

## *Thiomicrospira halophila* sp. nov., a moderately halophilic, obligately chemolithoautotrophic, sulfur-oxidizing bacterium from hypersaline lakes

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Enrichments at 2 M NaCl and pH 7.5–8, with thiosulfate or sulfide as electron donor, inoculated with sediments from hypersaline chloride–sulfate lakes of the Kulunda Steppe (Altai, Russia) resulted in the domination of two different groups of moderately halophilic, chemolithoautotrophic, sulfur-oxidizing bacteria. Under fully aerobic conditions with thiosulfate, bacteria belonging to the genus *Halothiobacillus* dominated while, under microaerophilic conditions, a highly motile, short vibrio-shaped phenotype outcompeted the halothiobacilli. Three genetically and phenotypically highly similar vibrio-shaped isolates were obtained in pure culture and one of them, strain HL 5<sup>T</sup>, was identified as a member of the *Thiomicrospira crunogena* cluster by 16S rRNA gene sequencing. The new isolates were able to grow with thiosulfate as electron donor within a broad salinity range from 0.5 to 3.5 M NaCl with an optimum at 1.5 M and within a pH range from 6.5 to 8.5 with an optimum at pH 7.5–7.8. Comparative analysis of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) gene sequences demonstrated that strain HL 5<sup>T</sup> possessed two genes, *cbbL-1* and *cbbL-2*, of the form I RuBisCO and a *cbbM* gene of the form II RuBisCO, similar to the other members of the *Thiomicrospira crunogena* cluster. On the basis of phenotypic and genetic comparison, the new halophilic isolates are proposed to be placed into a novel species, *Thiomicrospira halophila* sp. nov. (type strain HL 5<sup>T</sup>=DSM 15072<sup>T</sup>=UNIQEM U 221<sup>T</sup>).

Although hypersaline environments, such as inland salt lakes and sea salterns, are traditionally regarded as the domain of heterotrophic haloarchaea, which grow optimally at NaCl concentrations above 3 M (Oren, 2002), this is mostly true of the brines. In the sediments, which are separated from the brine by a thick salt crust, the microbial communities are more complex and include halophilic eubacteria of different physiology. In particular, halophilic sulfate-reducing bacteria can be active in these environments up to saturating salt concentrations (Ollivier *et al.*, 1994; Sørensen *et al.*, 2004). The latter can be a condition

for development of their counterpart, halophilic sulfur-oxidizing bacteria (SOB). Among the chemolithotrophic bacteria, SOB have a good chance to adapt to extreme conditions, such as high salt, owing to the high energy yield available during complete oxidation of sulfide/thiosulfate to sulfate (Oren, 1999).

The first and so far only neutrophilic, halophilic SOB able to grow at very high salt concentrations, up to 4 M NaCl, was discovered 15 years ago in an Australian hypersaline lake (Wood & Kelly, 1991) and is currently known as *Halothiobacillus halophilus* (Kelly & Wood, 2000). The organism belongs to the high-salt-tolerant moderate halophiles, with an optimum salt concentration for growth around 1 M. Since that time, no attempts have been made to peer more thoroughly into the diversity of halophilic SOB in hypersaline habitats. Our current research on haloalkaliphilic SOB inhabiting soda lakes, however, has demonstrated the widespread potential of SOB to grow at very high

**Abbreviations:** RSCU, relative synonymous codon usage; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SOB, sulfur-oxidizing bacterium.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *cbb* gene sequences of strain HL 5<sup>T</sup> are DQ390450 and DQ390451–DQ390453, respectively.

concentrations of sodium carbonates (Sorokin & Kuenen, 2005). This prompted us to start analogous research on the diversity of SOB in hypersaline chloride–sulfate inland lakes and salterns with neutral pH.

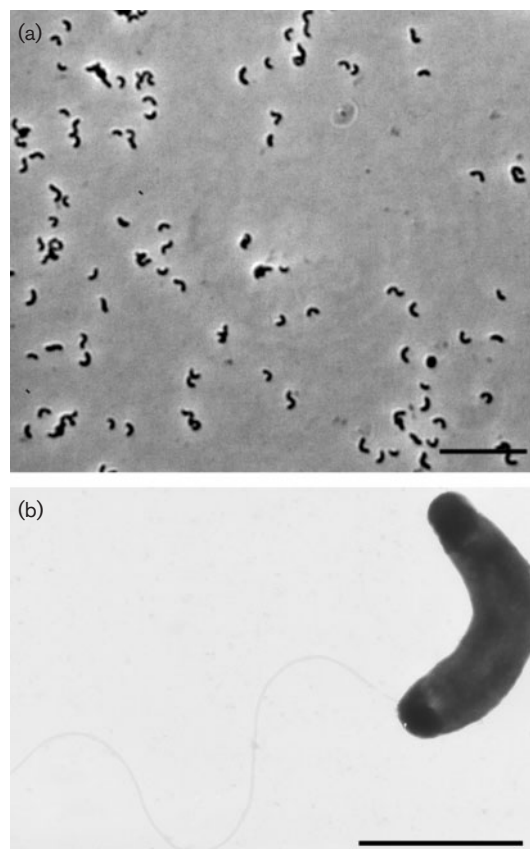
The Kulunda Steppe (Altai, Russia) harbours numerous salt lakes with a total salt content from 10 to 38%, a pH range of 7.0–8.5 and with  $\text{Na}^+$ ,  $\text{Cl}^-$  and  $\text{SO}_4^{2-}$  as the dominant ions in the brines. Samples were obtained from the top 10 cm of sediment with overlying brine water from 20 lakes and were grouped into three categories according to the salinity.

Halophilic SOB were enriched and isolated in pure culture using the following mineral base medium ( $\text{g l}^{-1}$ ):  $\text{NaCl}$ , 120;  $\text{K}_2\text{HPO}_4$ , 1.5;  $(\text{NH}_4)_2\text{SO}_4$ , 0.5. The pH was adjusted to 7.2. After sterilization, the medium was supplemented with 2 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 1 ml trace metal solution  $\text{l}^{-1}$  (Pfennig & Lippert, 1966) and 20–50 mM  $\text{NaHCO}_3$  (from a 1 M filter-sterilized solution). The final pH was 7.5–7.8 and the incubation temperature was 25 °C. Two types of enrichments were performed. Enrichments with thiosulfate as substrate (20 mM) were incubated in sealed 100 ml serum bottles with 10 ml medium under fully aerobic conditions (gas phase, air; rotary shaker at 150 r.p.m.) and under microaerophilic conditions with 10 ml medium in 250 ml bottles at 2%  $\text{O}_2$  (static). The second type of enrichment was based on gradient cultivation (Nelson & Jannasch, 1983) in 50 ml glass cylinders, whereby sulfide, as substrate, was diffusing upward from the bottom 2% (w/v) agar layer (10 ml, 0.1 mmol  $\text{Na}_2\text{S}$ ) overlaid by 30 ml of the above-mentioned mineral medium containing one-tenth of the amount of trace elements and 0.2% (w/v) agarose. Oxygen was diffusing downward from the 10 ml headspace. SOB usually developed within a thin layer approximately 1 cm below the surface of the top medium layer. The isolation strategy included several 1:100 transfers to stabilize the cultures, followed by serial dilutions and plating onto solid medium with the same composition (for thiosulfate enrichments). The plates were incubated either in plastic bags (aerobic) or in closed jars under 2%  $\text{O}_2$  (microaerophilic). For sulfide-gradient cultures, dilution series were performed in 20 ml stoppered tubes with the proportions of different layers similar to that used in the cylinders. Growth was monitored by measuring the  $\text{OD}_{600}$  (in case of intermediate sulfur formation, it was removed by centrifugation at 3000 r.p.m. for 1 min) and by monitoring thiosulfate consumption and pH. The pH was maintained above pH 7 by periodic addition of  $\text{NaHCO}_3$ . Sulfur compounds (thiosulfate, sulfide, tetrathionate and elemental sulfur), protein concentration and respiration rates of washed cells were measured as described previously (Sorokin *et al.*, 2001). Phase-contrast photomicrographs were obtained using a Zeiss Axioplan Imaging 2 microscope. For electron microscopy, cells were fixed with glutaraldehyde (final, 3% v/v) and positively contrasted with 1% (w/v) uranyl acetate. The isolation of DNA and subsequent determination of the G+C content of the DNA and DNA–DNA

hybridization were performed by the thermal denaturation/reassociation technique (Marmur, 1961; De Ley *et al.*, 1970).

The 16S rRNA genes were selectively amplified using general bacterial primers and the PCR products were purified from low-melting-temperature agarose using a Wizard PCR Prep kit (Promega) according to the manufacturer's instructions. To amplify bacterial genes of RuBisCO form I and form II large subunit (*cbbL* and *cbbM*, respectively), specially designed and previously tested primer pairs were employed (Spiridonova *et al.*, 2004). Purified PCR fragments were cloned into the pGEM-T vector (Promega) using competent cells of *Escherichia coli* DH5 $\alpha$  for transformation. Plasmid DNA was extracted and purified using the Wizard MiniPrep kit (Promega) according to the manufacturer's recommendations. Clones containing appropriately sized inserts were sequenced from universal M13 forward and reverse primers. Sequencing was performed using a Big Dye Terminator version 3.1 sequencing reaction kit with an ABI 3730 DNA automatic sequencer (Applied Biosystems).

Preliminary phylogenetic analysis of the new sequences was done with the NCBI BLAST server (<http://www.ncbi.nlm.nih>).



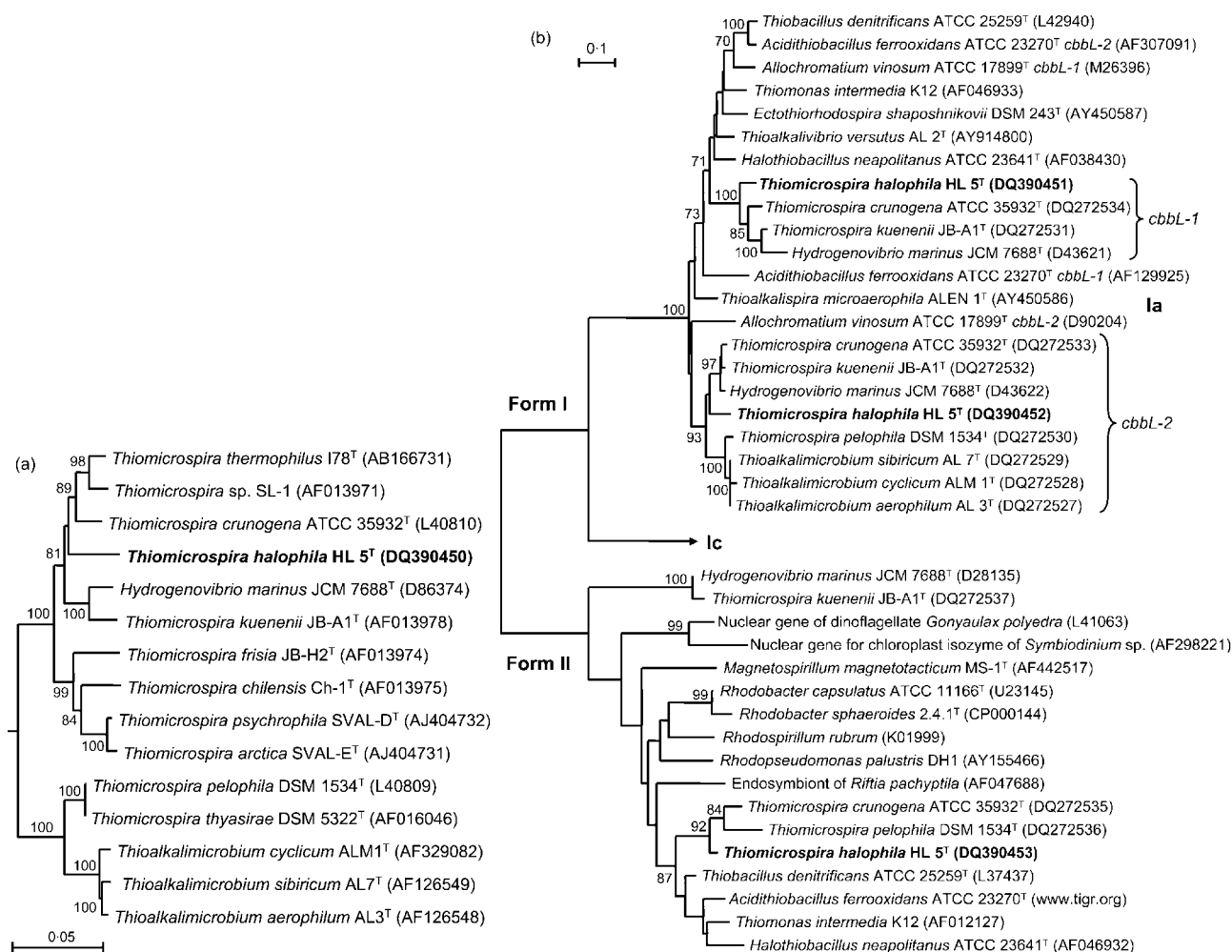
**Fig. 1.** Cell morphology of strain HL 5<sup>T</sup> grown at 2 M NaCl with thiosulfate. (a) Phase-contrast photomicrograph (bar, 10  $\mu\text{m}$ ). (b) Electron photomicrograph of positively stained cell (bar, 1  $\mu\text{m}$ ).

gov/blast/). Nucleotide and inferred amino acid sequences were aligned with sequences from GenBank using CLUSTAL W. Phylogenetic trees were reconstructed using the neighbour-joining algorithm within the TREECONW program package (Van de Peer & De Wachter, 1994).

Relative synonymous codon usage (RSCU) values of the RuBisCO genes were calculated using CodonW software (<http://www.molbiol.ox.ac.uk/cu>). To investigate major trends in codon usage in different species, CodonW was used to carry out a correspondence analysis. This resulted in a point in the codon space for each species, the positions of which sometimes suggested codon usage bias.

Aerobic enrichments with thiosulfate at 2 M NaCl resulted in rapid growth of rod-shaped, motile SOB. From these cultures, colonies of the rod-shaped SOB could be easily obtained on mineral plates with thiosulfate and two pure

cultures were identified by 16S rRNA gene sequencing as members of the genus *Halothiobacillus* (data not shown). However, in enrichments with thiosulfate under micro-oxic conditions and, especially in sulfide–oxygen gradient cultures, small and extremely motile vibrios developed more rapidly than halothiobacilli. Unfortunately, they did not readily form colonies on plates. As a consequence, the colony approach always resulted in the dominance of halothiobacilli, even if they were minor components in the liquid cultures. Only after several dilution series, when the vibrio phenotype became highly enriched, did tiny, sulfur-containing colonies appear on thiosulfate plates under micro-oxic conditions after 2–3 weeks incubation. Two pure cultures were obtained from single colonies originating from thiosulfate enrichments (lake salinity ranges 100–140 and 200–240 g l<sup>-1</sup>) and one culture after serial dilutions of a–1). The strains were designated HL 5<sup>T</sup>, HL 8 (isolated with



**Fig. 2.** Phylogenetic position of halophilic sulfur-oxidizing bacterium strain HL 5<sup>T</sup> within the Gammaproteobacteria based on analysis of 16S rRNA gene sequences (a) and translated amino acid sequences of *cbb* genes (b). Tree topology and evolutionary distances are given by the neighbour-joining method with Jukes & Cantor (for nucleotide sequences) and Poisson (for amino acid sequences) corrections. Numbers at nodes indicate percentages of bootstrap values for the clade of this group in 1000 replications.



it was shown that strain HL 5<sup>T</sup> possessed two different *cbbL* genes of the 'green-like' form I and a *cbbM* gene of the form II of this enzyme, similar to the other species in the *Thiomicrospira crunogena* cluster, such as *Hydrogenovibrio marinus* (Yoshizawa *et al.*, 2004), *Thiomicrospira crunogena* and *Thiomicrospira kuenenii* (Tourova *et al.*, 2006). However, the G + C content in the genome of all three halophilic isolates was 56.1–57.1 mol%, which is 5–15 mol% higher than in the *Thiomicrospira crunogena* group (39.2–51.7 mol%). DNA–DNA hybridization of the new isolates with *Thiomicrospira* sp. strain SL 1, the strain with the highest G + C content within the *Thiomicrospira crunogena* lineage (51.7 mol%) which originated from Solar Lake, Sinai, Egypt (Brinkhoff & Muyzer, 1997), and with *Thiomicrospira crunogena* DSM 12353<sup>T</sup> was below 20%. Nevertheless, analysis of both 16S rRNA gene sequences (Fig. 2a) and translated amino acid sequences of the RuBisCO genes (Fig. 2b) firmly placed strain HL 5<sup>T</sup> into the *Thiomicrospira crunogena* group.

The G + C content of the *cbb* genes revealed a strong linear correlation with the genomic G + C content, which is common (Muto & Osawa, 1987). The large differences in the G + C content of the total DNA between the new isolates and other members of the *Thiomicrospira crunogena* cluster were not an artefact, since the obtained fragments of RuBisCO genes from strain HL 5<sup>T</sup> also had a similarly high G + C content. The total G + C content for the *cbb* genes of this strain was 54.1–58.7 mol%, compared with 45.1–48.9 mol% for the corresponding genes of phylogenetically related species. Comparison of codon usage in the RuBisCO genes between strain HL 5<sup>T</sup> and *Thiomicrospira* species using RSCU data identified the major trends (Fig. 3): axis 2 is associated with the G + C<sub>3</sub> content (in the third position of codons) (Musto *et al.*, 1998) and

axis 1 shows the frequency of codons ending in C or U versus A or G (Fennoy & Bailey-Serres, 1993). The codon usage of all *Thiomicrospira* species was typical of A/T-biased micro-organisms, in which codons with an A or T in the third position are used preferentially (Ohtaka & Ishikawa, 1993). In contrast, the codon usage of strain HL 5<sup>T</sup> was typical of G/C-biased bacteria. Based on codon usage, the closest neighbours of the strain HL 5<sup>T</sup> were (halo)alkaliphilic sulfur-oxidizing gammaproteobacteria such as *Thioalkalispira microaerophila* and *Halothiobacillus neapolitanus*, with a similar G + C content of their genomic DNA (56.0 mol%) and the two types of RuBisCO genes (54.9–55.5 mol%).

On the basis of phenotypic and genetic comparison, the new halophilic SOB from hypersaline lakes are proposed to be assigned to a novel species of the genus *Thiomicrospira*, *Thiomicrospira halophila* sp. nov. Comparative properties of the new isolates and *Thiomicrospira* species with validly published names are presented in Table 1.

### Description of *Thiomicrospira halophila* sp. nov.

*Thiomicrospira halophila* (ha.lo'phi.la. Gr. n. *hals* salt; Gr. adj. *philos* loving; N.L. fem. adj. *halophila* salt-loving).

Cells are Gram-negative vibrios (0.3–0.5 × 1–2 µm), highly motile due to a single polar flagellum. Colonies on thio-sulfate agar at 1–2 M NaCl reach a maximum diameter of 1 mm and are convex, circular and silver-white (because of sulfur accumulation). Strictly aerobic, with a tendency for microaerophilism. Grows chemolithoautotrophically with thiosulfate and sulfide, oxidizing them to sulfate with elemental sulfur as an intermediate. Does not produce or oxidize polythionates. Autotrophic growth with thiosulfate is possible within the range of 0.5–3.5 M NaCl (optimum

**Table 1.** Comparative characteristics of strain HL 5<sup>T</sup> (*Thiomicrospira halophila* sp. nov.) and related species

Species: 1, *Thiomicrospira halophila*; 2, *Thiomicrospira crunogena* (data from Brinkhoff *et al.*, 2005); 3, *Thiomicrospira chilensis* (Brinkhoff *et al.*, 2005); 4, *Thiomicrospira frisia* (Brinkhoff *et al.*, 2005); 5, *Thiomicrospira kuenenii* (Brinkhoff *et al.*, 2005); 6, *Thiomicrospira arctica* (Knittel *et al.*, 2005); 7, *Thiomicrospira psychrophila* (Knittel *et al.*, 2005); 8, *Thiomicrospira thermophila* (Takai *et al.*, 2004); 9, *Hydrogenovibrio marinus* (Nishihara *et al.*, 1991). ND, Not determined.

Characteristic	1	2	3	4	5	6	7	8	9
Cell shape	Vibrio	Vibrio	Rod	Rod	Vibrio	Rod	Rod	Curved rod	Vibrio
DNA G + C content (mol%)	56.6	44.2	49.9	39.6	42.4	42.4	42.5	43.8	44.1
NaCl concentration range for growth (M)	0.50–3.50	0.045–ND	0.10–1.24	0.10–1.24	0.10–0.64	0.04–1.24	0.04–1.24	0.05–1.20	ND
Optimum NaCl concentration (M)	1.50	ND	0.47	0.47	0.47	0.25	0.25	0.30	0.50
Oxidation of tetrathionate	–	+	+	+	+	+	+	ND	+
S <sub>8</sub> as intermediate	+	+	+	–	–	+	+	+	+
H <sub>2</sub> as electron donor	–	–	–	–	–	–	–	–	+
Growth below 0 °C	–	–	–	–	–	+	+	–	+
Growth at > 50 °C	–	–	–	–	–	–	–	+	–

1.5 M) and at pH 6.5–8.5 (optimum pH 7.5–7.8). Inorganic carbon is assimilated through the Calvin cycle. Possesses three different RuBisCO genes. Differs from the other *Thiomicrospira* species by its high salt tolerance and much higher G+C content of genomic DNA. Represented by three closely related strains isolated from hypersaline inland lakes in south-western Siberia (Altai, Russia) with DNA G+C contents of between 56.1 and 57.1 mol%.

The type strain is HL 5<sup>T</sup> (=DSM 15072<sup>T</sup>=UNIQEM U 221<sup>T</sup>).

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