperscript{T} were short rods (0.5–0.9 \mu m in length and 0.4 \mu m in width) with a single polar flagellum (Fig. 1a and b). Under the microscope, they appeared as single cells, in pairs or sometimes as aggregates. Cell motility was observed, but spore formation was not. For the observation of ultrathin sections, cells were fixed with 0.1 \% (v/v) glutaraldehyde and 4 \% (v/v) osmium tetroxide, stained with platinum blue (Inaga \textit{et al.}, 2007) and lead citrate, and observed using an electron microscope (model H-7600; Hitachi) operating at 100 kV. Cells of strain BDA453\textsuperscript{T} possessed one or two inclusions like a carboxysome in the cell and a Gram-negative type of cell wall with an outer membrane (Fig. 1c). Cells were Gram-negative, catalase-negative and weakly oxidase-positive.

Cellular fatty acids were methylated by a 5 \% HCl/methanol solution (Sasser, 1990) and analysed with the MIDI microbial identification system (Microbial ID; Agilent Technologies) and GC-MS (GC system model 6890 and MSD model 5973; Agilent Technologies). Strain BDA453\textsuperscript{T} possessed C\textsubscript{18}:1\textsuperscript{\alpha}9\textsubscript{c} (38 \% of the total fatty acids) and C\textsubscript{16}:0 (34 \%) as major fatty acids and C\textsubscript{16}:1\textsuperscript{\omega7\textsubscript{c}} (18 \%), C\textsubscript{12}:0 (7 \%), C\textsubscript{12}:0 3-OH (2 \%) and C\textsubscript{18}:0 (2 \%) as minor fatty acids. An isoprenoid quinone was extracted from the cells according to the protocol of Nakagawa & Yamasato.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Phase-contrast (a; bar, 3 \mu m) and transmission electron (b and c; bars, 0.5 \mu m) micrographs of strain BDA453\textsuperscript{T}. Negatively stained cells and ultrathin cell sections are shown in (b) and (c), respectively.}
\end{figure}
In the basal medium containing sulfur compounds under an \(\text{N}_2/\text{CO}_2/\text{O}_2\) atmosphere, strain BDA453\(^T\) oxidized thiosulfate (5 mM), elemental sulfur (5 %), sulfide (2 and 5 mM) and tetrathionate (5 and 10 mM) as electron donors. The following substrates could not support growth of the strain: sulfite (2 and 5 mM), \(\text{CH}_4\) \([\text{N}_2/\text{CO}_2/\text{O}_2/\text{CH}_4, 50:20:20:10\ (\text{v/v/v/v})]\), \(\text{H}_2\) \([\text{H}_2/\text{CO}_2/\text{O}_2, 60:20:20\ (\text{v/v/v})]\), \(\text{K}_2\text{H}_2\text{PO}_4, 150\ \text{kPa}\), \(\text{H}_2 + \text{acetate}\) (10 mM), methanol (2 and 5 mM), formate (10 and 30 mM), acetate (10 and 30 mM), butyrate (10 mM), citrate (10 mM), fumarate (10 mM), glutamate (10 mM), lactate (10 mM), pyruvate (10 mM), malate (10 mM), succinate (10 mM), \(\text{L}-\text{arginine}\) (10 mM), \(\text{L}-\text{asparagine}\) (10 mM), \(\text{L}-\text{cysteine}\) (10 mM), \(\text{L}-\text{histidine}\) (10 mM), \(\text{L}-\text{leucine}\) (10 mM), \(\text{L}-\text{methionine}\) (10 mM), arabinose (5 mM), fructose (5 mM), galactose (5 mM), glucose (5 mM), inositol (5 mM), mannose (5 mM), raffinose (5 mM), sucrose (5 mM), xylose (10 mM), \(\text{Bacto yeast extract}\) (2 g l\(^{-1}\); Difco), polyethylene glycol (2 g l\(^{-1}\); Nihon Seiyaku) and \(\text{Bacto Casamino acids}\) (model 2695 with conductivity detector model 432 and IC-TNH-2612; ADVANTEC), and the decrease in the level of thiosulfate and increase in that of sulfate. The concentration of thiosulfate and sulfate under optimum growth conditions (45 \(^\circ\)C, pH 6.5 and no NaCl). Cell density was measured by direct counting of cells stained with 4',6-diamidino-2-phenylindole on membrane filters (0.2 \text{mu} \text{m} \text{pore size polycarbonate membrane filter}; ADVANTEC). 1 Cell density; \(\triangle\), thiosulfate concentration; \(\Delta\), sulfate concentration.

An almost-complete 16S rRNA gene sequence of strain BDA453\(^T\) was determined according to the procedure reported previously by Hattori \textit{et al.} (2000). After alignment with the \textsc{arb} program (Ludwig \textit{et al.}, 2004), phylogenetic trees were constructed by the neighbour-joining method using the \textsc{clustalx} program (Saitou \& Nei, 1987; Thompson \textit{et al.}, 1997) and the maximum-likelihood method using the \textsc{nuclml} program in \textsc{molphy} (Adachi \& Hasegawa, 1995; Hasegawa \textit{et al.}, 1985; Mori \textit{et al.}, 2003). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain BDA453\(^T\) belonged to the class \textit{Gammaproteobacteria}. However, the sequence of strain BDA453\(^T\) was distant from those of all known genera in the \textit{Gammaproteobacteria} and the similarities between strain BDA453\(^T\) and species with validly published names were less than 90 %. Furthermore, the sequence of strain BDA453\(^T\) resembled those of ‘\textit{Thiobacillus baregensis}’ (sequence similarity 92.6 %; GenBank accession number Y09280) and the following environmental clones: clone MO31 from a hot spring microbial mat in India (99.7 %; EU037210); clone BPC028 from a hydrocarbon seep sediment (96.8 %; AF154088); clone MS149BH1062003\(_5\) from the subsurface water of the Kalahari Shield, South Africa (95.5 %; DQ354745); clone zEL16 from a limestone-corroding stream biofilm in Frasassi Cave, Italy (95.1 %; DQ415810) (Macalady \textit{et al.}, 2006); and clone SILK74 from a sulfur-oxidizing biofilm in Frasassi Cave, Italy (95.2 %; EF467495). Phylogenetic analyses using both neighbour-joining and maximum-likelihood methods of strain BDA453\(^T\), related species in \textit{Gammaproteobacteria} and related clones demonstrated that strain BDA453\(^T\) was associated with members of the family \textit{Halothiobacillaceae} in the order \textit{Chromatiales}, although the sequence

\textit{Thiofaba tepidiphila} gen. nov., sp. nov. (1993) and analysed with an LCMS-QP 8000z spectrometer (Shimadzu). Strain BDA453\(^T\) contained ubiquinone-8. The genomic DNA G+C content was determined by HPLC using a Shodex ODS pack F-411 (Showa Denko K.K.) after nuclease P1 treatment using a DNA-GC kit (Yamas Shoyu) followed by alkaline phosphatase treatment (Kamagata \& Mikami, 1991). The DNA G+C content of strain BDA453\(^T\) was 66.0 mol%.

The optimum temperature, pH and NaCl concentration ranges for growth with thiosulfate (5 mM) and oxygen as electron donor and acceptor, respectively, were determined by examining the time-course of optical density (temperature gradient incubator with a bio-photorecorder, model TN-2612; ADVANTEC), and the decrease in the level of thiosulfate and increase in that of sulfate. The concentration of thiosulfate and sulfate were measured by HPLC (model 2695 with conductivity detector model 432 and IC-Pak Anion column; Waters). Strain BDA453\(^T\) was able to grow at 20–51 \(^\circ\)C, the optimum temperature being 45 \(^\circ\)C. The initial pH range for growth was 6.0–9.0 and the optimum pH was 6.5. The pH in the medium decreased during the cultivation. The isolate grew optimally in the presence of NaCl and growth did not occur above 2 % (w/v) NaCl. The growth curve of strain BDA453\(^T\) under optimum growth conditions (45 \(^\circ\)C, pH 6.5 and 0 % NaCl concentration) is shown in Fig. 2. Strain BDA453\(^T\) grew with a doubling time of 1.64 h and the final concentration of the cells was approximately 1 \times 10^6 cells ml\(^{-1}\).
similarities between strain BDA453<sup>T</sup> and species of the genera *Halothiobacillus* and *Thiovirga* were less than 90%. The tree constructed using the neighbour-joining method is shown in Fig. 3.

Characteristics of strain BDA453<sup>T</sup> and members of the genera *Halothiobacillus* and *Thiovirga* are summarized in Table 1. The genus *Halothiobacillus* comprises obligately chemolithoautotrophic, sulfur-oxidizing bacteria (Durand et al., 1993; Hutchinson et al., 1965, 1969; Kelly et al., 1998; McDonald et al., 1997; Sievert et al., 2000; Smith & Kelly, 1979; Visser et al., 1997; Wood & Kelly, 1991) and can be distinguished from *Thiobacillus* based on halophilicity and the results of a comparison of 16S rRNA gene sequences (Kelly & Wood, 2000). Strain BDA453<sup>T</sup> preferred the absence of NaCl and this finding differentiated it from members of the genus *Halothiobacillus*. The sole species of the genus *Thiovirga* was isolated from wastewater biofilms (Ito et al., 2004) and described as a new genus belonging to the *Halothiobacillaceae* (Ito et al., 2005). *Thiovirga sulfuroxydans* was not halophilic and could not grow at NaCl concentrations above 180 mM. Strain BDA453<sup>T</sup> was also a non-halophilic, sulfur-oxidizing bacterium, but it clearly differed from *Thiovirga sulfuroxydans* with regard to the optimum growth temperature and pH and genomic DNA G+C content (Table 1). These phenotypic differences support the phylogenetically solitary position of strain BDA453<sup>T</sup> in the family *Halothiobacillaceae* (Fig. 3). On the basis of physiological and phylogenetic findings, a novel taxon, *Thiofaba tepidiphila* gen. nov., sp. nov., belonging to the family *Halothiobacillaceae*, is proposed.

**Description of Thiofaba gen. nov.**

*Thiofaba* (Thi.o.fa’ba. Gr. n. thion sulfur; L. fem. n. faba bean; N.L. fem. n. *Thiofaba* sulfur bean).

Obligately aerobic and chemolithoautotrophic Gram-negative rods. Do not form spores. Grow by the oxidation of reduced sulfur compounds and the fixation of carbon dioxide. Non-halophilic. Contain ubiquinone-8 as a major respiratory quinone. Major cellular fatty acids are C<sub>18:1</sub>ω9c and C<sub>18:3</sub>ω3c.

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**Fig. 3.** Neighbour-joining tree based on 16S rRNA gene sequences of strain BDA453<sup>T</sup> and relatives. After alignment with the ARB program, 1075 positions were used for analyses. The numbers at the nodes represent the bootstrap values. Two numbers are indicated if identical topology was given by both trees (neighbour-joining tree/maximum-likelihood tree); a single number represents the bootstrap value calculated by the neighbour-joining method. Bar, 0.03 substitutions per nucleotide site.

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**Table 1.** Characteristics of strain BDA453<sup>T</sup> and members of the genera *Halothiobacillus* and *Thiovirga*.
Table 1. Characteristics of strain BDA453\(^T\) and species of Thiovirga and Halothiobacillus

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditions for optimum growth:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
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<td>30–34</td>
<td>28–32</td>
<td>30–32</td>
<td>35–40</td>
<td>37–42</td>
</tr>
<tr>
<td>pH</td>
<td>6.5</td>
<td>7.5</td>
<td>6.5–6.9</td>
<td>7.0–7.3</td>
<td>7.5–8.0</td>
<td>6.5</td>
</tr>
<tr>
<td>NaCl (mM)</td>
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<td>0</td>
<td>ND</td>
<td>800–1000</td>
<td>430</td>
<td>400–500</td>
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<tr>
<td>Upper NaCl concentration for growth (mM)</td>
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<td>&gt;860</td>
<td>4000</td>
<td>2000</td>
<td>2500</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>66.0</td>
<td>47.1</td>
<td>56.0</td>
<td>64.2</td>
<td>67.4</td>
<td>62.0</td>
</tr>
</tbody>
</table>

C\(_{16:\text{m}}\). Phylogenetic position based on 16S rRNA gene sequence is in the family Halothiobacillaceae of the class Gammaproteobacteria. The type species is Thiofaba tepidiphila.

**Description of Thiofaba tepidiphila sp. nov.**

*Thiofaba tepidiphila* (te.pi.di’phi.la. L. adj. tepidis moderately warm; Gr. adj. philos loving; N.L. fem. adj. tepidiphila loving lukewarm conditions).

Cells are short rods, approximately 0.4 \(\mu\)m wide and 0.5–0.9 \(\mu\)m long, with a single polar flagellum. Cells possess an inclusion and a Gram-negative type cell wall. Cells occur singly, in pairs or as aggregates. Motility is observed. Gram reaction is negative. Catalase-negative and weakly oxidase-positive. Obligately chemolithoautotrophic and grows aerobically by the oxidation of reduced sulfur compounds (thiosulfate, elemental sulfur, sulfide and tetrathionate) and the fixation of carbon dioxide. Anaerobic respiration and fermentation are not observed. Grows at 20–51 °C; optimum growth at 45 °C. The initial pH range for growth is 6.0–9.0, with optimum at pH 6.5; pH decreases during growth. Grows optimally in the absence of NaCl; does not grow at above 2 % (w/v) NaCl. Ubiquinone-8 is a major quinone. Major cellular fatty acids are C\(_{18:\text{c9c}}\) and C\(_{16:\text{e0}}\)C\(_{16:\text{t7c}}\), C\(_{12:\text{e0}}\), C\(_{12:\text{e0}}\) 3-OH and C\(_{18:\text{e0}}\) are also present as minor components. The DNA G+C content of the type strain is 66.0 mol%.

The type strain, BDA453\(^T\) (=NBRC 103218\(^T\)=DSM 19618\(^T\)), was isolated from water of a hot spring, Akayu, Fukushima Prefecture, Japan.

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**References**


