Thiohalobacter thiocyanaticus gen. nov., sp. nov., a moderately halophilic, sulfur-oxidizing gammaproteobacterium from hypersaline lakes, that utilizes thiocyanate

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A moderately halophilic, obligately chemolithoautotrophic, sulfur-oxidizing bacterium, designated strain HRh1^T, was obtained from mixed sediment samples from hypersaline chloride–sulfate lakes in the Kulunda Steppe, in south-western Siberia (Russia), using aerobic enrichment culture at 1 M NaCl with thiocyanate as substrate. Cells of the isolate were short, non-motile rods with a Gram-negative type of cell wall. The bacterium was an obligate aerobe capable of chemolithoautotrophic growth with thiocyanate and thiosulfate. With thiosulfate, it grew at NaCl concentrations of 0.2–3.0 M (optimum 0.5 M) and at pH 6.3–8.0 (optimum pH 7.3–7.5). During growth on thiocyanate, cyanate was identified as an intermediate. The dominant cellular fatty acids were C_{16:0} and C_{18:1ω7}. Phylogenetic analysis based on 16S rRNA gene sequencing placed the isolate in the class *Gammaproteobacteria* as an independent lineage, with an unclassified marine sulfur-oxidizing bacterium as the closest culturable relative (93% sequence similarity). A single *cbbL* gene (coding for the key enzyme of the Calvin–Benson cycle of autotrophic CO₂ assimilation) with relatively low similarity to any homologous genes found in chemolithoautotrophs was detected in strain HRh1^T. On the basis of our phenotypic and phylogenetic analysis, the halophilic isolate is proposed to represent a new genus and novel species, *Thiohalobacter thiocyanaticus* gen. nov., sp. nov. The type strain of *Thiohalobacter thiocyanaticus* is HRh1^T (=DSM 21152^T =UNIQEM U249^T).

Thiocyanate (N≡C–S[−]) is a unique inorganic compound that contains both a nitrogen atom and a reduced sulfur atom. Because of the latter, it can be used as an electron donor by a very limited number of lithoautotrophic sulfur-oxidizing bacteria (SOB) after primary degradation at either the nitrile (C≡N) or the carbon–sulfur (C–S) bond. Accordingly, two major mechanisms of primary thiocyanate degradation in bacteria are currently recognized (Kelly & Baker, 1990) based on the nature of the intermediates:

$\text{N}\equiv\text{C}-\text{S}^{-} + 2\text{H}_2\text{O} \rightarrow \text{O}=\text{C}=\text{S} + \text{NH}_3 + \text{OH}^{-}$ [the carbonyl sulfide (COS) pathway]

$\text{N}\equiv\text{C}-\text{S}^{-} + \text{H}_2\text{O} \rightarrow \text{N}\equiv\text{C}-\text{O}^{-} + \text{H}_2\text{S}$ (the cyanate pathway)

The COS pathway was confirmed to operate in the betaproteobacterium *Thiobacillus thioparus*. The enzyme thiocyanate hydrolase from this SOB hydrolyses the nitrile bond of thiocyanate with the formation of COS and ammonia. COS is hydrolysed further to hydrogen sulfide, which is finally utilized as the actual electron donor for lithoautotrophic growth (Katayama *et al.*, 1998). The evidence for an alternative pathway via cyanate is not so strong, and is based only on the presence of the enzyme cyanase. Recently, however, we found direct evidence of this pathway (i.e. cyanate formation from thiocyanate) in haloalkaliphilic, thiocyanate-utilizing SOB of the genus *Thioalkalivibrio* (Sorokin *et al.*, 2001).

Abbreviations: COS, carbonyl sulfide; RuBisCO, ribulose biphosphate carboxylase/oxygenase; SOB, sulfur-oxidizing bacteria.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *cbbL* gene sequences of strain HRh1^T are FJ482231 and FJ716702, respectively.

A comparison of the fatty acid profile of strain HRh1^T and related strains is available as supplementary material with the online version of this paper.

To date, microbial thiocyanate utilization at extremely high salt concentrations and neutral pH has not been reported. Our recent survey of hypersaline habitats with neutral pH revealed an unexpectedly rich diversity of culturable SOB represented by six different phylogenetic lineages of moderate and extremely halophilic SOB within the *Gammaproteobacteria* (Sorokin *et al.*, 2006; Sorokin, 2008). One of the moderately halophilic groups among them, represented by the novel genus and species *Thiohalophilus thiocyanatoxidans*, is capable of growth with thiocyanate, both aerobically and anaerobically with nitrite (at concentrations below 2 mM) at 1–4 M NaCl (Sorokin *et al.*, 2007a). It formed COS as an intermediate during thiocyanate degradation and the enzyme responsible for the reaction was identified as thiocyanate hydrolase (Bezudnova *et al.*, 2007). Aerobic enrichments from hypersaline lakes with thiocyanate as substrate were only positive at salt concentrations of 2 M and lower. One such enrichment from a mixture of lake sediments resulted in the isolation of strain HRh1^T, a thiocyanate-utilizing, moderately halophilic SOB which is characterized in this paper.

Surface sediments from several hypersaline chloride–sulfate lakes in the Kulunda Steppe (south-west Siberia, Altai, Russia) were used for the enrichment of halophilic SOB with total salt concentration of the brines from 20 to 38 % (w/v) and pH 7.5–8.5.

The mineral base medium used for enrichment and growth experiments contained 1–4 M NaCl and 10 mM K₂HPO₄. The pH was adjusted to 7.5 with 10 % KH₂PO₄. After sterilization, the medium was supplemented with 2 mM MgCl₂ · 6H₂O and 1 ml trace metal solution l⁻¹ (Pfennig & Lippert, 1966). Potassium thiocyanate (5 mM) or sodium thiosulfate (20 mM) were used as electron donors from filter-sterilized 1 M solutions. In the case of thiosulfate, the medium was also supplemented with 4 mM NH₄Cl. Filter-sterilized NaHCO₃ (1 M, pH 8.0) was added as a carbon source and a buffer at a final concentration of 10–40 mM. Routine aerobic incubation was performed in closed bottles with the liquid/gas ratio of 10:90 with shaking at 100 r.p.m. and at 30 °C for 5–20 days, depending on the substrate and salt concentration. Growth under denitrifying conditions was studied as described previously (Sorokin *et al.*, 2007a). Growth was monitored by following the optical density at 590 nm, substrate consumption and products formation. A pure culture was obtained from well-separated sulfur-containing colonies formed on solid medium with thiocyanate at 1 M NaCl after plating serially diluted enrichment culture stabilized by several 1:100 transfers. Culture purity was checked by microscopy, sequencing and by the absence of growth on rich organic medium in which thiocyanate was replaced by yeast extract.

Sulfur (thiocyanate, thiosulfate, tetrathionate and sulfate) and nitrogen (cyanate, NH₃) compounds were analysed as described previously (Sorokin *et al.*, 2001, 2007a). Cell

protein was measured by using the Lowry method (Lowry *et al.*, 1951) after removal of interfering sulfur compounds: soluble sulfur compounds were removed by washing (thiosulfate, tetrathionate) and insoluble sulfur was extracted from the cell pellet by overnight incubation with acetone. Phase-contrast photomicrographs were obtained using microscope model Zeiss Axioplan Imaging 2. For electron microscopy, the cells were fixed in glutaraldehyde (final concentration 3 %, v/v) in 0.5 M NaCl and, after removal of the fixative, were stained with 2 % (w/v) uranyl acetate. Other analyses (such as respiration tests and activity measurements) were performed as described previously (Sorokin *et al.*, 2007a).

Aerobic enrichments with thiocyanate as electron donor at 1–4 M NaCl gave positive results only at NaCl concentrations of 1–2 M. The cultures consumed 10 mM thiocyanate within a month with sulfate as the final product. Plating of the 1 M NaCl culture as the most active one after several repetitive transfers resulted in the formation of tiny sulfur-containing colonies with an identical cell morphology after 3 weeks of incubation. One such colony resulted in a positive liquid culture that grew with thiocyanate at 1 M NaCl, which was designated strain HRh1^T. The culture was represented by short, non-motile rods, often in chains and clusters (Fig. 1).

The novel bacterium was an obligately chemolithoautotrophic SOB, growing with either thiocyanate or thiosulfate as electron donor under strictly aerobic conditions. Growth with thiocyanate was very slow and, in many cases, the culture consumed only 5–10 mM substrate and then failed to grow any more on further additions. The final products of thiocyanate metabolism were sulfate and ammonia, and cyanate (but not COS) was identified as an intermediate (Fig. 2a). Growth with thiosulfate was much faster and resulted in much higher biomass. The final product was sulfate (Fig. 2b), with occasional intermediate formation of elemental sulfur because of oxygen limitation. The latter was utilized when the culture was supplied with extra air through an inserted air filter. Tetrathionate was formed from thiosulfate in minor amounts only at a highest salt concentration (3 M). The maximal experimentally measured growth yield and specific growth rate for strain HRh1^T during cultivation at 1 M NaCl on thiocyanate/thiosulfate were 4.0/4.4 mg protein mmol⁻¹ and 0.015/0.045 h⁻¹, respectively.

Apart from thiocyanate and thiosulfate, sulfide, tetrathionate and elemental sulfur could also be oxidized by washed cells of HRh1^T obtained from late-exponential-growth phase cultures pregrown either with thiocyanate or thiosulfate. However, attempts to grow the culture with these compounds failed. The rates of oxygen consumption [nmol O₂ (mg protein)⁻¹ min⁻¹; means of two independent experiments] at 1 M NaCl measured in two samples with cells grown with either thiocyanate or thiosulfate were 75 and 0 with thiocyanate, 65 and 350 with thiosulfate, 130 and 480 with sulfide, 20 and 200 with tetrathionate and 60

Fig. 1. Cell morphology of strain HRh1^T grown at 1 M NaCl with thiocyanate. (a) Phase-contrast microphotograph; (b) electron photomicrograph of fixed, intact cells. Bars, 10 µm (a) and 1 µm (b).

and 240 with sulfur (solution in acetone), respectively. Thiocyanate-degrading activity was therefore only manifested in cells grown with thiocyanate, while, with the other inorganic sulfur compounds, the activity was present in both preparations but was much higher in the thiosulfate-grown cells.

Strain HRh1^T was able to grow with thiosulfate at a NaCl concentration 0.2–3.0 M, with optimum growth at 0.5 M (Fig. 3a), meaning it is classified as a moderate halophile. Washed cells were active at up to 4 M NaCl, with an optimum at 1–1.5 M. Cells grown in 1 M NaCl were obviously less active at high salt than cells pregrown at a maximum salinity of 3 M (Fig. 3b). There was also a dramatic difference in product formation from thiosulfate oxidized by the washed cells: cells grown at 3 M NaCl performed complete oxidation of thiosulfate to sulfate over the entire salinity range, while cells grown at 1 M NaCl oxidized thiosulfate incompletely to tetrathionate at high salt (Fig. 3b). The latter fact pointed to a blockade of tetrathionate oxidation at high salt in cells grown at low salinity.

Washed cells obtained from thiocyanate-grown cultures were able to degrade thiocyanate only under aerobic

Fig. 2. Growth and product formation in autotrophic cultures of strain HRh1^T with thiocyanate at 1 M NaCl (a) and with thiosulfate at 2 M NaCl (b). ●, Biomass (as OD₅₉₀); ○, thiocyanate; ◇, thiosulfate; △, ammonia; ▲, cyanate; ◆, sulfate.

conditions. Ultrasonic cell disruption completely abolished the thiocyanate-degrading activity. Under aerobic conditions, washed cells oxidized thiocyanate to sulfate and ammonium at a maximum specific rate of 15 nmol (mg protein)⁻¹ min⁻¹. Cyanate was produced as a nitrogen intermediate at a maximum yield of 40 % from the metabolized thiocyanate nitrogen. However, cyanase activity was not detectable either in whole cells or in cell-free extracts. These data confirmed that HRh1^T uses the cyanate pathway for thiocyanate degradation and also show that the presence of cyanase activity cannot be taken as an indicator of the operation of this pathway, as was found previously for *Thioalkalivibrio* strains (Sorokin *et al.*, 2001).

Cellular fatty acids were extracted from freeze-dried biomass of strain HRh1^T grown at 2 M NaCl and 30 °C with thiosulfate by acidic methanol extraction and analysed by GC-MS according to Zhilina *et al.* (1997). The analysis demonstrated that two unsaturated species (C_{16:1}ω7 and C_{18:1}ω7) and saturated C_{16:0} made up more than 80 % of the total. In this, it resembled the fatty acid composition of the moderately halophilic *Thiohalomonas denitrificans* (Sorokin *et al.*, 2007b). On the other hand, the presence of the 10-methyl C_{16:0} species at a relatively high

Fig. 3. Influence of NaCl on growth of strain HRh1^T with thiosulfate at pH 7.5 (a) and on activity of thiosulfate oxidation by washed cells (b). ○, Biomass; ●, rate of thiosulfate consumption during growth; ◆, activity of thiosulfate oxidation by cells grown at 1 M NaCl; ◇, activity of thiosulfate oxidation by cells grown at 3 M NaCl.

concentration was more characteristic of extremely halophilic SOB of the genera *Thiohalospira* and *Thiohalorhabdus* isolated from the same habitats (Sorokin, 2008) (see Supplementary Table S1, available in IJSEM Online).

Spectroscopic analysis of cell membranes of strain HRh1^T showed a higher concentration of the *b*-type cytochromes in the cells grown with thiocyanate than those grown with thiosulfate. The CO-spectra indicated the presence of *cb*-type cytochrome oxidase in both preparations (most probably *cbb*₃) and, in addition, an *aa*₃-type in the thiocyanate-growing cells (data not shown).

Genomic DNA was extracted by using the phenol/chloroform method (Marmur, 1961) and the G+C content was analysed by using the thermal denaturation/reassociation technique (Marmur & Doty, 1962) with DNA from *Escherichia coli* K-12 as a standard. The DNA G+C content was 63.5 mol%. A nearly complete 16S rRNA gene (1455 bp) was amplified using the general bacterial primers GM3F and GM4R (Schäfer & Muyzer, 2001). To amplify a

cbbL gene fragment (800 bp), coding for the ribulose biphosphate carboxylase/oxygenase (RuBisCO) large sub-unit form I, a specially designed primer pair and protocol were used (Spiridonova *et al.*, 2004). The PCR products were purified from low-melting-point agarose using the Wizard PCR-Prep kit (Promega) according to the manufacturer's instructions. Phylogenetic trees were reconstructed using the TREECON W package (Van de Peer & De Wachter, 1994).

16S rRNA gene sequence analysis demonstrated that strain HRh1^T formed an independent lineage within the *Gammaproteobacteria* with an unidentified marine denitrifying SOB isolate as the closest cultured relative (93 % sequence similarity) (Fig. 4a). A search of the RDP database indicated a 100 % match to the *Gammaproteobacteria* and 85 % probability of affiliation with the *Chromatiales*. Among uncultured clones, a BLAST search using the sequence identified a group of unclassified marine gammaproteobacteria as the closest relatives, with 95 % sequence similarity (Hunter *et al.*, 2006).

A single *cbbL* gene of the 'green-like' form I RuBisCO was detected in strain HRh1^T. Phylogenetic analysis placed the sequence within a cluster of lithoautotrophic bacteria from the *Gammaproteobacteria* and *Betaproteobacteria* as an independent lineage (Fig. 4b).

The presented data demonstrate that the novel thiocyanate-utilizing halophilic SOB isolate HRh1^T differs in many respects from its counterparts from the same habitats (*Thiohalophilus* species), in both the mechanism of thiocyanate degradation and other essential phenotypic and phylogenetic properties (Table 1). On the basis of these unique features, a new genus and species *Thiohalobacter thiocyanaticus* gen. nov., sp. nov. are proposed here to accommodate strain HRh1^T.

Description of *Thiohalobacter* gen. nov.

Thiohalobacter (Thi'o.ha.lo.bac'ter. Gr. n. *thion* sulfur; Gr. n. *hals*, *halos* salt; N.L. masc. n. *bacter* a rod; N.L. masc. n. *Thiohalobacter* halophilic sulfur rod).

Gram-negative, aerobic, obligately chemolithoautotrophic sulfur-oxidizing bacteria. CO₂ is assimilated through the Calvin-Benson cycle. Oxidize inorganic sulfur compounds to sulfate. Moderately halophilic and neutrophilic. Major cellular fatty acids are C₁₆ and C₁₈ species. The known strain was isolated from a hypersaline habitat. The genus is a member of the *Gammaproteobacteria*. The type species is *Thiohalobacter thiocyanaticus*.

Description of *Thiohalobacter thiocyanaticus* sp. nov.

Thiohalobacter thiocyanaticus (thi'o.cy.a.na'ti.cus. N.L. n. *thiocyanas* -atis thiocyanate; L. masc. suff. -icus suffix used in adjectives with the sense of belonging to; N.L. masc. adj. *thiocyanaticus* related to thiocyanate, utilizing thiocyanate).

Fig. 4. Phylogenetic position of strain HRh1^T within the *Gammaproteobacteria* based on 16S rRNA gene sequence analysis (a) and based on inferred amino acid sequence of the *cbbL* gene (b). The trees were reconstructed from evolutionary distances by using the neighbour-joining method. In (a) the outgroup (*Desulfurivibrio alkaliphilus* AHT 2^T; GenBank accession no. EF422413) was pruned from the tree. In (a), halophilic SOB isolated from the same habitat as strain HRh1^T are highlighted. Bootstrap percentages were derived from 1000 resamplings; values greater than 70% were considered as significant. Bars, 0.05 (a) and 0.1 (b) substitutions per nucleotide position.

Table 1. Comparison of properties of strain HRh1^T with other moderately halophilic SOB from the *Gammaproteobacteria*

Strains/species: 1, HRh1^T; 2, *Thiohalophilus thiocyanatoxidans* (data from Sorokin *et al.*, 2007a); 3, *Thiohalomonas denitrificans* (Sorokin *et al.*, 2007b); 4, *Halothiobacillus halophilus* (Wood & Kelly, 1991; Kelly *et al.*, 1998). ND, No data available. All taxa oxidize thiosulfate, sulfide and inorganic sulfur.

Property	1	2	3	4
Cell morphology	Short rods	Long rods	Long rods	Short rods
Motility	—	—	—	+
Dominant fatty acids in membrane phospholipids	16:1 ω 7, 18:1 ω 7, 16:0	16:0, iso-17:1 ω 5, 16:1 ω 7	16:1 ω 7, 16:0, 18:1 ω 7	ND
Thiocyanate utilization	+ (via cyanate)	+ (via COS)	—	—
Denitrification	—	+ (nitrite)	+ (nitrate)	—
Oxidation of:				
Tetrathionate	+	+	—	+
Trithionate, hexathionate	—	—	—	+
Tetrathionate formation from thiosulfate	+	—	—	+
Salt tolerance (M NaCl)	3.0	4.0	3.0	4.0
RuBisCO form	I	I*	II*	I*
DNA G + C content (mol%)	63.5	58.2	58–60	64.2
Isolation source(s)	Hypersaline lakes	Hypersaline lakes	Hypersaline lakes and salterns	Hypersaline lakes

*Our unpublished data.

Shows the following properties in addition to those described for the genus. Cells are short non-motile rods, often in chains, 0.4 × 1–2 μ m. Colonies are up to 2 mm in diameter, silver-white from extracellular sulfur accumulation. The dominant cellular fatty acids include C_{16:1} ω 7, C_{18:1} ω 7 and C_{16:0}. Oxidase- and catalase-positive. Utilizes thiocyanate and thiosulfate as electron donors. Oxidizes sulfide, elemental sulfur and tetrathionate but not H₂. Thiocyanate is degraded under strictly aerobic conditions via cyanate. Grows at 0.2–3.0 M NaCl (optimum 0.5 M), pH 6.3–8.0 (optimum pH 7.3–7.5) and 20–40 °C (optimum 32 °C). The G + C content of the DNA of the type strain is 63.5 mol% (T_m).

The type strain, HRh1^T (=DSM 21152^T =UNIQEM U249^T), was isolated from sediments of hypersaline lakes in south-western Siberia.

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