Thermosulfurimonas dismutans gen. nov., sp. nov., an extremely thermophilic sulfur-disproportionating bacterium from a deep-sea hydrothermal vent

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An extremely thermophilic, anaerobic, chemolithoautotrophic bacterium (strain S95T) was isolated from a deep-sea hydrothermal vent chimney located on the Eastern Lau Spreading Center, Pacific Ocean, at a depth of 1910 m. Cells of strain S95T were oval to short Gram-negative rods, 0.5–0.6 μm in diameter and 1.0–1.5 μm in length, growing singly or in pairs. Cells were motile with a single polar flagellum. The temperature range for growth was 50–92 °C, with an optimum at 74 °C. The pH range for growth was 5.5–8.0, with an optimum at pH 7.0. Growth of strain S95T was observed at NaCl concentrations ranging from 1.5 to 3.5 % (w/v). Strain S95T grew anaerobically with elemental sulfur as an energy source and bicarbonate/CO2 as a carbon source. Elemental sulfur was disproportionated to sulfide and sulfate. Growth was enhanced in the presence of poorly crystalline iron(III) oxide (ferrihydrite) as a sulfide-scavenging agent. Strain S95T was also able to grow by disproportionation of thiosulfate and sulfite. Sulfate was not used as an electron acceptor. Analysis of the 16S rRNA gene sequence revealed that the isolate belongs to the phylum Thermodesulfotheria. On the basis of its physiological properties and results of phylogenetic analyses, it is proposed that the isolate represents the sole species of a new genus, Thermosulfurimonas dismutans gen. nov., sp. nov.; S95T (=DSM 24515T=VKM B-2683T) is the type strain of the type species. This is the first description of a thermophilic microorganism that disproportionates elemental sulfur.

Biogeochemical cycling of sulfur in aquatic environments includes the activities of different aerobic and anaerobic prokaryotes. Bacteria that disproportionate sulfur compounds such as thiosulfate or elemental sulfur (Bak & Cypionka, 1987; Thamdrup et al., 1993) are a unique group of sulfur cycle micro-organisms. Sulfur isotope data from early Archaean rocks and the presence of microfossils in 3.4-billion-year-old geological formations suggest that sulfur disproportionation could be one of the earliest modes of microbial metabolism (Philippot et al., 2007; Wacey et al., 2011). Inorganic sulfur fermentation has been reported for members of the mesophilic genera Desulfovibrio, Desulfobulbus, Desulfocapsa, Desulfonatronum, Desulfonatronaxispira and Desulfonatronovibrio in the Delta-proteobacteria (Bak & Pfennig, 1987; Lovley & Phillips, 1994; Janssen et al., 1996; Pikuta et al., 2003; Sorokin et al., 2008, 2011). Among thermophiles, Desulfotomaculum thermobenzoicum is the only micro-organism that has been reported to be capable of growth by thiosulfate disproportionation (Jackson & McInerney, 2000). Prior to this report, no thermophiles were known to disproportionate elemental sulfur. S0 is abundant in thermal ecosystems, including deep-sea hydrothermal vents, where it forms when hydrogen sulfide-rich hydrothermal fluid mixes with cold oxygenated seawater (Jannasch, 1984). In these extreme environments, microbial sulfur disproportionation may represent a previously unrecognized process of primary organic matter production.

Strain S95T was isolated from a sample of the actively venting hydrothermal sulfidic chimney-like deposit. The
sample was collected in June 2009 from the Mariner hydrothermal field (22° 10.82’ S 176° 36.09’ W, 1910 m deep) on the Eastern Lau Spreading Center, SW Pacific Ocean, using the ROV JASON II. Once collected, the samples were placed in an insulated box on the submersible’s basket. Upon reaching the ship, the samples were immediately divided up and small fragments were used as the inocula into anaerobic media. An enrichment culture was initiated by inoculation of 10% (w/v) of the sample into anaerobiocally prepared, bicarbonate-buffered, sterile liquid medium supplemented with elemental sulfur (sublimed, 150 mM) and poorly crystalline iron(III) oxide [ferrihydrite; 90 mmol Fe(III) l⁻¹]. The medium contained (g l⁻¹ distilled water): KH₂PO₄, 0.33; NH₄Cl, 0.33; KCl, 0.33; CaCl₂, 6H₂O, 0.33; MgCl₂, 6H₂O, 4.00; NaCl, 18.00; NaHCO₃, 2.00. The medium did not contain any organic substances except a vitamin solution (10 ml l⁻¹; Wolin et al., 1963). Trace element solution was added (1 ml l⁻¹) with the following composition (mmol l⁻¹): (NH₄)₂SO₄, FeSO₄, 6H₂O, 2.0; CoCl₂, 6H₂O, 1.0; NiCl₂, 2H₂O, 1.0; Na₂MoO₄, 2H₂O, 0.1; Na₂WO₄, 2H₂O, 0.1; ZnSO₄, 7H₂O, 0.5; CuCl₂, 2H₂O, 0.01; Na₂SeO₃, 0.5; H₂BO₃, 0.1; MnCl₂, 4H₂O, 1.0; SrSO₄, 0.05; CrO₃, 0.01; AlCl₃, 6H₂O, 0.1; BaCl₂, 0.1; Na₂SiO₃, 9H₂O, 0.5; KBr, 1.0; KI, 1.0; Na₂SO₃, 5.0. No reducing agents were added to the medium. pH of the autoclaved medium was 6.7–6.8 (measured at 25 °C). Ferrihydrite was prepared as described by Slobodkin et al. (1999). Medium (10 ml) was dispensed into 17 ml Hungate tubes; the headspace was filled with CO₂ (100 %). After incubation of the enrichments at 70 °C for 4 days, the colour of the ferrihydrite changed from brown to black, indicating Fe(III) reduction; elemental sulfur also changed colour from yellow to green. After three subsequent transfers and following serial 10-fold dilutions in the same medium only one morphological type was observed in the highest growth-positive dilution (10⁻⁹). Attempts to obtain separate colonies in agar- or Gelrite-blocks or by the roll-tube method were unsuccessful either at 70 °C or at 50 °C with 1 % Gelrite gellan gum or with 1 % agar as the solidifying agent in the medium with or without ferrihydrite. A pure culture of strain S95T was obtained by multiple serial dilutions in the same medium.

Physiological studies on substrate utilization, and on temperature, pH and salinities for growth, were carried out in the medium used for isolation unless noted otherwise. All organic substrates were tested in the presence of 0.2 g yeast extract l⁻¹. In electron acceptor utilization experiments, elemental sulfur and ferrihydrite were omitted. Sulfide was measured colorimetrically with dimethylp-phenylenediamine (Trüper & Schlegel, 1964). Sulfate was analysed with a Stayer ion chromatograph (Aquilon) equipped with an IonPack AS4-ASC column (Dionex) and conductivity detector; the eluant was bicarbonate (1.36 mM)/carbonate (1.44 mM) and the flow rate was 1.5 ml min⁻¹. Cellular fatty acid (CFA) profiles were determined by GC-MS of methyl ester derivatives prepared from 5 mg dry cell material (Sasser, 1990). CFA content was determined as percentages of total ion current peak area. Light and electron microscopy, Gram staining, Fe(II) analysis, DNA extraction and determination of DNA G+C content were performed as described previously (Slobodkin et al., 1999). The 16S rRNA gene was selectively amplified from genomic DNA by PCR using primers 11F and 1492R (Lane, 1991). PCR was carried out in a 50 μl reaction mixture containing 50 ng DNA template, 5 pmol (each) primers, 12.5 mmol (each) dNTPs and 3 U Taq DNA polymerase (Fermentas) in Taq DNA polymerase reaction buffer (Fermentas). The temperature cycling was done by using the following program: first cycle of 9 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, then 30 amplification cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. The final extension was carried out at 72 °C for 7 min. The PCR products were purified using the Wizard PCR Preps kit (Promega) as recommended by the manufacturer. Both strands of the almost complete 16S rRNA gene were sequenced using Big Dye Terminator v3.1 (Applied Biosystems) as described in the manufacturer’s instructions and resolved using an ABI PRISM 3730 DNA Analyser (Applied Biosystems). The sequences were manually assembled and checked for accuracy using the alignment editor BioEdit v5.0.9 (Hall, 1999). A multiple sequence alignment was generated using the NAST Alignment Tool (DeSantis et al., 2006a) and the Greengenes Database (DeSantis et al., 2006b; http://greengenes.lbl.gov/cgi-bin/nph-index.cgi). The alignment generated was then checked manually and only unambiguously aligned positions were used (1359 bp). The phylogeny was inferred by using the maximum-likelihood analysis (Tamura & Nei, 1993). Evolutionary analyses were conducted in MEGA version 5 (Tamura et al., 2011). Pairwise similarity values were calculated by means of EzTaxon (Chun et al., 2007).

Cells of strain S95T were oval to short rods, 0.5–0.6 μm in diameter and 1.0–1.5 μm in length, growing singly or in pairs (Fig. 1a). Cells were motile due to a single polar flagellum (Fig. 1b). Formation of endospores was not observed. The cells stained Gram-negative in both the exponential and stationary growth phases. Ultrathin sections of strain S95T revealed a Gram-negative cell wall type (Fig. 1c). The temperature range for growth was 50–92 °C, with an optimum at 74 °C (Fig. S1, available in IJSEM Online). No growth was detected at 47 or 94 °C after incubation for 2 weeks. The pH range for growth was 5.5–8.0, with an optimum at 7.0. No growth was detected at pH 5.0 or 8.5. Growth of strain S95T was observed at NaCl concentrations ranging from 1.5 to 3.5% (w/v), but no growth was evident at 1.0 or 4.0% (w/v) NaCl. Strain S95T grew lithoautotrophically with elemental sulfur as the only energy source and HCO₃⁻/CO₂ as the only carbon source (Fig. 2). When ferrihydrite was omitted from the cultivation medium, growth of strain S95T was observed only in one type of cultivation vessel used (90 ml flat anaerobic mattress bottles; Bellco Glass), which was filled with 10 ml medium and incubated vertically. Attempts to get a sustainable growth of strain S95T in other glassware made
malate (10 mM) or maleinate (10 mM) caused a twofold increase in the maximal cell yields of strain S95T. In the presence of malate and maleinate, strain S95T also grew due to sulfur disproportionation as was evident from the production of sulfate and sulfide in the same ratio as was seen in the absence of these compounds. Addition of H2/CO2 (80:20, v/v), formate, methanol, ethanol, n-propanol, i-propanol, butanol, acetate, propionate, butyrate, lactate, pyruvate, fumarate, succinate, tartrate, oxalate, citrate, glycerol, glycine, alanine (each substrate at 10 mM) and peptone (2.5 g l⁻¹) did not stimulate growth of strain S95T in the presence of elemental sulfur and ferrihydrite. Strain S95T grew with thiosulfate (10 mM) and H2/CO2 (80:20, v/v in the gas phase) in the absence of ferrihydrite up to maximal cell densities of 1.2–1.4 × 10⁸ cells ml⁻¹. While molecular hydrogen was partially consumed under these conditions, thiosulfate was also converted to sulfide and sulfate. Addition of organic substrates such as ethanol, fumarate, succinate and malate did not stimulate growth of strain S95T with thiosulfate. The strain did not ferment glucose, fructose, maltose, sucrose, cellobiose, arabinose or malate (each substrate at 10 mM). Strain S95T did not reduce sulfate, nitrate, fumarate, 9,10-anthraquinone 2,6-disulfonate, iron(III) citrate (each substrate at 10 mM), ferrihydrite [90 mM Fe(III)] or oxygen [3.0 or 20 % (v/v) in the gas phase] with acetate, lactate, ethanol, pyruvate, malate, peptone or H₂ as electron donors. CFAs of strain S95T comprised a mixture of saturated and monounsaturated straight-chain and branched (iso- and anteiso-) fatty acids (Table S1). The major CFAs were: C₁₈ : ₀, anteiso-C₁₇ : ₀, C₁₆ : ₀ (21.3, 15.91 and 13.4 % of total CFA, respectively); C₁₈ : ₁ω7 (8.88 %), iso-C₁₈ : ₁ (8.58 %), iso-C₁₆ : ₀ (6.26) and C₁₇ : ₀ (5.04 %) were also present. Other fatty acids were present in low or trace amounts (less than 5 %).

The G+C content of the genomic DNA of strain S95T was 52 mol% (Tₘ). A comparison of 1532 nt of the 16S rRNA gene sequence of strain S95T with those available in GenBank showed that strain S95T had the highest similarity with members of the phylum Thermodesulfovibacteria (90.7–92.5 %) (Fig. 3). Using maximum-likelihood, neighbour-joining or parsimony methods, strain S95T formed a distinct phylogenetic lineage within the class Thermodesulfovibacteria. Data using EzTaxon (Chun et al., 2007) showed that strain S95T was most closely related to ‘Geothermobacterium ferrireducens’ FW-1a and Caldimicrobium rimae DS⁴T, with 92.2 and 92.5 % 16S rRNA gene sequence similarity, respectively. Similarity with representatives of the class Aquificae was less than 87 % (Thermosulfidibacter takaii ABI7056⁷, 86.2 % and members of the Desulfurobacteriaceae, 81.8–82.8 %).

The novel isolate described herein represents the first reported thermophile that is capable of elemental sulfur disproportionation. To our knowledge, microbial sulfur disproportionation in hydrothermal environments has not been detected using geochemical methods. However, elemental sulfur is abundant in different thermal ecosystems and it is not surprising that this metabolism could be
important in the biogeochemical cycling of sulfur at elevated temperatures. The fast rate of lithoautotrophic growth of strain S95T verifies the capacity of sulfur-disproportionation as an important contributor to primary production. Additionally, we searched in our 16S rRNA multiplexed barcoded pyrosequences from numerous deep-sea vent sites and detected sequences similar to the 16S rRNA gene sequence from S95T in deep-sea hydrothermal vent samples from the Mid-Atlantic Ridge (Flores et al., 2011), Guaymas Basin and Eastern Lau Spreading Center (data not shown). Thus, it is possible that microbial sulfur disproportionation by thermophiles such as strain S95T might be widespread in deep-sea vents.

Currently, the phylum Thermodesulfobacteria includes species of three genera with validly published names, Thermodesulfobacterium, Thermodesulfatator and Caldimicrobium, as well as ‘Geothermobacterium ferrireducens’. Strain S95T does not belong to the genera Thermodesulfobacterium and Thermodesulfatator based on its inability to reduce sulfate and its significant phylogenetic difference (90.7–92.3 % 16S rRNA gene sequence similarity). Furthermore, S95T differs from Caldimicrobium rimaec in its inability to utilize organic compounds as electron donors for growth, and from ‘Geothermobacterium ferrireducens’ by its lack of dissimilatory Fe(III) reduction. In addition to phylogenetic differences, strain S95T differs from its closest phylogenetic relatives in a number of other physiological characteristics such as salinity and temperature range for growth and genomic DNA G+C content (Table 1). The capacity for disproportionation of sulfur compounds has not been reported for any species of the phylum Thermodesulfobacteria, and we confirmed that Caldimicrobium rimaec strain DS1 was not able to disproportionate elemental sulfur, thiosulfate or sulfite. The major fatty acids present in strain S95T differed from those of other members of the phylum Thermodesulfobacteria. In contrast to Thermodesulfobacterium commune, unsaturated fatty acids were found in strain S95T; however, they did not include C19:0, as found in Thermodesulfatator atlanticus,

![Fig. 2. Growth of (○) and production of sulfide (□) and sulfate (▲) by strain S95T in medium with elemental sulfur without ferrihydrite. Cultivation in vessels with large headspace volume (see text).](image-url)

![Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences indicating the position of strain S95T within representatives of the phylum Thermodesulfobacteria. Bootstrap values are shown at the base of nodes and were based on 100 replicates. Bar, 0.02 substitutions per site.](image-url)
Description of Thermosulfurimonas gen. nov.

Thermosulfurimonas (Ther.mo.sul.fu.ri.mo’nas. Gr. adj. thermos hot; L. n. sulfur sulfur; Gr. fem. n. monas a unit, monad; N.L. fem. n. Thermosulfurimonas thermophilic sulfur monad).


Description of Thermosulfurimonas dismutans sp. nov.

Thermosulfurimonas dismutans (dis.mu’tans. L. inseparable particle dis in two; L. part. adj. mutans altering, changing; N.L. part. adj. dismutans dismutating, splitting).

Shows the following properties in addition to those given in the genus description. Cells are oval or short rods, 0.5–0.6 μm in diameter and 1.0–1.5 μm in length, growing singly or in pairs. Cells are motile with single polar flagellum. Gram-stain-negative. The temperature range for growth is 50–92 °C, with an optimum at 74 °C. The pH range for growth is 5.5–8.0, with an optimum at pH 7.0. Grows in 1.5–3.5 % (w/v) NaCl. Grows with elemental sulfur as an energy source and bicarbonate/CO₂ as a carbon source. Elemental sulfur is disproportionated to sulfide and sulfate. Growth is enhanced with poorly crystalline iron(III) oxide (ferrihydrite) as a sulfide-scavenging agent. Able to grow by disproportionation of thiosulfate and sulfate. In the presence of elemental sulfur and ferrihydrite, malate and maleate stimulate growth. In the presence of thiosulfate, molecular hydrogen stimulates growth. Formate, methanol, ethanol, n-propanol, i-propanol, butanol, acetate, propionate, butyrate, lactate, pyruvate, fumarate, succinate, tartrate, oxalate, citrate, glycerol, glycine, alanine, peptone, yeast extract and H₂/CO₂ do not stimulate growth in the presence of elemental sulfur and ferrihydrite. Does not ferment glucose, fructose, maltose, sucrose, cellulose, arabinose or malate. Does not reduce sulfate, nitrate, poorly crystalline iron(III) oxide, iron(III) citrate, 9,10-anthraquinone 2,6-disulfonate, fumarate and oxygen with acetate, lactate, ethanol, pyruvate, malate, peptone and H₂ as electron donors.

The type strain is S95ᵀ (DSM 24515ᵀ = VKM B-2683ᵀ), isolated from a deep-sea hydrothermal vent along the Eastern Lau Spreading Center, SW Pacific Ocean. The G+C content of the genomic DNA of the type strain is 52 mol% (Tₘ).
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References


