Thiomicrospira thermophila sp. nov., a novel microaerobic, thermotolerant, sulfur-oxidizing chemolithomixotroph isolated from a deep-sea hydrothermal fumarole in the TOTO caldera, Mariana Arc, Western Pacific

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A novel thermotolerant bacterium, designated strain I78^T, was isolated from a self-temperature-recording *in situ* colonization system deployed in a hydrothermal diffusing flow (maximal temperature 78 °C) at the TOTO caldera in the Mariana Arc, Western Pacific. Cells were highly motile curved rods with a single polar flagellum. Growth was observed at 15–55 °C (optimum 35–40 °C; 60 min doubling time) and pH 5.0–8.0 (optimum pH 6.0). The isolate was a microaerobic chemolithomixotroph capable of using thiosulfate, elemental sulfur or sulfide as the sole energy source, and molecular oxygen as the sole electron acceptor. The isolate was able to grow chemolithoautotrophically with carbon dioxide. Various organic substrates such as complex proteinaceous compounds, carbohydrates, organic acids, amino acids and sugars could also support growth as the carbon source instead of carbon dioxide with sulfur oxidation. The G + C content of the genomic DNA was 43.8 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the isolate belonged to the genus *Thiomicrospira* and was most closely related to *Thiomicrospira crunogena* strain TH-55^T and *Thiomicrospira* sp. strain L-12, while DNA–DNA hybridization demonstrated that the novel isolate could be genetically differentiated from previously described strains of *Thiomicrospira*. On the basis of its physiological and molecular properties the isolate is representative of a novel *Thiomicrospira* species, for which the name *Thiomicrospira thermophila* sp. nov. is proposed (type strain, I78^T = JCM 12397^T = DSM 16397^T).

The genus *Thiomicrospira* accommodates obligate chemolithoautotrophs within the γ -*Proteobacteria*, except for *Thiomicrospira denitrificans* strain DSM 1251^T, which is within the ϵ -*Proteobacteria*. Since the type species *Thiomicrospira pelophila* strain DSM 1534^T was first isolated from the intertidal mud flats of the Dutch Wadden Sea (Kuenen &

Veldkamp, 1972), six species and many strains of the genus *Thiomicrospira* have been isolated from various marine habitats, including deep-sea hydrothermal environments (Ruby & Jannasch, 1982; Ruby *et al.*, 1981; Jannasch *et al.*, 1985; Eberhard *et al.*, 1995). In addition to the type species *Thiomicrospira pelophila* strain DSM 1534^T, *Thiomicrospira crunogena* strain TH-55^T was isolated from the tubes of vestimentiferan tube worms in the East Pacific Rise (EPR) deep-sea hydrothermal environment (Jannasch *et al.*, 1985) and *Thiomicrospira thyasirae* strain DSM 5322^T was isolated from the gill extract of the marine bivalve *Thyasira flexuosa* inhabiting shallow bays and cold seepage sites (Wood & Kelly, 1989, 1993). Both *Thiomicrospira kuenenii* strain JB-A1^T and *Thiomicrospira frisia* strain JB-A2^T were obtained from an intertidal mud flat of the German Wadden Sea (Brinkhoff *et al.*, 1999a). The most recently described species is *Thiomicrospira chilensis* strain Ch-1^T, from a

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Abbreviations: EPR, East Pacific Rise; STR-ISCS, self-temperature-recording *in situ* colonization.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain I78^T is AB166731.

The effects of temperature, pH and NaCl concentration on the growth of strain I78^T are shown in a supplementary figure available in IJSEM Online.

sediment sample dominated by filamentous sulfur bacteria of the genus *Thioploca* in the Chilean coastal shelf (Brinkhoff *et al.*, 1999b). In addition to the recognized members of the genus *Thiomicrospira*, members of the closely related genera *Hydrogenovibrio* (Nishihara *et al.*, 1989, 1991) and *Thioalkalimicrobium* (Sorokin *et al.*, 2001, 2002) have also been isolated. Recent phylogenetic analysis (Sorokin *et al.*, 2002) has revealed that *Thiomicrospira pelophila* strain DSM 1534^T and *Thiomicrospira thyasirae* strain DSM 5322^T are only distantly related to other *Thiomicrospira* species and cluster with *Thioalkalimicrobium* species; *Hydrogenovibrio marinus* strain MH-110^T is phylogenetically related to *Thiomicrospira kuenenii* strain JB-A1^T and should be classified within the genus *Thiomicrospira*.

The abundance of members of the genus *Thiomicrospira* in both deep and shallow hydrothermally active marine environments has been well characterized using not only cultures and isolates but also cultivation-independent molecular techniques (Ruby & Jannasch, 1982; Ruby *et al.*, 1981; Jannasch *et al.*, 1985; Eberhard *et al.*, 1995; Brinkhoff & Muyzer, 1997; Brinkhoff *et al.*, 1999c). However, phylogenetic and physiological diversity of members of the genus *Thiomicrospira* together with their biogeography in the global deep-sea hydrothermal systems were unclear because investigations had been limited to isolates from the EPR and the Mid Atlantic Ridge hydrothermal environments (Jannasch *et al.*, 1985; Wirsén *et al.*, 1998). Recent extensive analyses of sulfur- and hydrogen-oxidizing chemolithoautotrophs in a variety of microbial habitats in the deep-sea hydrothermal systems of the Okinawa Trough and Central Indian Ridge have provided new information regarding the phylogenetic and physiological diversity of the ϵ -*Proteobacteria*. However, little has emerged with regard to deep-sea hydrothermal *Thiomicrospira* species potentially inhabiting similar ecological environments (but see Inagaki *et al.*, 2003, 2004; Takai *et al.*, 2003a, 2004a, b, c). In this study, we report the isolation of a novel *Thiomicrospira* strain, I78^T, from a deep-sea hydrothermal field in the TOTO caldera, Mariana Arc, Western Pacific margin. It was isolated from a self-temperature-recording *in situ* colonization (STR-ISCS) device (Takai *et al.*, 2003a) deployed in a hydrothermal diffusing flow (maximal temperature 78 °C). We report here on the taxonomic study of strain I78^T and propose that it represents a novel species for which the *Thiomicrospira thermophila* sp. nov. is proposed.

Sample collection, enrichment and purification

An STR-ISCS, a newly constructed microbial habitat consisting of a stainless steel pipe with numerous small holes (5 mm in diameter) and a substratum of very porous, natural pumice (Takai *et al.*, 2003a), was deployed for 4 days in a diffusing hydrothermal flow with a maximum fluid temperature of 78 °C and a pH of 5.3 at the TOTO caldera (12° 42' 8007" N, 143° 32' 3415" E), at a depth of 2922 m by means of the manned submersible *Shinkai 6500* (Dive no. 772) in August 2003. After deployment, it was recovered to the sea surface in a sample box attached to

the submersible (Dive no. 776). The TOTO caldera deep-sea hydrothermal field is characterized by highly acidic hydrothermal fluids resulting from oxidation of volatile volcanic gas (H₂S) to sulfate (Gamo *et al.*, 2004). During a series of dives, we sampled a white smoker hydrothermal vent with an unusually low pH value of 1.6 in the TOTO caldera, suggesting that the deep-sea hydrothermal activity in the TOTO caldera is a novel system driven by sub-seafloor mixing between oxygenated sea water and superheated volcanic gas, as was previously proposed for the DESMOS caldera in the Manus Basin (Gamo *et al.*, 1997). The STR-ISCS was deployed in one of the diffusing hydrothermal flows derived from the highly acidic hydrothermal fluid with further dilution of sea water. During the 4-day deployment, the temperature of the substratum in the colonization device fluctuated between 20 and 40 °C in the first 24 h, gradually increased up to 70 °C for the next 24 h and remained stable at 65–70 °C for the last 2 days.

Immediately after recovery of the STR-ISCS device on the ship, the substratum was suspended in 20 ml sterilized MJ synthetic sea water (Takai *et al.*, 1999) containing 0.05 % (w/v) sodium sulfide in a 100 ml glass bottle (Schott) tightly sealed with a butyl-rubber cap under a gas phase of 100 % N₂ (100 kPa). The suspended slurry was used to inoculate a series of media including MMJHS medium (Takai *et al.*, 2003a) under a gas phase of 80 % H₂/19 % CO₂/1 % O₂ (200 kPa), and the cultures were then incubated at 30 °C in a dry oven.

Growth of motile, slightly curved rods was observed with production of colloidal elemental sulfur in MMJHS medium after 2 days incubation at 30 °C. A pure culture was obtained by using the dilution-to-extinction technique at 30 °C with the same medium as used for enrichment (Takai & Horikoshi, 2000). The pure culture was streaked onto MMJHS plates solidified with 1.2 % (w/v) agar. After 2 days incubation at 30 °C under a gas phase of 80 % H₂/19 % CO₂/1 % O₂ (200 kPa), only one colony type with a white to cream colour and elemental sulfur particles was noted. An isolated colony was picked and inoculated into fresh liquid MMJHS medium under a gas phase of 80 % H₂/19 % CO₂/1 % O₂ (200 kPa). This culture was designated strain I78^T. Purity was confirmed by microscopic examination and by repeated partial sequencing of the 16S rRNA gene using several PCR primers.

Morphology

Cells were observed under a phase-contrast Olympus BX51 microscope with the SPOT RT Slider CCD camera system (Diagnostic Instruments Inc.). Transmission electron microscopy of negatively stained cells was carried out as described by Zillig *et al.* (1990). Cells grown in MMJHS medium (described below) under microaerobic conditions (1 % partial pressure of O₂) at 30 °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.

Fig. 1. An electron micrograph of a negatively stained cell of *Thiomicrospira thermophila* strain I78^T in the exponential phase of growth. Bar, 1.0 μm.

Cells of strain I78^T were Gram-negative, slightly curved rods about 0.4–0.7 μm in diameter, 0.8–1.5 μm in length (Fig. 1), and were motile with a polar flagellum (Fig. 1). In contrast to other *Thiomicrospira* isolates, we observed neither spiral-shaped cells (Kuenen & Veldkamp, 1972) nor the formation of aggregates (Brinkhoff *et al.*, 1999a, b). When strain I78^T was heterotrophically grown in MMJS medium with organic substrates such as yeast extract and sugars instead of carbon dioxide, the cells were enlarged straight rods (0.6–1.0 μm in diameter and 1.0–2.0 μm in length). No spore formation was observed in any of the growth conditions examined. Morphological features of strain I78^T were thus very similar to those of *Thiomicrospira crunogena* strain TH-55^T (Jannasch *et al.*, 1985) and *Thiomicrospira* sp. strain L-12 (Ruby & Jannasch, 1982), which were examined using the same medium and conditions as used in this study.

Thiomicrospira crunogena strain TH-55^T (Jannasch *et al.*, 1985) and *Thiomicrospira* sp. strain L-12 (Ruby & Jannasch, 1982) were kindly donated by Jan Kuever, Institute of Material Testing, Bremen, Germany, and by Stefan Sievert, Woods Hole Oceanic Institute, Woods Hole, MA, USA. These strains were routinely cultivated with MMJS medium (described below) under microaerobic conditions (1% partial pressure of O₂) at 30 °C.

Growth characteristics

Strain I78^T was routinely cultivated in MMJS medium. MMJS medium consists of (per litre of distilled, deionized water) 20.0 g NaCl, 0.14 g K₂HPO₄, 0.8 g CaCl₂, 1.0 g NH₄Cl, 4.0 g MgSO₄·7H₂O, 3.0 g MgCl₂·6H₂O, 0.33 g KCl, 0.5 mg NiCl₂·6H₂O, 0.5 mg Na₂SeO₃·5H₂O, 0.1 mg Na₂WO₄, 0.01 g Fe(NH₄)₂(SO₄)₂·6H₂O, 10 ml trace mineral solution (Balch *et al.*, 1979), 10 ml vitamin

solution (Balch *et al.*, 1979), 2.0 g NaHCO₃ and 5 mM Na₂S₂O₃·5H₂O. To prepare MMJS medium, materials other than vitamin solution (Balch *et al.*, 1979) and NaHCO₃ were dissolved, and the pH of the medium was adjusted to about 6.0 with HCl before autoclaving. After autoclaving under an air atmosphere, a concentrated solution of vitamins and NaHCO₃ was added to the medium under gas purging of 80% N₂/20% CO₂, and the pH was readjusted to 6.0 with HCl at room temperature if necessary. A concentrated sodium bicarbonate solution was separately sterilized by autoclaving and the vitamin solution was filter-sterilized. The medium was dispensed at 20% of the bottle (Schott Glaswerke, Mainz, Germany) or tube (Iwaki glass, Tokyo, Japan) volume, and tightly sealed with a butyl-rubber stopper under a gas phase consisting of 80% N₂/19% CO₂/1% O₂ at 200 kPa unless stated otherwise.

Growth of strain I78^T was measured by direct cell counting after staining with 4',6-diamidino-2-phenylindole (DAPI) (Porter & Feig, 1980) using a phase-contrast Olympus BX51 microscope. Cultures were prepared in duplicate. The cultures were grown in 100 ml glass bottles (Schott Glaswerke) with shaking (100 r.p.m.) in a temperature-controlled dry incubator. With MMJS medium, strain I78^T grew over the temperature range of about 15–55 °C, showing optimal growth at 35–40 °C; the generation time at 40 °C, pH 6.0, was about 60 min (supplementary figure available in IJSEM Online). The effect of pH on growth was tested at 35 °C, using MMJS medium adjusted to various pH values with 30 mM acetate/acetic acid buffer (pH 4–5), MES (pH 5–6), PIPES (pH 6–7), HEPES (pH 7–7.5) and Tris (pH 8–9.5) at room temperature (supplementary figure available in IJSEM Online). Growth occurred at pH 5.0–8.0, with optimum growth at about pH 6.0. The pH was found to be stable during the cultivation period and no apparent inhibitory effect on growth was seen with any of the buffer systems. Strain I78^T, when tested in MMJS medium with variable NaCl content, grew over the NaCl concentration range 3–70 g l⁻¹, with optimum growth at 12–20 g l⁻¹ at 30 °C and pH 6.0 (supplementary figure available in IJSEM Online). The optimum temperature and the temperature range for growth of strain I78^T were considerably higher than those of any previously described species of the genus *Thiomicrospira* (Kuenen & Veldkamp, 1972; Jannasch *et al.*, 1985; Wood & Kelly, 1989, 1993; Brinkhoff *et al.*, 1999a, b) and for *Thiomicrospira* sp. strain L-12 (Ruby & Jannasch, 1982) (Table 1). The optimum pH for growth of the strain I78^T was lower than those of *Thiomicrospira crunogena* strain TH-55^T (Jannasch *et al.*, 1985) and *Thiomicrospira* sp. strain L-12 (Ruby & Jannasch, 1982) from deep-sea hydrothermal environments (Table 1).

The effect of oxygen concentration in the gas phase on growth of strain I78^T was tested with MMJS medium under a gas mixture of 80% N₂/20% CO₂, 80% N₂/19.9% CO₂/0.1% O₂, 80% N₂/19.5% CO₂/0.5% O₂, 80% N₂/19% CO₂/1% O₂, 80% N₂/15% CO₂/5% O₂, 75% N₂/15%

Table 1. Comparison of properties among *Thiomicrospira thermophila* strain I78^T, *Thiomicrospira crunogena* strain TH-55^T, *Thiomicrospira* sp. strain L-12, *Thiomicrospira kuenenii* strain JB-A1^T and *Hydrogenovibrio marinus* strain MH-110^T

Taxa: 1, *T. thermophila* strain I78^T (data from this study); 2, *T. crunogena* strain TH-55^T (Jannasch *et al.*, 1985); 3, *Thiomicrospira* sp. strain L-12 (Ruby & Jannasch, 1982); 4, *T. kuenenii* strain JB-A1^T (Brinkhoff *et al.*, 1999a); 5, *H. marinus* strain MH-110^T (Nishihara *et al.*, 1989, 1991). +, Positive; −, negative; ND, not determined.

Characteristic	1	2	3	4	5
Source of isolate	Deep-sea hydrothermal fumarole in the West Pacific	Deep-sea hydrothermal vent chimneys and animals in the East Pacific	Deep-sea hydrothermal vent chimneys and animals in the East Pacific	Intertidal mud flat in the German Wadden Sea	Coastal sea water in Japan
Temperature for growth (°C):					
Range (optimum)	15–55 (35–40)	14–38.5 (28–32)	10–35 (25)	3.5–42 (29–33.5)	>5 to <45 (37)
pH for growth:					
Range (optimum)	5.0–8.0 (6)	5.0–8.5 (7.5–8.0)	6.0–8.5 (8)	4.0–7.5 (6)	ND (6.5)
NaCl concentration for growth (%):					
Range (optimum)	0.3–7.0 (1.2–2.0)	>0.25 (ND)	>0.45 (1.1–2.3)	0.56–6.9 (2.6)	ND (2.9)
O ₂ concentration for growth (%):					
Optimum (upper limit)	0.5–1.0 (10)	Microaerobic (>20)	Microaerobic (>20)	4–20 (>20)	5–10 (>40)
Electron donor:					
H ₂	−	−	−	−	+
Reduced sulfur compounds	+	+	+	+	+
Accumulation of S ⁰ during growth	+	+	+	−	−
Heterotrophic growth	+	+	+	ND	−
Utilization of N ₂ as nitrogen source	+	−	−	ND	−
Vitamin dependence	−	−	−	−	−
G+C content of genomic DNA (mol%)	43.8	44.2	ND	42.4	44.1

*Property confirmed in this study.

CO₂/10% O₂ or 65% N₂/15% CO₂/20% O₂ at 200 kPa. In the absence of oxygen, either 10 mM nitrate or 10 mM fumarate was added to MMJS medium as a potential, alternative electron acceptor. The maximum cell yield of strain I78^T was approximately $8-9 \times 10^8$ cells ml^{−1} under a gas phase in the presence of 0.5 or 1% O₂, whereas slightly lower yields ($2-5 \times 10^8$ cells ml^{−1}) were seen with 0.1, 5 and 10% O₂. No growth was observed in the absence of O₂ or with 20% O₂ in a gas phase. These results indicated that strain I78^T grew under microaerobic (up to 10% O₂) conditions.

Heterotrophic growth was tested in MMJS medium without NaHCO₃ under a gas phase of 99% N₂/1% O₂ (200 kPa), containing the following as potential organic carbon sources: 0.1% (w/v) yeast extract, 0.1% (w/v) peptone, 0.1% (w/v) tryptone, 0.1% (w/v) casein, 0.1% (w/v) starch, 0.1% (w/v) carboxymethylcellulose (CMC), 0.1% (w/v) Casamino acids, 5 mM formate, 5 mM acetate, 5 mM glycerol, 5 mM citrate, 5 mM tartrate, 5 mM fumarate, 5 mM malate, 5 mM succinate, 5 mM propionate, 5 mM lactate, 5 mM oxalate, 5 mM pyruvate, 5 mM of each of 20 amino acids, 0.02% (w/v) glucose, 0.02% (w/v) galactose, 0.02% (w/v) sucrose, 0.02% (w/v)

fructose, 0.02% (w/v) lactose, 0.02% (w/v) maltose and 0.02% (w/v) trehalose. It was able to grow with any of yeast extract, peptone, tryptone, casein, starch, CMC, Casamino acids, glycerol, fumarate, glycine, leucine, isoleucine, lysine, glucose, galactose, sucrose, fructose, lactose, maltose and trehalose, providing a maximum cell yield of $3.0-5.0 \times 10^8$ cells ml^{−1}. A somewhat lower yield ($1-2 \times 10^8$ cells ml^{−1}) was obtained from heterotrophic growth on malate, citrate, alanine, valine, cysteine, methionine, arginine, histidine, asparagine, glutamine, aspartate and glutamate. No other organic carbon source supported the heterotrophic growth using thiosulfate as an energy source and molecular oxygen as an electron acceptor. Simultaneous experiments with *Thiomicrospira crunogena* strain TH-55^T (Jannasch *et al.*, 1985) and *Thiomicrospira* sp. strain L-12 (Ruby & Jannasch, 1982) in MMJS medium indicated that both were able to grow heterotrophically on any of the organic carbon compounds, such as yeast extract, tryptone, casein, starch, Casamino acids, glucose, galactose, sucrose, fructose, lactose, maltose, xylose and trehalose.

Utilization of these organic compounds as alternative energy sources instead of thiosulfate was also examined in MMJS medium in the absence of thiosulfate under a gas

phase of 80 % N₂/19 % CO₂/1 % O₂ (200 kPa). None of the organic compounds sustained the growth of strain I78^T. In an attempt to determine potential electron donors other than thiosulfate for autotrophic growth, sulfide (0.25, 0.5, 1, 2 or 5 mM), sulfite (1 or 5 mM), elemental sulfur (3 %; w/v), cysteine/HCl (0.25, 0.5, 1, 2 or 5 mM) or tetrathionate (1 or 5 mM) were tested instead as a sole electron donor in MMJS medium with a gas phase of 80 % N₂/19 % CO₂/1 % O₂ (200 kPa). Molecular hydrogen was also examined in MMJS medium with a gas phase of 80 % H₂/19 % CO₂/1 % O₂ (200 kPa). Tetrathionate produced a similar maximum cell yield to thiosulfate (7×10^8 cells ml⁻¹), whereas sulfide and elemental sulfur gave considerably lower yields ($2\text{--}4 \times 10^8$ cells ml⁻¹). In addition, a very narrow concentration range of sulfide (1 and 2 mM) produced positive growth. Other reduced sulfur compounds and hydrogen did not serve as the sole electron donor. To test for the utilization of electron acceptors, nitrate (10 mM), nitrite (1 or 5 mM), ferric citrate (20 mM), ferrihydrite (20 mM), selenate (5 mM), arsenate (5 mM) or fumarate (10 mM) were tested with MMJS medium under 80 % N₂/20 % CO₂ (200 kPa). None of the electron acceptors other than O₂ supported the growth of strain I78^T. The potential nutrients required for growth such as selenite, tungstate and vitamins were examined with MMJS medium with and without the specified nutrients. Nitrogen sources (NH₄Cl, NaNO₂, N₂, NaNO₃ or yeast extract) for growth and no nitrogen source were also examined with MMJS medium. Strain I78^T utilized nitrate, ammonium, yeast extract and molecular nitrogen as nitrogen sources but could not utilize nitrite. Selenium, tungsten and vitamins were not required for growth. These results indicated that strain I78^T was a chemolithomixotroph, utilizing the reduced sulfur compounds such as thiosulfate, tetrathionate, elemental sulfur and sulfide as energy sources, and molecular oxygen (up to 10 %; v/v) as the sole electron acceptor. According to Brinkhoff *et al.* (1999a, b), all previously described members of the genus *Thiomicrospira* are strict chemolithoautotrophs. However, the chemolithoautotrophy described for recognized *Thiomicrospira* species suggested they were not able to utilize organic carbon compounds as both energy and carbon sources; they should therefore not have been described as chemo-organotrophs (J. Kuever, personal communication). In this study, strain I78^T and the reference strains of *Thiomicrospira crunigena* strain TH-55^T (Jannasch *et al.*, 1985) and *Thiomicrospira* sp. strain L-12 (Ruby & Jannasch, 1982) were not able to grow chemo-organotrophically but were able to grow heterotrophically on various organic carbon sources with sulfur oxidation. Thus, strain I78^T and at least two reference strains of *Thiomicrospira crunigena* strain TH-55^T (Jannasch *et al.*, 1985) and *Thiomicrospira* sp. strain L-12 (Ruby & Jannasch, 1982) are chemolithomixotrophs (Table 1).

The time-course of oxidation of thiosulfate and concomitant bacterial growth of strain I78^T were examined with MMJS medium under a gas phase of 80 % N₂/19 % CO₂/1 %

Fig. 2. Time-course of oxidation of thiosulfate (○), the production of sulfate (■) and elemental sulfur (Δ) and concomitant bacterial growth (●) of *Thiomicrospira thermophila* strain I78^T.

O₂ (200 kPa) (Fig. 2). The concentrations of thiosulfate, sulfite and sulfate were analysed by ion chromatography using a Shim-pack IC column (Shimadzu, Kyoto, Japan) and the production of elemental sulfur during growth was monitored as described by Takai *et al.* (2001). Thiosulfate was consumed and sulfate and elemental sulfur were produced during the growth of strain I78^T (Fig. 2). Production of sulfite was not observed during the growth, in contrast to growth in *Thiomicrospira* strain L-12 (Ruby & Jannasch, 1982). In addition, the accumulation of elemental sulfur during growth was a distinct characteristic of strain I78^T and *Thiomicrospira crunigena* strain TH-55^T (Jannasch *et al.*, 1985) and *Thiomicrospira* sp. strain L-12 (Ruby & Jannasch, 1982); other *Thiomicrospira* species show the nearly complete oxidation of thiosulfate to sulfate (Brinkhoff *et al.*, 1999a, b). The control (uninoculated) medium showed no oxidation of thiosulfate and no production of either elemental sulfur or sulfate. Thus, strain I78^T was found to be a respiratory sulfur-oxidizing, oxygen-reducing chemolithomixotroph.

Strain I78^T was found to be sensitive to a variety of antibiotics, including chloramphenicol (50 µg ml⁻¹), streptomycin (50 µg ml⁻¹), kanamycin (50 µg ml⁻¹), ampicillin (50 µg ml⁻¹), vancomycin (50 µg ml⁻¹) and rifampicin (50 µg ml⁻¹) (data not shown).

Fatty acid analysis

The cellular fatty acid composition was analysed from cells grown in MMJS medium at 35 °C in the late-exponential phase of growth. The cellular fatty acid composition of *Thiomicrospira crunigena* strain TH-55^T (Jannasch *et al.*, 1985) was also determined from cells grown in MMJS medium at 30 °C in the late-exponential phase of growth. Lyophilized cells (100 mg) were placed in a Teflon-lined, screw-capped tube containing 3 ml anhydrous methanolic HCl that was heated at 100 °C for 3 h. Extraction and

analysis of fatty acid methyl esters were as described by Takai *et al.* (2003b). The major cellular fatty acids of strain I78^T were found to be C_{14:0} (7.4%), C_{16:0} (16.3%), C_{16:1} (37.2%), anteiso-C_{17:0} (4.0%), C_{18:0} (21.3%) and C_{18:1} (13.8%), whereas those of *Thiomicrospira crunogena* strain TH-55^T were C_{14:0} (2.0%), C_{16:0} (49.2%), C_{16:1} (21.4%), anteiso-C_{17:0} (0.9%), C_{18:0} (15.2%) and C_{18:1} (11.8%). As compared with *Thiomicrospira crunogena* strain TH-55^T, strain I78^T contained higher proportions of unsaturated fatty acids and longer chains of fatty acids. This may be associated with the higher temperature range for growth of strain I78^T.

Nucleic acid analyses

Genomic DNA of strain I78^T was prepared as described by Marmur & Doty (1962). The DNA G+C content was determined by direct analysis of deoxyribonucleotides by HPLC (Tamaoka & Komagata, 1984). The G+C content of the genomic DNA of strain I78^T was found to be 43.8 mol%, which is similar to those of *Thiomicrospira crunogena* strain TH-55^T (44.2 mol%) (Jannasch *et al.*, 1985) and *Thiomicrospira kuenenii* strain JB-A1^T (42.4 mol%) (Brinkhoff *et al.*, 1999a) (Table 1).

The 16S rRNA gene sequence was amplified by the PCR using primers Bac 27F and 1492R (DeLong, 1992; Lane, 1985) as described previously (Takai *et al.*, 2001). The nearly complete sequence (1478 bp) of the 16S rRNA gene from strain I78^T was directly sequenced by both strands using the dideoxynucleotide chain-termination method with a DNA sequencer model 3100 (Perkin Elmer/Applied Biosystems). The rRNA gene sequence was analysed using the gapped-BLAST search algorithm (Altschul *et al.*, 1997; Benson *et al.*, 1998) and was found to be most closely related to the sequences of *Thiomicrospira* sp. strain L-12 (97.2%)

(Ruby & Jannasch, 1982) and *Thiomicrospira crunogena* strain TH-55^T (95.6%) (Jannasch *et al.*, 1985), isolated from deep-sea hydrothermal environments; rRNA gene sequence similarity with other *Thiomicrospira* species was below 95%. The nearly complete sequence was manually re-aligned according to the secondary structures using ARB (Ludwig *et al.*, 2004). Phylogenetic analyses were restricted to nucleotide positions that could be chosen by using the γ -*Proteobacteria* filter of Hugenholtz (2002). Evolutionary distance matrix analysis (using the Jukes–Cantor correlation method) and neighbour-joining analysis were performed using ARB (Fig. 3). Bootstrap analysis was performed to provide confidence estimates for phylogenetic tree topologies. The phylogenetic tree indicated that strain I78^T was most closely related to *Thiomicrospira* sp. strain L-12 (Ruby & Jannasch, 1982) and *Thiomicrospira crunogena* strain TH-55^T (Jannasch *et al.*, 1985) (Fig. 3).

DNA–DNA hybridization was carried out at 42 °C for 3 h and was measured fluorometrically using photobiotin according to the method of Ezaki *et al.* (1989) among the genomic DNA of strain I78^T, *Thiomicrospira* sp. strain L-12 and *Thiomicrospira crunogena* strain TH-55^T. Mean hybridization values were 24.0% between strain I78^T and *Thiomicrospira crunogena* strain TH-55^T and 37.3% between strain I78^T and *Thiomicrospira* sp. strain L-12, indicating that strain I78^T could be genotypically differentiated from both these strains.

Comparison with related species

Strain I78^T was isolated from an STR-ISCS device deployed in a hydrothermal diffusing flow of a deep-sea hydrothermal fumarole at a depth of 2922 m at the TOTO caldera in the Mariana Arc. This is the first *Thiomicrospira* strain obtained from the Western Pacific region. Phylogenetic analysis

Fig. 3. Phylogenetic tree of representative *Thiomicrospira* strains and *Proteobacteria* potentially related to *Thiomicrospira thermophila* strain I78^T inferred from 16S rRNA gene sequences using the neighbour-joining method on the 1208 homologous sequence positions for each organism. The number at each node represents the bootstrap percentage (1000 replicates). Bar, 10 substitutions per 100 nucleotides.

indicated that strain I78^T is most closely related to *Thiomicrospira crunogena* strain TH-55^T, which was isolated from the outer tube of ventimentiferan tube worms in the deep-sea hydrothermal vent of the EPR (Jannasch *et al.*, 1985), and *Thiomicrospira* sp. strain L-12, which was isolated from a hydrothermal mussel colony on the Galapagos Rift (Ruby & Jannasch, 1982). However, many of the physiological characteristics of strain I78^T are different from those of deep-sea hydrothermal vent *Thiomicrospira* strains and even from other previously described species such as *Thiomicrospira kuenenii* strain JB-A1^T (Brinkhoff *et al.*, 1999a) and *Hydrogenovibrio marinus* strain MH-110^T (Nishihara *et al.*, 1989, 1991) obtained from coastal environments (Table 1). Strain I78^T is highly thermophilic, growing optimally at 35–40 °C and up to 55 °C, much higher than the optimum and highest temperatures for growth of any previously described members of the genus *Thiomicrospira* and of *Hydrogenovibrio marinus* strain MH-110^T (Nishihara *et al.*, 1989, 1991) (Table 1). In comparison with phylogenetically related *Thiomicrospira* sp. strain L-12 (Ruby & Jannasch, 1982) and *Thiomicrospira crunogena* strain TH-55^T from deep-sea hydrothermal environments, strain I78^T has a lower optimal pH for growth (pH 6.0, Table 1). Another distinctive feature is its ability to use molecular nitrogen as the nitrogen source (Table 1). These are distinctive physiological features that clearly differentiate strain I78^T from previously described deep-sea hydrothermal vent *Thiomicrospira* strains. In addition, DNA hybridization analysis clearly reveals that the novel isolate can be genetically differentiated from *Thiomicrospira* sp. strain L-12 (Ruby & Jannasch, 1982) and *Thiomicrospira crunogena* strain TH-55^T at the species level. On the basis of these physiological and genetic properties, we suggest that strain I78^T is representative of a novel species of the genus *Thiomicrospira*, for which the name *Thiomicrospira thermophila* sp. nov. is proposed.

The physiological properties of *Thiomicrospira thermophila* described in this study may provide a key to understanding the potential ecological niches of *Thiomicrospira thermophila* in the deep-sea hydrothermal field of the TOTO caldera. *Thiomicrospira thermophila* was obtained from an STR-ISCS device deployed in a hydrothermal diffusing flow with a maximum temperature of 78 °C and a pH of 5.3. The substratum trapping a viable population of *Thiomicrospira thermophila* was exposed to a temperature of about 70 °C for at least 2 days. *Thiomicrospira thermophila* was probably entrained by the diffusing flow from the sub-seafloor and survived in the substratum. The relative thermophily, thermotolerance and acidophily of *Thiomicrospira thermophila* among the genus *Thiomicrospira* and its potential for nitrogen fixation are consistent with its occurrence in the sub-seafloor hydrothermal mixing zones with oxygenated sea water. Because the TOTO caldera hydrothermal system is characterized by strongly acidified fluids enriched with volatile volcanic gas components, the sub-seafloor mixing zones would provide microaerobic, acidic, mesophilic to thermophilic microbial habitats with

abundant reduced sulfur compounds and molecular nitrogen provided from the volcanic gas input. Indeed, successful cultivation of *Thiomicrospira thermophila* was only via the ISCS, not from any other samples such as the ambient sea waters, hydrothermal plumes, chimney structures and rocks (data not shown). Localization of the viable population and the physiology of *Thiomicrospira thermophila* will point to the existence of novel sub-seafloor habitats beneath the TOTO caldera deep-sea hydrothermal field.

Description of *Thiomicrospira thermophila* sp. nov.

Thiomicrospira thermophila (ther.mo'phi.la. Gr. fem. n. *therme* heat; Gr. adj. *philos* loving; N.L. fem. adj. *thermophila* heat-loving).

Cells occur singly, as Gram-negative, motile, straight to curved rods with a polar flagellum, with a mean length of 0.8–1.5 µm and a diameter of approximately 0.4–0.7 µm. Cells are microaerobic, tolerating up to 10 % O₂ in the gas phase. The temperature range for growth is 15–55 °C (optimum 35–40 °C). The pH range for growth is 5.0–8.0 (optimum pH 6.0). NaCl in the concentration range 3–70 g l⁻¹ is an absolute growth requirement; optimum growth occurs at 12–20 g l⁻¹. Chemolithoautotrophic growth occurs with reduced sulfur compounds such as thiosulfate, elemental sulfur and sulfide as electron donors and molecular oxygen as an electron acceptor. Heterotrophic growth is possible with 0.1 % (w/v) yeast extract, 0.1 % (w/v) peptone, 0.1 % (w/v) tryptone, 0.1 % (w/v) casein, 0.1 % (w/v) starch, 0.1 % (w/v) CMC, 0.1 % (w/v) Casamino acids, 5 mM glycerol, 5 mM fumarate, 5 mM malate, 5 mM citrate, 5 mM glycine, 5 mM alanine, 5 mM leucine, 5 mM isoleucine, 5 mM valine, 5 mM lysine, 5 mM cysteine, 5 mM methionine, 5 mM arginine, 5 mM histidine, 5 mM asparagine, 5 mM glutamine, 5 mM aspartate, 5 mM glutamate, 0.02 % (w/v) glucose, 0.02 % (w/v) galactose, 0.02 % (w/v) sucrose, 0.02 % (w/v) fructose, 0.02 % (w/v) lactose, 0.02 % (w/v) maltose or 0.02 % (w/v) trehalose as the sole carbon source. Thiosulfate is oxidized to sulfate and elemental sulfur during growth. Nitrate, ammonium, organic nitrogen compounds and molecular nitrogen are utilized as nitrogen sources. Vitamins, selenium and tungsten are not required for growth. The major cellular fatty acids are C_{14:0} (7.4 %), C_{16:0} (16.3 %), C_{16:1} (37.2 %), anteiso-C_{17:0} (4.0 %), C_{18:0} (21.3 %) and C_{18:1} (13.8 %). The DNA G + C content is 43.8 mol% (by HPLC). Isolated from an *in situ* colonization device deployed in the hydrothermal diffusing flow (maximally 78 °C) at the TOTO caldera in the Mariana Arc, Western Pacific.

The type strain is I78^T (= JCM 12397^T = DSM 16397^T).

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