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; Stöhr 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 OPB37 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; Stöhr et al., 2001), Thiomonas (Shooper 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

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain C55T is AB180657.

Micrographs of cell morphology and a diagram showing the effects of temperature, pH and Na+ concentration on growth of C55T are available as supplementary figures in IJSEM Online.
facultatively autotrophic or strictly heterotrophic organisms. *Thermothrix azorensis* is obligately chemolithoautotrophic, using reduced sulfur compounds as the energy source. The new isolate showing obligately chemolithoautotrophic growth by the oxidation of reduced sulfur compounds is phylogenetically and physiologically compared with members of the genera within the β-Proteobacteria.

Sample collection, enrichment and purification

A hot (70–1°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 transportation 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 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 microscopic 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 solution (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), 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 microscopy 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 negatively stained with 2% (w/v) uranyl acetate and observed under a JEOL JEM-1210 electron microscope at an accelerating 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 counting 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 containing 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 x 10^9 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 containing 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/CO2/O2 of 80:20:0, 80:19:5:0:5, 80:1:19:8:2, 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 x 10^8 to 2 x 10^9 cells ml^-1. The maximum increase in growth of strain C55T was seen under 2 or 5% O2 with 1–2 x 10^9 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 microaerophilic 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 C55\textsuperscript{T} 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, NaHCO\textsubscript{3} and CO\textsubscript{2} in a gas phase.

To determine potential electron donors other than thiosulfate for autotrophic growth, 1 or 5 mM each of Na\textsubscript{2}S\textsubscript{2}, cysteine hydrochloride, disulfate (Na\textsubscript{2}S\textsubscript{2}O\textsubscript{7}) 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 % N\textsubscript{2}, 18 % CO\textsubscript{2} and 2 % O\textsubscript{2} (200 kPa). Molecular hydrogen was also examined in TSmj medium without thiosulfate with a gas phase of 80 % H\textsubscript{2}, 18 % CO\textsubscript{2} and 2 % O\textsubscript{2} (200 kPa). Elemental sulfur as an electron donor resulted in a similar maximum increase in cell numbers to that obtained with thiosulfate (1 × 10\textsuperscript{10} cells ml\textsuperscript{−1}), whereas Na\textsubscript{2}S (1 mM) produced lower cell numbers (1–2 × 10\textsuperscript{10} cells ml\textsuperscript{−1}) and other reduced sulfur compounds and hydrogen did not support growth of strain C55\textsuperscript{T} as the sole electron donor. Na\textsubscript{2}S at 5 mM seemed to be toxic to strain C55\textsuperscript{T}. No electron acceptor tested [NaNO\textsubscript{3} (2 or 10 mM), NaNO\textsubscript{2} (1 or 5 mM), ferric citrate (20 mM), ferrihydrite (20 mM), Na\textsubscript{2}SO\textsubscript{3} (5 mM), Na\textsubscript{2}SO\textsubscript{4} (5 mM)] supported growth of strain C55\textsuperscript{T}. These results indicate that strain C55\textsuperscript{T} 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 C55\textsuperscript{T} utilized nitrate, ammonium and Casamino acids, but could not utilize NaNO\textsubscript{2} or N\textsubscript{2}.

The time-course of oxidation of thiosulfate during growth of strain C55\textsuperscript{T} was monitored with TSmj medium at pH 6.5 under a gas phase of 80 % N\textsubscript{2}, 18 % CO\textsubscript{2} and 2 % O\textsubscript{2} (200 kPa) at 55 °C (Fig. 1). Concentrations of thiosulfate, sulfite and sulfate were analysed using the P/ACE MDQ capillary electrophoresis system (Beckman Coulter). Consumption of thiosulfate and production of sulfate were both observed during the growth of strain C55\textsuperscript{T}. However, some inconsistency was observed in the stoichiometry of thiosulfate consumption and resulting sulfate production by strain C55\textsuperscript{T}. 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 C55\textsuperscript{T}, 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 C55\textsuperscript{T} were found during fatty acid analysis (see below). From the concentrated fatty acid sample extracted from whole cells of strain C55\textsuperscript{T}, 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 C55\textsuperscript{T} 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 C55\textsuperscript{T} is a respiratory sulfur-oxidizer, producing sulfate as an end product.

Sensitivity to a variety of antibiotics in strain C55\textsuperscript{T} was examined with liquid TSmj medium containing each compound. Cell growth of strain C55\textsuperscript{T} was inhibited by chloramphenicol (10 µg ml\textsuperscript{−1}), streptomycin (10 µg ml\textsuperscript{−1}), kanamycin (1 µg ml\textsuperscript{−1}), ampicillin (1 µg ml\textsuperscript{−1}), rifampicin (1 µg ml\textsuperscript{−1}) and vancomycin (60 µg ml\textsuperscript{−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 methanol 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 C55\textsuperscript{T} were C\textsubscript{16:0} (72.8 %), C\textsubscript{16:1} (23.1 %),


The genomic DNA of strain C55\(^T\) 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 C55\(^T\) was 66 mol\%, a value similar to that of other thermophilic members of the \(\beta\)-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 C55\(^T\) was amplified by PCR and directly sequenced from both strands with a DNA sequencer model 3100 (Perkin Elmer/Applied Biosystems). Similarity analysis indicated that strain C55\(^T\) is related to members of the \(\beta\)-Proteobacteria, such as *Hydrogenophilus hirschi* (58\%; Stöhr et al., 2001), *Tepidimonas ignava* (49\%; Moreira et al., 2000) or *Tepidiphilus margaritifer* (43\%; Manaia et al., 2003).

Sequence similarities to members of other thermophilic microorganisms within the \(\beta\)-Proteobacteria, such as *Hydrogenophilus hirschi* (58\%; Stöhr et al., 2001), *Tepidimonas ignava* (49\%; Moreira et al., 2000) or *Tepidiphilus margaritifer* (43\%; Manaia et al., 2003). Genomic DNA of strain C55\(^T\) 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 C55\(^T\) was 66-9 mol\%, a value similar to that of other thermophilic members of the \(\beta\)-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).

### Table 1. Comparison of properties among thermophilic members of the \(\beta\)-Proteobacteria

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Comparison with related genera

In recent years, a number of workers using culture-independent molecular analyses have reported the detection of related micro-organisms using the neighbour-joining algorithm in CLUSTAL X version 1.81. Similarity analysis indicated that strain C55\(^T\) is related to members of the \(\beta\)-Proteobacteria, and comparative evolutionary distance analysis demonstrated that the isolate represents a separate lineage of descent within the \(\beta\)-Proteobacteria (Fig. 2). The highest similarity (98\%) was observed between the 16S rRNA gene sequences of strain C55\(^T\) and the environmental clone phAub-J previously detected from the same hot aquifer water (Takai et al., 2002). The sequence of strain C55\(^T\) 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 C55\(^T\) was distantly related to members of other genera within the \(\beta\)-Proteobacteria, such as *Azoarcus buckelii* U120\(^T\) (92-9\%; Mechichi et al., 2002), *Sterolibacterium dentrificans* Chol-1\(^T\) (92-1\%; Tarlera & Denner, 2003), *Thiobacillus aquaesulis* ATCC 43788\(^T\) (91-8\%; Wood & Kelly, 1988), *Tepidiphilus margaritifer* N2-214\(^T\) (90-8\%; Manaia et al., 2003), *Tepidimonas ignava* SPS-1037\(^T\) (90-6\%; Moreira et al., 2000), *Hydrogenophilus thermoluteolus* HT-1\(^T\) (89-1\%; Hayashi et al., 1999) and *Thiomonas thermosulfata* ATCC 51520\(^T\) (85-5\%; Shooner et al., 1996).

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C\(_{18:0}\) (2-3\%), iso-C\(_{18:0}\) (1-3\%) and C\(_{18:1}\) (0-4\%). The ratio of n-C\(_{18}\) fatty acids (95-9\% of the total fatty acids) in strain C55\(^T\) was high in comparison with other thermophilic micro-organisms within the \(\beta\)-Proteobacteria, such as *Hydrogenophilus hirschi* (58\%; Stöhr et al., 2001), *Tepidimonas ignava* (49\%; Moreira et al., 2000) or *Tepidiphilus margaritifer* (43\%; Manaia et al., 2003).
Thiobacter subterraneus gen. nov., sp. nov.

**Description of Thiobacter subterraneus gen. nov.**

*Thiobacter* (Thi.o.bac’ter. Gr. neut. n. thion sulfur; N.L. masc. n. bacter a rod; N.L. masc. n. 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 CO₂ as a carbon source. Phylogenetically affiliated to the \(\beta\)-Proteobacteria. The type species is *Thiobacter subterraneus*.

**Description of Thiobacter subterraneus sp. nov.**

*Thiobacter subterraneus* (sub.ter.ra’ne.us. L. masc. adj. *subterraneus* under the earth, indicating the source of isolation).

Cells are straight with a polar flagellum, 1.1–1.9 µm long and 0.4–0.5 µm wide. Microaerobic (up to 10 % O₂ 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). Na⁺ 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 CO₂ as the sole carbon source. Nitrate and ammonium are used for nitrogen source. The major cellular fatty acids are C₁₆:0 (72-8 %), C₁₆:1 (23-1 %), C₁₈:0 (2-3 %), iso-C₁₈:0 (1-3 %) and C₁₈:1 (0-4 %). The DNA G+C content is 66±9 or 2 mol% (by HPLC).

The type strain, C₅₅ᵀ (= JCM12421ᵀ = DSM 16629ᵀ = ATCC BAA-941ᵀ), was isolated from subsurface hot aquifer water in the Hishikari gold mine, Kagoshima Prefecture, Japan.

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References


