

**Thiobacter subterraneus** gen. nov., sp. nov., an obligately chemolithoautotrophic, thermophilic, sulfur-oxidizing bacterium from a subsurface hot aquifer

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A novel, thermophilic, obligately chemolithoautotrophic, sulfur/thiosulfate-oxidizing bacterium was isolated from subsurface geothermal aquifer water (temperature approximately 70 °C) in the Hishikari gold mine, Japan. Cells of the isolate, designated strain C55T, were motile, straight rods with a single polar flagellum. Growth was observed at temperatures between 35 and 62 °C (optimum 50–55 °C; 60 min doubling time) and pH between 5.2 and 7.7 (optimum pH 6.5–7.0). High growth rate of strain C55T was observed on either thiosulfate or elemental sulfur as a sole energy source, with molecular oxygen as the only electron acceptor. None of the organic compounds tested supported or stimulated growth of strain C55T. The G+C content of the genomic DNA was 66.9 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain C55T was affiliated to the β-Proteobacteria, but was distantly related to recognized genera. On the basis of its physiological and molecular properties, strain C55T (=JCM12421T = DSM 16629T = ATCC BAA-941T) is proposed as the type strain of *Thiobacter subterraneus* gen. nov., sp. nov.

During the last decade, studies using culture-dependent isolation techniques or culture-independent molecular analytical methods have suggested that thermophilic, chemolithotrophic, hydrogen- and/or sulfur-oxidizing micro-organisms within the order Aquificales and β-Proteobacteria are prevalent in terrestrial hot springs at high temperatures with neutral to alkaline pH (Huber et al., 1998; Hugenholtz et al., 1998; Reysenbach et al., 1994; Stoehr et al., 2001; Takai et al., 2001, 2002; Yamamoto et al., 1998). Previous culture-independent analyses of microbial communities in subsurface geothermal aquifer waters (70–73 °C) in a Japanese gold mine identified two predominant phylotypes, pHAuB-D within the Aquificales and pHAuB-J within the β-Proteobacteria, representing novel phylogenetic affiliations distantly related to previously cultivated strains (Takai et al., 2002). Numerous cultivation experiments to identify these previously uncultivated phylotypes were conducted by focusing on thermophilic chemolithotrophs capable of using inorganic substrates enriched in the aquifer, and they resulted in successful isolation of several potential novel thermophilic species and the description of a novel hydrogen- and sulfur-oxidizing bacterium, *Sulfurihydrogenobium subterraneus* (Takai et al., 2002, 2003; Inagaki et al., 2003). In this study, isolation and characterization of another novel thermophilic sulfur/thiosulfate-oxidizing bacterium within the β-Proteobacteria are described. The 16S rRNA gene sequence of this bacterium was similar to those of the previously detected environmental clones pHAuB-J from the mine and OBP37 from sulfide-rich sediment in the Obsidian Pool (75–95 °C) in Yellowstone National Park, USA (Hugenholtz et al., 1998).

A number of thermophilic, hydrogen- and/or sulfur-oxidizing members of the β-Proteobacteria have been described, including genera such as *Hydrogenophilus* (Hayashi et al., 1999; Stoehr et al., 2001), *Thiomonas* (Shooner et al., 1996), *Thermothrix* (Caldwell et al., 1976; Odintsova et al., 1996), *Tepidimonas* (Moreira et al., 2000) and *Thiobacillus* (Wood & Kelly, 1988). These organisms have been isolated from terrestrial hot-spring environments or wastewater-treatment plants, and most of them are...
facultatively autotrophic or strictly heterotrophic organisms. *Thermothrix azorensis* is obligately chemolithoauto-
trophic, using reduced sulfur compounds as the energy
source. The new isolate showing obligately chemolithoauto-
trophic growth by the oxidation of reduced sulfur com-
pounds is phylogenetically and physiologically compared
with members of the genera within the β-Proteobacteria.

**Sample collection, enrichment and purification**

A hot (70-4 °C) subsurface aquifer water from AW-S hole in
the main deposit of the Hishikari gold mine, Kagoshima
Prefecture, Japan, was obtained at the dewatering station in
the mine (Izawa et al., 1990; Takai et al., 2002). At the time
of sampling, 1 ml of the hot aquifer water was inoculated
into 3 ml TSmj medium (see below) under a gas phase of
80 % N₂, 15 % CO₂ and 5 % O₂ (200 kPa). After trans-
portation of the inoculated medium to the laboratory
without temperature control, cultivation was performed at
55 °C in a dry oven. Growth of motile, straight rods was
observed after 3 days of incubation. A pure culture was
observed after 3 days of incubation. A pure culture was
obtained by using the repeated dilution-to-extinction
technique (Baross, 1995) at 55 °C with the same medium
as used for the enrichment. This culture was designated
strain C55T. Its purity was confirmed routinely by micro-
scopic examination and by repeated partial sequencing of
the 16S rRNA gene using several PCR primers.

**Culture medium and conditions**

Strain C55T was routinely cultivated in TSmj medium. TSmj
medium consists of 1 g Na₂S₂O₃.5H₂O, 0-5 g NaHCO₃,
0-5 g NH₄Cl, 1 g Na₂SiO₃.9H₂O and 10 ml vitamin mixture
(Balch et al., 1979) per litre of mj water (Takai et al., 2001).
The mj water consists of (per litre of distilled, deionized
water) 3-0 g NaCl, 14 mg KH₂PO₄, 80 mg CaCl₂, 0-34 g
MgSO₄.7H₂O, 0-42 g MgCl₂.6H₂O, 33 mg KCl, 0-05 mg
NiCl₂.6H₂O, 0-05 mg Na₂SeO₃.5H₂O, 0-01 mg Na₂WO₄,
2 mg Fe(NH₄)₂(SO₄)₂.6H₂O and 1 ml trace mineral solu-
tion (Balch et al., 1979). To prepare TSmj medium, all
chemical reagents other than vitamin solution and NaHCO₃
were dissolved, and the pH of the medium was adjusted
to around 7-0 with HCl before autoclaving. After autoclaving
under an air atmosphere, a concentrated solution of vitamins and NaHCO₃ was added to the medium. The
concentrated NaHCO₃ solution was separately sterilized
by autoclaving and the vitamin solution was filter-sterilized.
The medium, dispensed at 20 % of the bottle (Schott
Glasswerke) or tube (Iwaki Glass) volume, was then purged
with 80 % N₂ and 20 % CO₂. The bottle or tube was tightly
sealed with a butyl rubber stopper and the headspace was
then pressurized with a gas mixture (80 % N₂, 18 % CO₂
and 2 % O₂) at 200 kPa unless otherwise indicated.

**Morphology**

Cells were observed under a phase-contrast Olympus BX51
microscope with the SPOT RT Slider CCD camera system
(Diagnostic Instruments). Transmission electron microcopy
of negatively stained cells was carried out as described by
Zillig et al. (1990). Cells grown in TSmj medium under
microaerobic conditions (2 % partial pressure of O₂) at
55 °C in the mid-exponential phase of growth were nega-
tively stained with 2 % (w/v) uranyl acetate and observed
under a JEOL JEM-1210 electron microscope at an acceler-
ating voltage of 120 kV. Cells of strain C55T were Gram-
negative rods, about 1-1-1-9 μm long and 0-4-0-5 μm wide,
and were motile with a polar flagellum (see Supplementary
Figs A and B in IJSEM Online).

**Growth characteristics**

Growth of strain C55T was measured by direct cell count-
ing after staining with 4',6-diamidino-2-phenylindole using
a phase-contrast Olympus BX51 microscope. Duplicate
cultures were prepared in 100 ml glass bottles each con-
taining 20 ml medium, with shaking (100 r.p.m.) in a
temperature-controlled dry oven. In TSmj medium, strain
C55T grew at the temperature range of 35–62 °C, with
optimal growth at 50–55 °C. No growth was observed below
30 °C or above 65 °C (see Supplementary Fig. C in IJSEM
Online). The effect of pH on growth was tested at 55 °C.
The pH of TSmj medium was readjusted with HCl or
NaOH immediately before inoculation. The pH of the
TSmj medium used for this experiment was found to be
stable during cultivation up to a density of 2 × 10⁹ cells
ml⁻¹, and therefore growth was monitored in cultures
with a density below this value. Growth of strain C55T
occurred at pH 5-2–7-7, with optimum growth at pH 6-5–
7-0 (Supplementary Fig. D). No growth was observed at
pH 5-1 or 8-5.

To determine the effect of mineral salt concentration on
growth, variously diluted or concentrated mj waters con-
taining constant amounts of Na₂S₂O₃.5H₂O, NaHCO₃,
NH₄Cl, Na₂SiO₃,9H₂O, vitamin mixture and trace mineral
solution were tested. Growth of strain C55T was determined
with several Na⁺ concentrations in the medium. Strain
C55T grew at [Na⁺] between 20 and 280 mM. Optimum
growth was seen at 70 mM [Na⁺], 55 °C and pH 6-5, with a
60 min doubling time (Supplementary Fig. E).

The effect of oxygen concentration in the gas phase was
tested with TSmj medium under a series of gas mixtures of
N₂/O₂ of 80:20:0, 80:19:5:0:5, 80:19:1:80:18:2, 80:15:5,
75:15:10 or 65:15:20, at 200 kPa. Growth of strain C55T
was observed at 0-5–10 % O₂ with an increase
in cell numbers from 3 × 10⁸ to 2 × 10⁹ cells ml⁻¹. The
maximum increase in growth of strain C55T was seen
under 2 or 5 % O₂ with 1–2 × 10⁹ cells ml⁻¹, whereas no growth
was observed either in the absence of O₂ or under 20 % O₂.
These results indicated that strain C55T is a microaero-
philic organism.

Heterotrophic growth was examined in TSmj medium
without NaHCO₃ under a gas phase of 98 % N₂ and 2 % O₂
(200 kPa), containing potential organic carbon sources:
0-1 % (w/v) each of yeast extract, peptone, tryptone and
Casamino acids, 5 mM each of formate, acetate, citrate, tartrate, fumarate, malate, succinate, lactate, oxalate and pyruvate, 0-02 % (w/v) each of glucose, galactose, sucrose, maltose and 0-01 % methanol. Strain C55ᵀ was not able to grow with any of the organic compounds tested as sole carbon sources. Furthermore, no stimulation of growth was observed with the addition of yeast extract or tryptone to TSmj medium containing thiosulfate, NaHCO₃ and CO₂ in a gas phase.

To determine potential electron donors other than thiosulfate for autotrophic growth, 1 or 5 mM each of Na₂S₄, cysteine hydrochloride, disulfate (Na₂S₂O₇) or elemental sulfur (3 %; w/v) was added to TSmj medium instead of thiosulfate as a sole electron donor with a gas phase of 80 % N₂, 18 % CO₂ and 2 % O₂ (200 kPa). Molecular hydrogen was also examined in TSmj medium without thiosulfate with a gas phase of 80 % H₂, 18 % CO₂ and 2 % O₂ (200 kPa). Elemental sulfur as an electron donor resulted in a similar maximum increase in cell numbers to that obtained with thiosulfate (1 × 10⁹ cells ml⁻¹), whereas Na₂S (1 mM) produced lower cell numbers (1–2 × 10⁸ cells ml⁻¹) and other reduced sulfur compounds and hydrogen did not support growth of strain C55ᵀ as the sole electron donor. Na₂S₄ at 5 mM seemed to be toxic to strain C55ᵀ. No electron acceptor tested [NaNO₃ (2 or 10 mM), NaNO₂ (1 or 5 mM), ferric citrate (20 mM), ferrihydride (20 mM), Na₂SO₃ (5 mM), Na₂SO₄ (5 mM)] supported growth of strain C55ᵀ. These results indicate that strain C55ᵀ is a chemolithoautotroph utilizing reduced sulfur compounds (thiosulfate, elemental sulfur or sulfide) as an energy source and molecular oxygen as the sole electron acceptor.

With regard to nitrogen sources for growth, strain C55ᵀ utilized nitrate, ammonium and Casamino acids, but could not utilize NaNO₂ or N₂.

The time-course of oxidation of thiosulfate during growth of strain C55ᵀ was monitored with TSmj medium at pH 6.5 under a gas phase of 80 % N₂, 18 % CO₂ and 2 % O₂ (200 kPa) at 55 °C (Figs. 1). Concentrations of thiosulfate, sulfite and sulfate were analysed using the P/ACE MDQ capillary electrophoresis system (Beckman Coulter). Consumption of thiosulfate, sulfite and production of sulfate were both observed during the growth of strain C55ᵀ. However, some inconsistency was observed in the stoichiometry of thiosulfate consumption and resulting sulfate production by strain C55ᵀ. During the early growth phase (0–3 h), 1–4 mM thiosulfate was consumed but only 0–2 mM sulfate was produced (7 % of the theoretical value) (Fig. 1). During the mid-exponential growth phase (3–5 h), 0-8 mM thiosulfate was consumed and 1 mM sulfate was produced (63 % of the theoretical value). In contrast, in the late exponential growth phase (5–7.5 h), consistency in stoichiometry was found, with 2–1 mM thiosulfate consumed and 4–3 mM sulfate produced (100 % of the theoretical value). However, in the stationary growth phase (7.5–16.5 h), only 0.1 mM thiosulfate was consumed whereas 1–2 mM sulfate was produced (600 % of the theoretical value). This result indicates possible accumulation of sulfur compounds in the cells of strain C55ᵀ, especially in the early stages of growth, because no obvious elemental sulfur precipitation was observed in the medium during growth. Accumulated sulfur compounds in the cells of strain C55ᵀ were found during fatty acid analysis (see below). From the concentrated fatty acid sample extracted from whole cells of strain C55ᵀ, considerable amounts of sulfur compounds were precipitated. Further GC-MS analysis of the sample detected a cyclic polysulfur (8S) peak among the peaks of fatty acids. Therefore, strain C55ᵀ seemed to transport thiosulfate into the cells and accumulate it as polysulfur, especially in the early stage of growth, for utilization as an energy source in the stationary phase. The production of sulfite was not observed during growth. The control medium (uninoculated) did not exhibit either thiosulfate oxidation or sulfate production. These results indicate that strain C55ᵀ is a respiratory sulfur-oxidizer, producing sulfate as an end product.

Sensitivity to a variety of antibiotics in strain C55ᵀ was examined with liquid TSmj medium containing each compound. Cell growth of strain C55ᵀ was inhibited by chloramphenicol (10 μg ml⁻¹), streptomycin (10 μg ml⁻¹), kanamycin (1 μg ml⁻¹), ampicillin (1 μg ml⁻¹), rifampicin (1 μg ml⁻¹) and vancomycin (60 μg ml⁻¹).

**Fatty acid composition and G+C content of genomic DNA**

The cellular fatty acid composition of cells grown in TSmj medium at 55 °C in the late exponential growth phase was determined. Lyophilized cells (100 mg) were placed in a Teflon-lined, screw-capped tube containing 3 ml anhydrous methanolic HCl and heated at 100 °C for 3 h. Extraction and analysis of fatty acid methyl esters were done as described by Takai et al. (2003). The major cellular fatty acids of strain C55ᵀ were C₁₆:₀ (72.8 %), C₁₆:₁ (23.1 %),

![Fig. 1. Time-courses of oxidation of thiosulfate (■), the production of sulfate (●) and concomitant bacterial growth (▲) of strain C55ᵀ.](image-url)
C₁₈:₀ (2.3%), iso-C₁₈:₀ (1.3%) and C₁₈:₁ (0.4%). The ratio of n-C₁₈ fatty acids (95.9% of the total fatty acids) in strain C55ᵀ was high in comparison with other thermophilic micro-organisms within the β-Proteobacteria, such as Hydrogenophilus hirsutus (58%; Stöhr et al., 2001), Tepidimonas ignava (49%; Moreira et al., 2000) or Tepidiphilus margaritifer (43%; Manaia et al., 2003). Genomic DNA of strain C55ᵀ was prepared as described by Marmur & Doty (1962). The DNA G+C content was determined by direct analysis of deoxyribonucleotides using HPLC (Tamaoka & Komagata, 1984). The G+C content of the genomic DNA of strain C55ᵀ was 66 mol%, a value similar to that of other thermophilic members of the β-Proteobacteria, including Hydrogenophilus thermoluteolus (63.5 mol%; Hayashi et al., 1999), Tepidiphilus margaritifer (64.8 mol%; Manaia et al., 2003), Thiobacillus aquaesulis (65.7 mol%; Wood & Kelly, 1988) and Tepidimonas ignava (69.7 mol%; Moreira et al., 2000) (Table 1).

### 16S rRNA gene sequence and phylogenetic analysis

The nearly complete sequence (1452 bp) of the 16S rRNA gene of strain C55ᵀ was amplified by PCR and directly sequenced from both strands with a DNA sequencer model 3100 (Perkin Elmer/Applied Biosystems). Similarity of the 16S rRNA gene sequence to the nucleotide sequence sequenced from both strands with a DNA sequencer model 3100 (Perkin Elmer/Applied Biosystems). Similarity of the 16S rRNA gene sequence and phylogenetic analysis indicated that strain C55ᵀ is related to members of the β-Proteobacteria, and comparative evolutionary distance analysis demonstrated that the isolate represents a separate lineage of descent within the β-Proteobacteria (Fig. 2). The highest similarity (98%) was observed between the 16S rRNA gene sequences of strain C55ᵀ and the environmental clone pHAuB-J previously detected from the same hot aquifer water (Takai et al., 2002). The sequence of strain C55ᵀ was also similar to that of environmental clone OBP37 (95%), which was detected from sulfide-rich sediment in the Obsidian Pool (75–95 °C) in Yellowstone National Park, USA (Hugenholtz et al., 1998). Other than these environmental clone sequences, strain C55ᵀ was distantly related to members of other genera within the β-Proteobacteria, such as Azoarcus buckelii U120ᵀ (92-9%; Mechichi et al., 2002), Sterolibacterium dentriticans Chol-1Sᵀ (92-1%; Tarlera & Denner, 2003), Thiobacillus aquaesulis ATCC 43788ᵀ (91-8%; Wood & Kelly, 1988), Tepidiphilus margaritifer N2-214ᵀ (90-8%; Manaia et al., 2003), Tepidimonas ignava SPS-1037ᵀ (90-6%; Moreira et al., 2000), Hydrogenophilus thermoluteolus HT-1ᵀ (89-1%; Hayashi et al., 1999) and Thiomonas thermosulfata ATCC 51520ᵀ (85-5%; Shooner et al., 1996).

### Table 1. Comparison of properties among thermophilic members of the β-Proteobacteria

Strains: 1, Thiobacter subterraneus gen. nov., sp. nov. C55ᵀ; 2, Thiobacillus aquaesulis ATCC 43788ᵀ (data from Wood & Kelly, 1988); 3, Tepidimonas ignava SPS-1037ᵀ (Moreira et al., 2000); 4, Tepidiphilus margaritifer N2-214ᵀ (Manaia et al., 2003); 5, Hydrogenophilus thermoluteolus TH-1ᵀ (Hayashi et al., 1999); 6, Thiomonas thermosulfata ATCC 51520ᵀ (Shooner et al., 1996). ND, Not described.

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In recent years, a number of workers using culture-independent molecular analyses have reported the
dominance of \textit{\beta\text{-}Proteobacteria} in hot springs and other high temperature environments (Hugenholtz \textit{et al.}, 1998; LaPara \textit{et al.}, 2000; Yamamoto \textit{et al.}, 1998). Yet relatively few thermophilic micro-organisms within the \textit{\beta\text{-}Proteobacteria} have been isolated and described, and almost none of these has been an obligate chemolithoautotroph (Table 1). \textit{Thiobacillus aquaeulis} ATCC 43788\textsuperscript{T} and \textit{Thiomonas thermosulfata} ATCC 51520\textsuperscript{T} are facultatively chemolithoautotrophic sulfur-oxidizers, whereas \textit{Tepidimonas ignava} SPS-1037\textsuperscript{T} is an obligate heterotroph with the ability to use reduced sulfur compounds as an energy source. Species of the genus \textit{Hydrogenophilus} are facultative chemolithioautotrophs by the oxidation of \textit{H}_2, and a chemo-organoheterotrophic \textit{Tepidiphilus margaritifer} strain N2-214\textsuperscript{T} is reported to show a positive hydrogenase activity. In comparison with these thermophilic species of \textit{\beta\text{-}Proteobacteria} isolated from terrestrial hot-spring environments or wastewater-treatment plants, strain C55\textsuperscript{T} represents obligately chemolithoautotrophic growth by the oxidation of reduced sulfur compounds, and is the first thermophilic isolate from a subsurface geothermal environment within the \textit{\beta\text{-}Proteobacteria}. On the basis of its physiological and molecular properties, we consider that strain C55\textsuperscript{T} represents a novel genus, \textit{Thiobacter} gen. nov., with type species \textit{Thiobacter subterraneus} sp. nov.

**Description of \textit{Thiobacter} gen. nov.**

\textit{Thiobacter} (\text{Thi.o.bac.}ter. Gr. neut. n. \text{thion} sulfur; N.L. masc. n. \text{bacter} a rod; N.L. masc. n. \text{Thiobacter} sulfur rod).

Cells are Gram-negative, motile and rod-shaped. Thermophilic aerobe. Growth occurs chemolithoautotrophically with reduced sulfur compounds as electron donors and with oxygen as an electron acceptor using \textit{CO}_2 as a carbon source. Phylogenetically affiliated to the \textit{\beta\text{-}Proteobacteria}. The type species is \textit{Thiobacter subterraneus}.

**Description of \textit{Thiobacter subterraneus} sp. nov.**

\textit{Thiobacter subterraneus} (sub.ter.ra'ne.us. L. masc. adj. \textit{subterra}neus under the earth, indicating the source of isolation).

Cells are straight with a polar flagellum, 1-1-1-9 \text{\textmu}m long and 0-4-0-5 \text{\textmu}m wide. Microaerobic (up to 10 % \textit{O}_2 in a gas phase, optimum 2-5 %). Temperature range for growth is 35–62 °C (optimum 50–55 °C), pH range for growth is 5-2–7-7 (optimum 6-5–7-0). \textit{Na}\textsuperscript{+} concentration range for growth is 20–280 mM (optimum 70 mM). Chemolithoautotrophic growth occurs with elemental sulfur and reduced sulfur compounds, such as thiosulfate and sulfide, as electron donors and with molecular oxygen as the sole electron acceptor. Obligately autotrophic using \textit{CO}_2 as the sole carbon source. Nitrate and ammonium are used for the nitrogen source. The major cellular fatty acids are \textit{C}_{16:0} (72-8 \%), \textit{C}_{16:1} (23-1 \%), \textit{C}_{18:0} (2-3 \%), iso-C_{18:1} (1-3 \%) and \textit{C}_{18:1} (0-4 \%). The DNA \textit{G}+\textit{C} content is 66-9±0-2 mol\% (by HPLC).

The type strain, C55\textsuperscript{T} (=JCM12421\textsuperscript{T}=DSM 16629\textsuperscript{T}=ATCC BAA-941\textsuperscript{T}), was isolated from subsurface hot aquifer water in the Hishikari gold mine, Kagoshima Prefecture, Japan.

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References


